MINI-REVIEW

Microbial acetylcholinesterase inhibitors for Alzheimer's therapy: recent trends on extraction, detection, irradiation-assisted production improvement and nano-structured drug delivery

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Abstract
Neurodegenerative disorders especially Alzheimer's disease (AD) are significantly threatening the public health. Acetylcholinesterase (AChE) inhibitors are compounds of great interest which can be used as effective agents for the symptomatic treatment of AD. Although plants are considered the largest source for these types of inhibitors, the microbial production of AChE inhibitors represents an efficient, easily manipulated, eco-friendly, cost-effective, and alternative approach. This review highlights the recent advances on the microbial production of AChE inhibitors and summarizes all the previously reported successful studies on isolation, screening, extraction, and detecting methodologies of AChE inhibitors from the microbial fermentation, from the earliest trials to the most promising anti-AD drug, huperzine A (HupA). In addition, improvement strategies for maximizing the industrial production of AChE inhibitors by microbes will be discussed. Finally, the promising applications of nano-material-based drug delivery systems for natural AChE inhibitor (HupA) will also be summarized.

Key Points
• *AChE inhibitors are potential therapies for Alzheimer's disease.*
• *Microorganisms as alternate sources for prospective production of such inhibitors.*
• *Research advances on extraction, detection, and strategies for production improvement.*
• *Nanotechnology-based approaches for an effective drug delivery for Alzheimer's disease.*

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Introduction

Acetylcholinesterase (AChE) inhibitors are chemical compounds which considered being among the most therapeutic tools for many neurodegenerative disorders such as Alzheimer's disease (AD), glaucoma, Parkinson's disease, myasthenia gravies, and Down syndrome. AChE inhibitors belong to many classes such as carbamate derivatives which participate in the action mechanism of insecticidal drugs, while those belonging to parathion and malathion play a significant role in nerve gases (Erdogan Orhan et al. 2011).

AD is a serious neurodegenerative disease, mostly targeting people above 65 years of age (Mohamed and Rao 2011). AD patients are suffering mainly from sequential memory loss and deficiency of their cognitive functions (Khairallah and Kassem 2011). AD threatens the public health

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and society as the epidemiological data indicated that the number of people affected with AD worldwide by 2050 is expected to increase, predicting that, every 33 s, there will be one new AD case (Prince et al. 2013; Alzheimer's Association 2017).

About 5% of the people above 65 years and more than 20% above 80 years are subjected to the risk of AD. After cardiovascular diseases and cancer, AD is considered to be the third main reason of death in the developing countries. Pathogenesis of AD is complex and includes genetic and environmental factors (Williams et al. 2011).

This disorder is characterized by the progressive irreversible loss of neurons in specific brain areas, mainly the hippocampus, accumulation of β-amyloid protein (Aβ) plaques and neurofibrillary tangles (NFTs) in brain tissues, hyperphosphorylation of Tau protein in neurons, and collapsing of cognitive functions leading to death (Khairallah and Kassem 2011; Mohamed and Rao 2011).

Surprisingly, oxidative stress and the formation of reactive oxygen species (ROS) are considered to be the main causes of this disease (Behl and Moosmann 2002). As AD is a multifactorial disease, many therapeutic approaches were suggested for its treatment. Therapeutic approaches were then classified into two main effective routes, cholinergic and non-cholinergic. In patients with AD, the disorder in the cholinergic neurotransmission is causing a decline in the neurotransmitter acetylcholine (ACh) (Konrath et al. 2013).

As a result, acetylcholinesterase (AChE) inhibitors could rebalance the level of acetylcholine by inhibiting the AChE activity. Consequently, acetylcholine could be accumulated in the synaptic cleft (Su et al. 2017). This route is called cholinergic approach and considered as the major symptomatic treatment for AD.

Another treatment approach depends on preventing the accumulation of β-amyloid peptide (Aβ). Thus, any compound, able to reduce or prevent the aggregation of Aβ between neurons, could be a potential anti-AD drug candidate which can reduce the disease progression (Jiang et al. 2003). Other suggested approaches included many compounds and routes such as antioxidants, vitamins, anti-hypertensive drugs, selective phosphodiesterase inhibitors, non-steroidal anti-inflammatory drugs, transition metal chelators, inhibitory drugs of Tau proteins hyper-phosphorylation and intracellular NFT accumulation, use of brain-derived neurotropic factor (BDNF), stem cell therapy, and hormonal therapy (Dey et al. 2017). Additionally, stimulatory therapies such as physical exercise, cognitive training, socialization, and music have been reported. Moreover, nutritional supplements, medicinal plants, phyto-chemicals, minerals, and omega-3 fatty acids have been also tested (Wollen 2010).

AChE inhibitors can be obtained either by chemical synthesis or by extraction from natural plants and microorganisms. Microbial production of AChE inhibitors is gaining a lot of attention due to its advantages over both phyto-extraction and chemical synthesis. On the one hand, extraction processes are limited by lacking of natural plant resources due to their excessive harvesting, and chemical synthesis is threatening the environment by pollution and hazardous materials (Wang et al. 2015b).

On the other hand, marked-available AChE inhibitors derived from plants and chemical synthesis showed many disadvantages such as low bioavailability and other abdominal side effects (Erdogan Orhan et al. 2011). Thus, exploring other alternatives of AChE inhibitors derived from microbial sources with different niches is a must.

Huperzine A (HupA) is a Lycopodium alkaloid, which can be naturally extracted from the traditional Chinese medicinal plant, *Huperzia serrata*. HupA has attracted intense attention after discovering its activity as an effective cholinesterase inhibitor (Ishiuchi et al. 2013). Compared with the well-known and commercially available AChE inhibitors, HupA is a more effective reversible inhibitor.

Additionally, it can effectively penetrate the blood–brain barrier and showed better oral bioavailability and longer inhibitory effect (Wang et al. 2006). Consequently, since 1996, HupA appeared in the Chinese drug market in a form of tablets named Shuangyiping for symptomatic treatment of AD, while in the USA and Europe, HupA is present as a dietary supplement (*H. serrata* powder in a capsule format) for limiting further memory disorders (Ma and Gang 2008).

This review collects a variety of soil, marine, and endophytic microorganisms which considered promising producers of anti-AD drugs that showed in vitro anti-AChE activity. In addition, it summarizes recent reports on the production, extraction, and detection methodologies of the most effective anti-AD drug candidate HupA with the established and recommended enhancement strategies for scaling up the microbial production of AChE inhibitors, to open the way towards the large-scale production. Moreover, incorporation of these active compounds with nano-structured drug delivery systems to increase their selectivity and reactivity will be also discussed.

Acetylcholinesterase and AChE inhibitors

The enzyme acetylcholinesterase selectively catalyzes the ester bond in acetylcholine via hydrolysis at the synaptic cleft to stop its impulse transmitting role. Accordingly, the activated cholinergic neurons return to the resting state (Williams et al. 2011). In addition, AChE regulates the cholinergic neurotransmission in vertebrates by inactivating acetylcholine immediately after presynaptic neurons releasing (Pope and Brimijoin 2018).

AChE inhibitors began to be very attractive to be used in AD symptomatic therapy, after the initial discovery of physostigmine, a *Physostigma venenosum* Balf (*Fabaceae*) seed-

derived alkaloid, which can reverse scopolamine inducing cognition disruption in animal models. Although physostigmine shows a quick, selective, and reversible AChE inhibitory effect, its current use is limited by many limitations such as its gastrointestinal effects, short half-life, and narrow therapeutic image (Konrath et al. 2013).

AChE inhibitors could not only improve the cholinergic transmission but also they showed a protective role against free radical injury of brain cells and interfere against aggregation and deposition of amyloid beta protein as the second suggested mechanism of AD (Almasi et al. 2018). Because of the medicinal value of these inhibitors, there is a massive research interest focusing on the development of new AChE inhibitors.

The approved AChE inhibitors for treating AD

Many drugs have been approved by the US Food and Drug Administration (FDA) for symptomatic treatment of AD patients. Most of these drugs are AChE inhibitors. For example, Tacrine was approved in 1993, but it was withdrawn from market in 2012 because of its hepato-toxicity which affected more than 29% of patients. In addition, Donepezil was approved in 1996 as a reversible AChE inhibitor and a fully synthetic compound with good inhibitory effect and low toxic effects than Tacrine. Moreover, galantamine was approved in 2001 as a natural alkaloid extracted from *Galanthus nivalis* L. and related plants in *Amaryllidaceae* family (Heinrich and Teoh 2004; Marco and Carreiras 2006). More and above, Rivastigmine which is a semi-synthetic derivative of physostigmine, was approved in 2000. Although it did not show liver toxicity like Tacrine, it showed other side effects such as nausea and vomiting (Zhao et al. 2004).

Microbial production of AChE inhibitors

Plants represent the main significant source of AChE inhibitors. However, few researches reported the ability of some microorganisms to produce similar inhibitors (Pandey et al. 2014). Searching for natural, cost-effective, and sustainable source of effective AChE inhibitors became an attractive scope for many researchers. Hence, great efforts have been dedicated for investigating the production of AChE inhibitors by microbial strains isolated from soil and marine environments, and unusual sources such as plant-associated microbes known as endophytes (Singh et al. 2012). Table 1 summarizes most-recent reported data on the microbial anti-AChE activity and the identified microbial AChE inhibitors by various microorganisms from different niches.

Endophytic fungi as new sources for AChE inhibitors

Endophytic fungi are mutualistic microorganisms, occupy the internal tissues of healthy plants through certain life stages or throughout their whole life, without leading to any significant disease symptoms on the host (Strobel 2003; Ismaiel et al. 2017). They are known to provide a plethora of fitness benefits to the host as previously reported endophytes are biologically active and can efficiently enhance the growth of their host, and promote host resistance to diseases-causing phytopathogens and environmental stress (Aly et al. 2011).

Interestingly, they can also produce identical or similar bioactive substances as host plants (Strobel 2003). After Stierle et al. (1993) reported the discovery of an endophytic fungus, *Taxomyces andreanae*, which can produce the anticancer-active substance, paclitaxel, the endophytic fungi producing pharmaceutically active compounds have gained significant consideration worldwide.

The hypothesis of horizontal gene transfer has revealed the capability of endophytes to produce the same active compounds which produced by host plants. Moreover, they could produce some novel medicines against some incurable diseases (Strobel 2003; Strobe and Daisy 2003; Staniek et al. 2008). However, the unmanaged harvesting of plant resources for extracting AChE inhibitors would lead to their depletion. For protecting these resources, the capability of producing similar AChE inhibitors by endophytes of medicinal plants has been widely investigated. Endophytic fungi proved their efficiency as valuable, novel, and alternate resources with good AChE inhibitory activity (Wang et al. 2015b; Ali et al. 2016).

Endophytic fungi with good anti-AChE activity are presented in Table 1. Recovery of endophytic fungi from healthy plant tissues led to a massive argument whether these entophytes are plant pathogen or not. According to Chen et al. (2010) and Mohinudeen et al. (2019), endophytic fungi may appear at two phases, non-pathogenic and pathogenic. Many non-pathogenic endophytes are dormant pathogens and may become pathogenic under certain environmental stress conditions or after plant aging. Other possible reason may be attributed to the nature of host species as endophytes can be useful and growth stimulating to certain host species while they can be pathogenic to other plants.

HupA identity and structural elucidation

Huperzine A is a naturally occurring sesquiterpene alkaloid compound found in the extract of the Chinese club moss botanically known as *Huperzia serrata* (also known as *Lycopodium serratum*). It can be also found with varying quantities in other *Huperzia* species, including *H. elmeri*, *H. carinat*, and *H. aqualupian* (Lim et al. 2010). *H. serrata* grow at high alleviations and in cold climates. It has been used

Table 1 Microbial strains producing various AChE inhibitors

Microbial strain	Isolation source	AChE inhibitor	Reference
Bacillus subtilis M18SP4P	*Fasciospongia cavernosa*	Methanolic extract	Pandey et al. 2014
Bacillus subtilis	Oyster	No identified compound	Wang et al. 2014
Streptomyces sp. AH-4	Soil sample	Physostigmine	Murao and Hayashi 1986
Streptomyces antibioticus	Not clear	Two organophosphates	Neumann and Peter 1987
Streptomyces lavendulae NK901093	Not clear	Cyclophostin (1)	Kurokawa et al. 1993
Streptomyces sp. LB173	Marine sediment	Geranylphenazinediol	Ohlendorf et al. 2012
Rubrobacter radiotolerans	*Petrosia* sp.	Dimeric indole derivatives	Li et al. 2015
Actinobacterial isolate N98-1021	Not clear	Terferol	Yue-sheng et al. 2002
Streptosporangium sp.	Not clear	7,4′-Dihydroxy flavone	Binghuo et al. 2005
Streptomyces sp. UTMC 1334	Marine samples	Pyrrole derivatives	Almasi et al. 2018
Penicillium sp. FO-4259	Soil sample	Arigsugacin	Omura et al. 1995
Penicillium citrinum	Soil sample	Quinolactacins A1and A2	Kim et al. 2001
Xylaria sp.	Marine sample	Xyloketal A	Lin et al. 2001
Chrysosporium sp.	Not clear	14 (2′,3′,5′- trihydroxyphenyl) tetradecan-2-ol	Sekhar Rao et al. 2001
Aspergillus flavus cf-5	Marine red alga	Fungal extract	Qiao et al. 2011
Hyalodendriella sp. Ponipodef12	*Populus deltoides*	Palmariol B, 4-hydroxymellein, Alternariol 9-methyl ether, Botrallin	Meng et al. 2012
Macroscopic fungi (mushrooms)	Different areas	Fungal extract	Patočka 2012
Alternaria sp. Cas1	*Ricinus communis*	Fungal extract	Singh et al. 2012
Paecilomyces lilacinus	A soil sample	Paecilomide	Paula et al. 2013
Penicillium sp. sk5GW1L	*Kandelia candel*, China	Arigsugacin I, Arigsugacins F, Territrem B	Huang et al. 2013
Aspergillus terreus (No. GX7-3B)	*Bruguiera gymnoihiza*	Anhydrojavanicin, 8-O-methyljavanicin, NGA0187, Beauvericin	Deng et al. 2013
Talaromyces sp. strain LF458	*Axinella verrucosa*	Talaromycesone A, isopentenylxanthenone, talaroxanthenone	Wu et al. 2014
Phomopsis sp. Cs-c2	*Senna spectabilis*	Cytochalasin H	Chapla et al. 2014
Endophytic fungus	*Huperzia serrata*	Avertoxin B	Wang et al. 2015a
Haddowia longipes	Tropical areas	Lanostanoids	Zhang et al. 2015d
Alternaria alternata VS-10	*Catharanthus roseus*	Altenuene	Bhagat et al. 2016

for centuries in the Chinese Folk Medicine (known as Qian Ceng Ta). The chemical stability of HupA is very good, and it possesses good resistant to structural changes in both acidic and alkaline solutions, which indicated that HupA has a relatively longer shelf life. The chemical structure of HupA is displayed in Fig. 1.

Fig. 1 The chemical structure of HupA (**a**), structure of acetylcholinesterase complex with HupA at 2.35A resolution (**b**), and 3D molecular spaces fill of HupA (**c**)
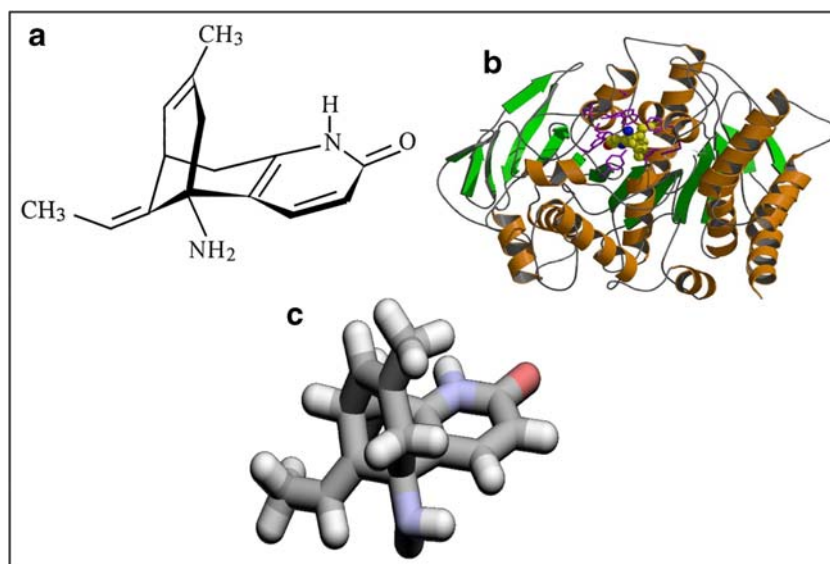
HupA has been extensively investigated as a treatment for neurological conditions such as Alzheimer's disease; a meta-analysis concluded that previous studies were of poor methodological quality and the findings should be interpreted with caution (Yang et al. 2013). HupA inhibits the breakdown of the neurotransmitter acetylcholine by acetylcholinesterase enzyme, and this is the same mechanism of action of AD-treating pharmaceutical drugs such as galantamine and donepezil. HupA is commonly available over the counter as a nutrient supplement, and was marketed as a cognitive enhancer for improving memory and concentration (Ma X, Gang DR 2008).

HupA [IUPAC name: (1R,9S,13E)-1-amino-13-ethylidene-11-methyl-6-azatricyclo-[7.3.1.02,7]-trideca-2(7),3,10-trien-5-one; commercially known as CogniUp] is an alkaloid, an AChE inhibitor, and N-methyl-d-aspartate receptor (and glutamate receptor) antagonist (Table 2; (Wang et al. 2008).

The acute oral toxicity (LD_{50}) of HupA was found to be 4.6 mg/kg body weight in mice. Considering a therapeutic oral dose of 0.2 mg HupA/kg body weight, it shows a wide margin of safety (Bagchi and Barilla 1998). Extensive laboratory testing of HupA has been performed, demonstrating its non-mutagenic properties as revealed by Ames' bacterial reverse mutation assay (Li et al. 2012).

Biosynthesis of HupA: related genes and enzymes

Distribution of gene functions and biochemical pathways in *Colletotrichum gloeosporioides* (HupA-producing fungus) was based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) assignments, particularly in the categories of molecular function and metabolism (Zhang et al. 2015b). These annotations provide valuable resources for the investigation of gene functions, and cellular structures and processes in *C. gloeosporioides*.

Among the 308 metabolic pathways (annotated by KEGG), three pathways were involved in alkaloid biosynthesis: lysine biosynthesis, biotin metabolism, and (tropane, piperidine, and pyridine) alkaloid biosynthesis. A total of 30 unigenes in this library showed similarities to the non-characterized enzymes that might be associated with the biosynthesis of HupA (Table 3).

Based on these three pathways and more literature survey (Luo et al. 2010; Choi et al. 2012; Liu et al. 2012), a more detailed biosynthetic pathway was deduced as shown in Fig. 2 with related enzymes and genes as shown in Table 3. The discovery of new transcripts will further facilitate the elucidation of the biosynthetic mechanisms of HupA at the molecular level in *C. gloeosporioides* (Zhang et al. 2015a, b, c, d).

Table 2 Physicochemical characteristics of HupA (Wang et al. 2008)

Physicochemical characteristics	Data
Molecular formula	$C_{15}H_{18}N_2O$
Melting point	422.6 °F (217 °C)
Molar mass	242.32 g/mol
Solubility	Dimethyl sulfoxide (DMSO) or Ethanol

Table 3 Suggested encoding enzymes and unigenes involved in HupA biosynthetic pathway

Biosynthetic pathway	Encoding enzyme	Unigenes
Biotin metabolism	Biotin—protein ligase [EC:6.3.4.9, EC:6.3.4.10, EC: 6.3.4.11, EC: 6.3.4.15]	Unigene15126_c1_seq1, unigene15126_c1_seq2, unigene15126_c1_seq3, unigene15126_c1_seq4, unigene15126_c1_seq5
	EC:3.4.-.- (hydrolysis enzymes acting on peptides bonds)	Unigene17901_c0_seq1, unigene9788_c0_seq1
Lysine biosynthesis	Homocitrate synthase [EC:2.3.3.14]	Unigene15734_ c0_seq1
	Homoaconitatehydratase [EC:4.2.1.36]	Unigene11242_c0_seq1
	Homoisocitratedehydrogenase [EC:1.1.1.87]	Unigene17283_c0_seq1
	Aromatic amino acid aminotransferase [EC:2.6.1.57]	Unigene14978_c1_seq1
	L-aminoadipate semialdehydedehydrogenase [EC:1.2.1.31]	Unigene9165_c0_seq1
	Saccharopine dehydrogenase (NADP+,L-glutamate forming) [EC:1.5.1.10]	Unigene14707_c0_seq1
	Saccharopine dehydrogenase (NAD+, L-lysine forming) [EC:1.5.1.7]	Unigene6171_c0_seq1
Tropane piperidine, pyridine alkaloid biosynthesis	Primary-amine oxidase [EC:1.4.3.21]	Unigene10060_c0_seq1, unigene10314_c0_seq1, unigene112700_c0_seq1, unigene12610_c0_seq1, unigene12610_c1_seq1, unigene12610_c2_seq1, unigene12610_c2_seq2, unigene13099_c0_seq1, unigene13099_c1_seq1, unigene14974_c0_seq1 unigene16440_c0_seq1, unigene49225_c0_seq1, unigene5254_c0_seq1, unigene8472_c0_seq1, unigene9217_c0_seq1, unigene9322_c0_seq1

HupA production and supply problems

There is a serious problem obstructing the large-scale production of this highly effective drug as *Huperziaceae* plant species specially, *Huperzia serrata* which are the major source of HupA, give low extracted yield and their vegetative cycle is very long (Ma and Gang 2008). It means that these plants are not considered to be effective source for HupA production at the commercial scale. As a result, researchers tried to find other methods to produce HupA such as in vitro plant tissue cultivation and chemical synthesis. Tissue culturing was not considered as a successful technique for HupA production at a large scale (Ma and Gang 2008; Ishiuchi et al. 2013). Although HupA was totally synthesized from (R)-pulegone (Ding et al. 2012), the chemical synthesis of HupA showed some limitations such as complex interactions, continual pollution, and expensive processes (Koshiba et al. 2009). As these trials have not achieved satisfactory results to be applied at a large industrial scale, the discovery of HupA-producing endophytic fungi from the tissues of *Huperziaceae* plant species provides an alternative solution.

Although the production of HupA drug from endophytes would reduce the price of traditional drugs and effectively protect the ecological environment, only a small number of endophytic fungi that produce HupA were reported. Li et al. (2007) reported the recovery of HupA-producing endophytic fungal species from tissues of *Huperzia serrata*. This finding

stimulated many researchers to isolate and screen many endophytic fungi from *Huperzia serrata* and other related plants such as *Phlegmariurus*, and this research interest is still increasing till today. The reported HupA-producing endophytes with varying HupA yield are highlighted in Table 4.

Isolation of endophytic fungi from *Huperziaceae* plant species

Since, endophytic fungi were suggested as prospective sources for HupA production, isolation of endophytic fungi from stems, leaves, and roots of healthy *Huperziaceae* species was the first serious challenge. The main principle of endophytic fungal isolation involved the recovery of fungal species at the internal plant tissues which was successfully achieved through surface sterilization of the tested plant samples.

Surface sterilizing sequence differed from report to another as demonstrated in Table 5. Ju et al. (2009) used 70% ethanol for seconds, 0.2% mercuric chloride ($HgCl_2$) for 1 min, 2% sodium hypochlorite, then rinsing in distilled water, while 75% ethanol for 2 min, 0.1% $HgCl_2$ for 8 min, then successive rinsing in sterile water were reported (Wang et al. 2011a; Zhang et al. 2015a).

Additionally, 75% ethanol for 5 min and 0.1% $HgCl_2$ for 8 min were reported (Zhu et al. 2010). Another report used 75% ethanol for 2 min and 0.1% $HgCl_2$ up to 10 min (Wang
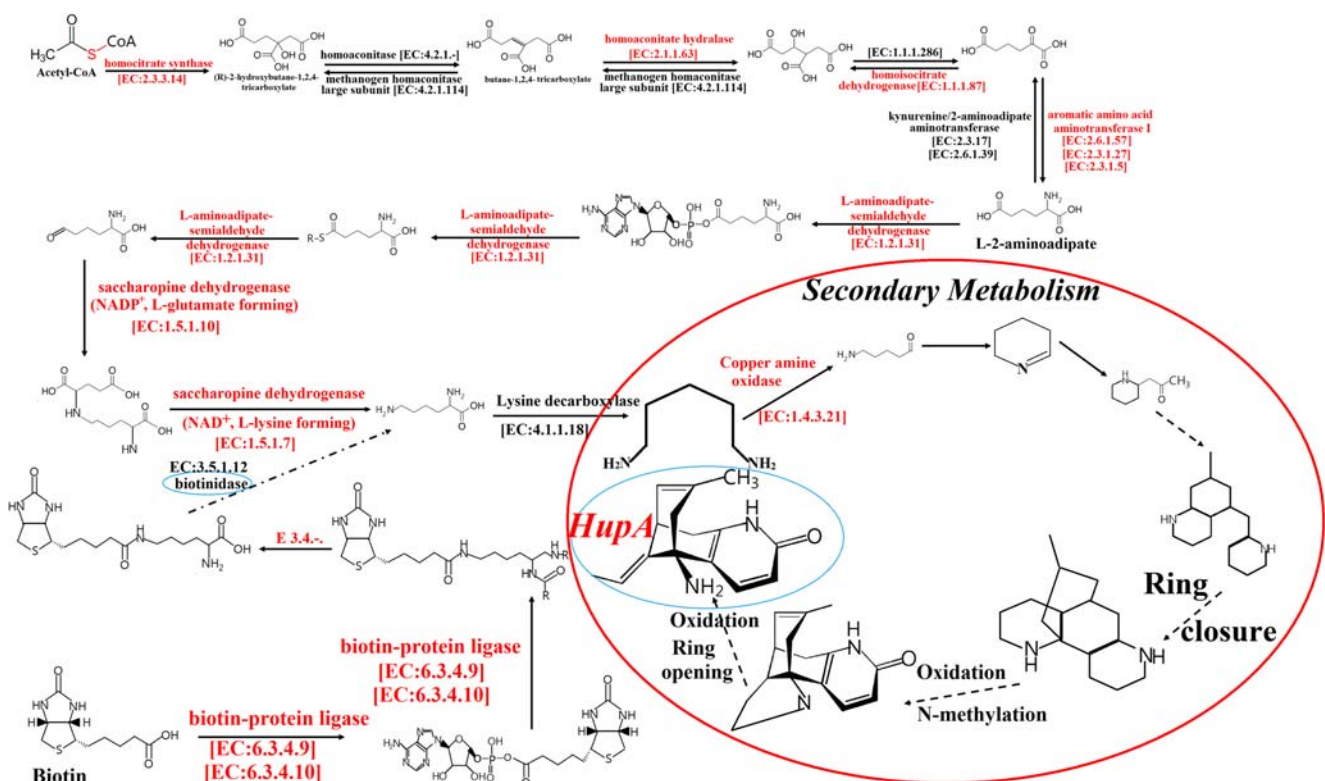
Fig. 2 Proposed biosynthetic pathway for huperzine A in fungal cells. Enzymes that were annotated in the transcriptome of fungal cells are presented in red, and those that could not be annotated are presented in black. Two arrows used for reversible reaction, and an arrow used for irreversible reaction

et al. 2011b). Similarly with slight difference, 75% ethanol for 5 min and 0.2% $HgCl_2$ for 1.5 min were used (Shu et al. 2014). Differently, 0.1% $HgCl_2$ for 15 min was used to disinfect the stems while, 0.1% $HgCl_2$ for 10 min was used for leaves and roots (Dong et al. 2014).

Additionally, 75% ethanol for 30 s, 10% sodium hypochlorite for 5 min, and 75% ethanol for 30 s again were reported (Han et al. 2015). Similarly, 75% ethanol for 1 min, 3.4% sodium hypochlorite for 10 min, and 75% ethanol for 30 s again were also reported (Cruz-Miranda et al. 2019). Finally, ethanol (70%) for 1 min and 1% available chlorine for 3 min were also investigated (Zaki et al. 2019). After surface tissue sterilization, the plant parts were cut into small segments and directly cultured on a suitable media; potato dextrose agar (PDA) was mostly chosen, which was enriched by streptomycin, 60 μg/ml, and ampicillin, 100 μg/ml, to avoid bacterial contamination (Zhu et al. 2010; Wang et al. 2011a, b; Zhang et al. 2015a; Zaki et al. 2019; Cruz-Miranda et al. 2019). Shu et al. (2014) and Dong et al. (2014) used Murashige and Skoog (MS) media for endophytes' isolation. Indeed, more than one type of media can be used to encourage the emergence of more endophytic strains by using both PDA and MS media as reported by Ju et al. (2009).

HupA extraction and detection from endophytic fungal culture

HupA is an intracellular metabolite of endophytic fungi and is mainly extracted from the fungal biomass. Thus, the HupA extraction method depends primarily on some sequenced steps of biomass grinding, acidic soaking, and ultrasonication in order to weaken the fungal cell walls and facilitate the extraction of the targeted intracellular metabolite (Pinu et al. 2017).

As HupA is an alkaloid compound by nature, it could be extracted by the conventional acid-water method. However, Weigang et al. (2013) and Ting et al. (2015) studied the significant extraction factors and determined the optimum levels for maximizing the HupA extracted yield from *H. serrata* using Box-Behnken factorial design. Currently, there are no reported extraction optimization studies for maximizing the extraction of HupA from fungal biomass and the different extraction conditions have been reported and summarized in Table 5.

Ju et al. (2009) used 2% tartaric acid on the fungal dried biomass followed by ultrasonic extraction for 2 h. Then, high-speed centrifugation at 10.000 rpm for 10 min; finally, supernatant was screened for HupA. Wang et al. (2011a) and Dong

Table 4 The reported HupA-producing endophytic fungal strains isolated from graphically distributed *Huperziaceae* plant species

Endophytic microorganism	*Huperziaceae* plant source	HupA yield	Reference
Acremonium sp.	*H. serrata*, China.	8.32 μg/L	Li et al. 2007
Penicillium chrysogenum	*H. serrata*, China.	7.21 μg/ml	Zhou et al. 2009
Blastomyces sp. and *Botrytis* sp.	*Phlegmariuruscryptomerianus*, Liancheng Country, Fujian Province.	20–30 μg/g dcw	Ju et al. 2009
Shiraia sp. Slf14	Leaves of *H. serrata* from Lushan Botanical Garden in Jiangxi Province, central China.	327.8 μg/L or 142.6 μg/g dcw	Zhu et al. 2010
Cladosporium cladosporioides LF70	Leaves of *H. serrate*, China.	56.84 μg/L 39.61 μg/g dcw	Zhang et al. 2011
*Aspergillus flavus*LF40	*H. serrata* from Laoshan Botanical Garden of the Chinese Academy of Sciences in Jiangxi Province.	80.1 μg/g dcw	Wang et al. 2011a
Aspergillus flavus, Penicillium griseofulvum Penicillium sp., *Mycoleptodiscus* sp., *Leptosphaeria* sp., *Acremonium implicatum, Cladosporium cladosporioides*	*H. serrata* from Lushan Botanical Garden in Jiangxi Province, central China.	Not clear	Wang et al. 2011b
*Colletotrichum gloeosporioides*ES026	*H serrata* from Fobaoshan Mountain, Enshi City, Hubei Province, China.	32.75 μg/g dcw, 1 μg/g dcw	Zhao et al. 2013; Shu et al. 2014
Trichoderma sp. L44	*H. serrata* from Tianmu Hangzhou Mountains, Zhejiang.	37.63 μg/g dcw	Dong et al. 2014
Hypoxylon investiens MY311 *Ceriporia lacerate* MY183	*Phlegmariurus phlegmaria* from Guan Zai Shan Garden, Fujian province.	40.53 μg/L	Zhang et al. 2015a
Paecilomyces tenuis	*H. serrata* from the mountainous region of Fujian Province, China.	21.0 μg/L	Su and Yang 2015
Penicillium sp.	Wild *H. serrate.*	Not clear	Han et al. 2015
Penicillium polonicum	*H. serrata* in Shaoyang, Hunan, China.	Not clear	Kang et al. 2016
Alternaria brassicae AGF041	*H. serrata* from Meihua Mountain in Fujian Province, P.R. China.	25.3 μg/g dcw 42.89 μg/g dcw	Zaki et al. 2019
Fusarium sp. C17	*P. taxifolius* from Coatepec, Mexico.	3.2 μg/g dcw	Cruz-Miranda et al. 2019

et al. (2014) exposed the collected cells to sonication with immersion in 95% ethanol overnight, then the extract was concentrated under reduced pressure.

Other reports soaked the dried cells (1 g) overnight in 50 mL of 0.5–1.5% (v/v) hydrochloric acid (Zhao et al. 2013; Shu et al. 2014;Han et al. 2015; Zaki et al. 2019) or 2% tartaric acid (Zhang et al. 2015a), then cells were disrupted by ultrasonication for 40 min. After that, ammonia solution was used to alkalize the water phase (pH 9); thus, alkaloids containing HupA left the water phase and transferred to the chloroform layer upon vigorous shaking.

After that, the chloroform extract was evaporated, and the obtained residue was dissolved in methanol. Zhu et al. (2010) and Cruz-Miranda et al. (2019) applied sequential extraction; the dried cells underwent extraction with 75% ethanol for 30 min in a 40 °C ultrasonic bath. Then, alcoholic extracts were allowed to evaporate under reduced pressure. After that, 2.5% hydrochloric acid was added to dissolve any dried residues. Then, ammonia solution was added followed by chloroform extraction and evaporation. Finally, the obtained dried residues were dissolved in 1 ml solvent (mostly methanol) for HupA identification.

HupA in an alkaloid extract from the endophytic fungi could be mainly identified and quantified by using spectroscopic and chromatographic analyses such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) analyses. Each analysis conditions and the composition of tested mobile phases were reviewed in Table 5 as the following: the preliminary identification step for HupA is application of TLC analysis,

Table 5 Different protocols for the isolation of HupA-producing endophytic fungi, extraction, and detection of HupA from fungal cultures

Isolation protocols	Extraction of HupA	Detection of HupA
Different plant surface sterilization solutions were applied as the following: 70% ethanol for seconds, 0.2% mercuric chloride ($HgCl_2$) for 1 min, 2% sodium hypochlorite, then rinsing in distilled water (Ju et al. 2009). 75% ethanol for 2 min, 0.1% of $HgCl_2$ for 8 min, then successive rinsing in sterile water (Wang et al. 2011a; Zhang et al. 2015a). 75% ethanol was used for 5 min, and 0.1% $HgCl_2$ for 8 min (Zhu et al. 2010). 75% ethanol for 2 min and then 0.1% $HgCl_2$ up to 10 min (Wang et al. 2011b). 75% ethanol for 5 min and 0.2% $HgCl_2$ for 1.5 min were used (Shu et al. 2014). 0.1% $HgCl_2$ for 15 min was used to disinfect the stems while 0.1% $HgCl_2$ for 10 min was used for leaves and roots (Dong et al. 2014). 75% ethanol for 30 s, 10% sodium hypochlorite for 5 min, and 75% ethanol for 30 s were used (Han et al. 2015). 75% ethanol for 1 min, 3.4% sodium hypochlorite for 10 min, and 75% ethanol for 30 s were also tested (Cruz-Miranda et al. 2019). Ethanol (70%) for 1 min, and 1% available chlorine for 3 min was also tested (Zaki et al. 2019).	Ju et al. (2009) used 2% tartaric acid on the fungal dried biomass followed by ultrasonic extraction for 2 h, then high-speed centrifugation at 10.000 rpm for 10 min, finally, the supernatant was screened for HupA. Wang et al. (2011a) and Dong et al. (2014) exposed the collected cells to sonication with immersion in 95% ethanol overnight, then the extract was concentrated under reduced pressure. Soaking the dried cells (1 g) overnight in 50 mL of 0.5–1.5% (v/v) hydrochloric acid (Zhao et al. 2013; Shu et al. 2014; Han et al. 2015; Zaki et al. 2019) or 2% tartaric acid (Zhang et al. 2015a), the next steps were as follows: 1. The cells were disrupted by ultrasonication for 40 min. 2. Ammonia solution was added to alkalize the water phase (pH 9); thus, alkaloids containing HupA will leave the water phase and be transferred to the chloroform layer upon vigorous shaking. 3. The chloroform extracts were evaporated, and the obtained residue was dissolved in 1 mL methanol. Zhu et al. (2010) and Cruz-Miranda et al. (2019) used sequential extraction. Primarily, the dried cells underwent extraction with 75% ethanol for 30 min in a 40 °C ultrasonic bath. The alcoholic extracts were then allowed to evaporate under reduced pressure. After that, 2.5% hydrochloric acid was added to dissolve the obtained dry residues.	For TLC analysis: The developed run reagents composed of acetone: chloroform: isopropanol (4:4:2 v/v/v) (Wang et al. 2011a, 2011b; Han et al. 2015). Acetone: chloroform: isopropanol: ammonia (4:4:2:0.12) (Zhu et al. 2010; Zaki et al. 2019). Acetone: chloroform: isopropanol: ammonia (4:4:1.5:0.15); acetic acid: 1-Butanol: water (3:3:2); 1-Butanol: isopropanol: water (10:5:4); 1-Butanol: isopropanol: acetic acid: water (7:5:2:4); chloroform: acetone: methanol (65:35:5) (Zhu et al. 2010). For HPLC analysis: A mobile phase of acetonitrile: 0.02 M KH_2PO_4 (10:90), flow rate 1 ml/min, 20 µl injected sample, column temperature (25 °C), and detecting wavelength of 310 nm was used (Li et al. 2007). Methanol: 0.8% ammonium acetate solution (33: 67, p H 6.0) (Ju et al. 2009), while 10% acetonitrile, and 35 °C column temperature (Zhou et al. 2009). Methanol: water (85:15) (Zhu et al. 2010; Wang et al. 2011a; Zaki et al. 2019). Ammonium acetate (80 mM, or 0.1 M, pH 6.0): methanol (7:3 or 6:4 or 36:64 v/v) (Zhao et al. 2013; Dong et al. 2014; Su and Yang 2015). Methanol: 0.1% formic acid (75:25 v/v) (Han et al. 2015), Acetonitrile: water (acidified with 0.0125% trifluoro-acetic acid) (15:85 v/v) (Cruz-Miranda et al. 2019).

where the extracted HupA and standard samples were loaded on silica gel-coated plates with developing run reagent that differed from one report to another (Table 5), as it was composed of acetone: chloroform: isopropanol (4:4:2 v/v/v) (Wang et al. 2011a, b; Han et al. 2015), acetone: chloroform: isopropanol: ammonia (4:4:2:0.12) (Zhu et al. 2010; Zaki et al. 2019); acetone: chloroform: isopropanol: ammonia (4:4:1.5:0.15); acetic acid:1-Butanol:water (3:3:2); 1-Butanol: isopropanol: water (10:5:4); 1-Butanol: isopropanol: acetic acid: water (7:5:2:4); chloroform: acetone: methanol (65:35:5) (Zhu et al. 2010).

Then, HupA spot was visualized using UV-fluorescence at 254 nm (Su and Yang 2015) or by spraying 3% (w/v) potassium permanganate as a chromogenic reagent forming yellow spots containing HupA compound against a violet background (Zhu et al. 2010). HupA extracted from the positive producing strains recorded the closest migration rate to the standard HupA with a retention factor (R_F) between 0.70 and 0.73 (Zhu et al. 2010).

Subsequent characterization and quantification of HupA were performed using different HPLC methodologies as shown in Table 5. The extracted HupA content can be quantified using a standard curve that was created by analyzing the concentration range of HupA standard sample, and represented by µg/g dcw (dried mycelia).

Li et al. (2007) used HPLC with a mobile phase of acetonitrile: 0.02 M KH_2PO_4 (10:90), flow rate 1 ml/min, 20 µl injected sample, column temperature (25 °C), and detecting wavelength of 310 nm, while HPLC mobile phase of methanol: 0.8% ammonium acetate solution (33: 67, p H 6.0), flow rate: 1 ml/min, column temperature: 25 °C, detection wavelength: 310 nm, and the injection volume: 20 µL was reported by Ju et al. (2009).

Additionally, Zhou et al. (2009) used a run solvent of 10% acetonitrile, 35 °C column temperature, and 308 nm detecting wavelength. Other reports (Zhu et al. 2010; Wang et al. 2011a; Zaki et al. 2019) performed HPLC with a mobile phase of methanol: water (85: 15), 1.0 mL/min as flow rate, 25 °C column temperature, 10 µL injected volume, and 310 nm detecting wavelength.

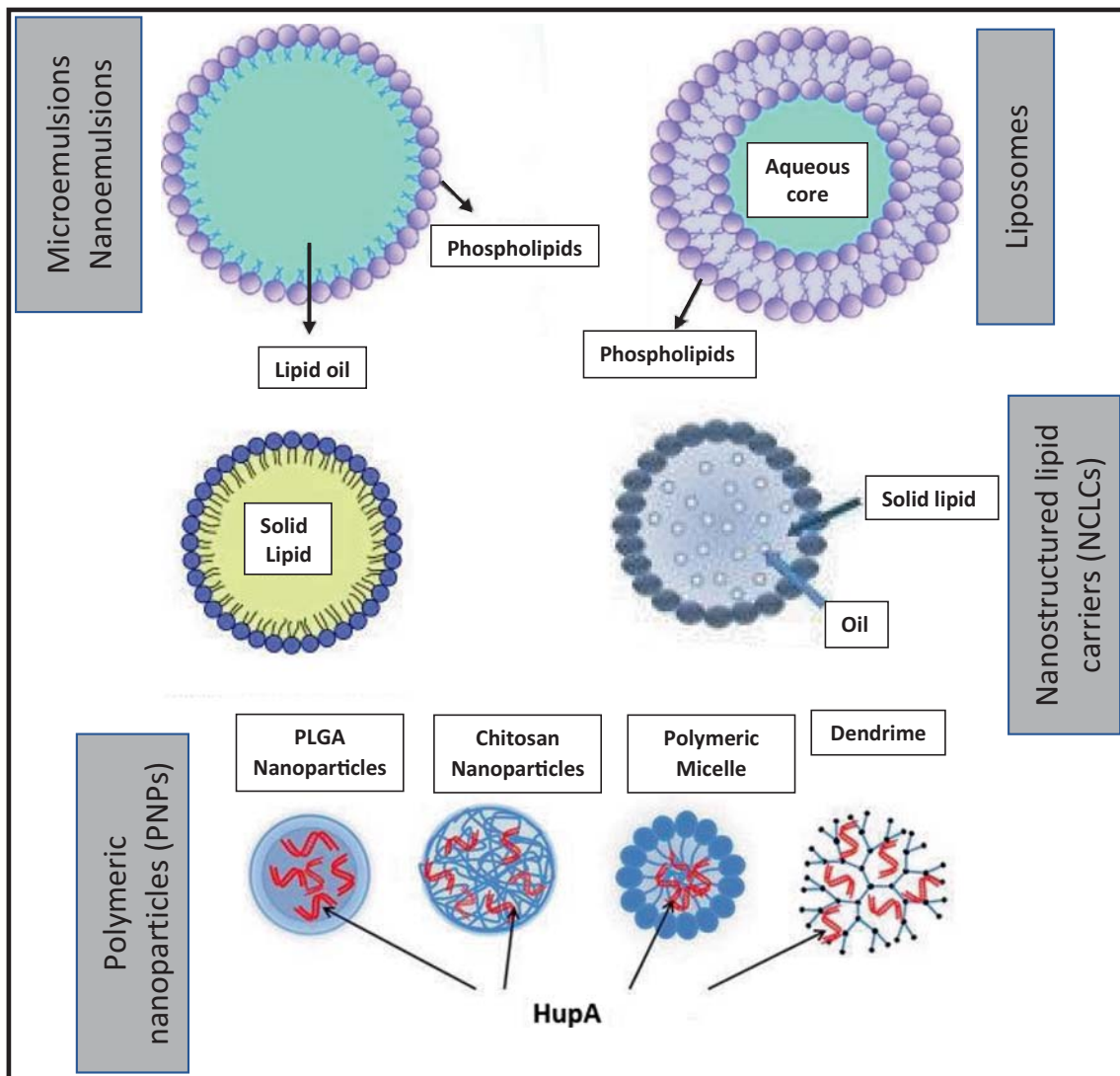Also, a mobile phase composed of ammonium acetate (80 mM, or 0.1 M, pH 6.0): methanol (7: 3 or 6: 4 or 36:

Fig. 3 Different nano-material-based delivery systems for HupA drug as a potential treatment of AD

64 v/v) was also tested (Zhao et al. 2013; Dong et al. 2014; Su and Yang 2015). In addition, Han et al. (2015) applied a mobile phase of methanol: 0.1% formic acid (75: 25 v/v). Different mobile phases of acetonitrile: water (acidified with 0.0125% trifluoro-acetic acid) (15: 85 v/v) were also investigated (Cruz-Miranda et al. 2019).

Confirmatory analyses combining both liquid chromatography (LC) and mass analysis by mass spectrometry (MS) using LC/MS and UPLC/MS analyses were also performed, where the alkaloid extract underwent chromatographic separation using a suitable run solvent as methanoic acid (0.1%): acetonitrile (9: 1 v/v) at a flow rate of 1.0 mL/min (Su and Yang 2015), 10 mM ammonium acetate (pH 3.5) and methanol with 5: 56: 56% gradient elution (T: 0: 20: 30 min) (Shu et al. 2014), methanol: 0.2% aqueous formic acid (50: 50 v/v), flow rate of 0.15 mL/min, 308 nm as a detecting wavelength,

and 5 μL injected volume (Zhang et al. 2015a), and acetonitrile: 0.025% formic acid 15: 85 v/v, 0.25 mL/ min as a flow rate (Cruz-Miranda et al. 2019), then the elute of the chromatography was subjected to MS analysis.

Improvement strategies for enhancing the production of microbial AChE inhibitors

Till now, there are no commercial products of AChE inhibitors depending on the microbial fermentation processes. Hence, enhancement of microbial strains for large-scale production through process optimization and irradiation-assisted strain improvement will lead to a cost-effective fermentation.

In any microbial fermentation process, optimization of process variables such as medium designing is considered to be one of the most important and critical scopes. The constituents
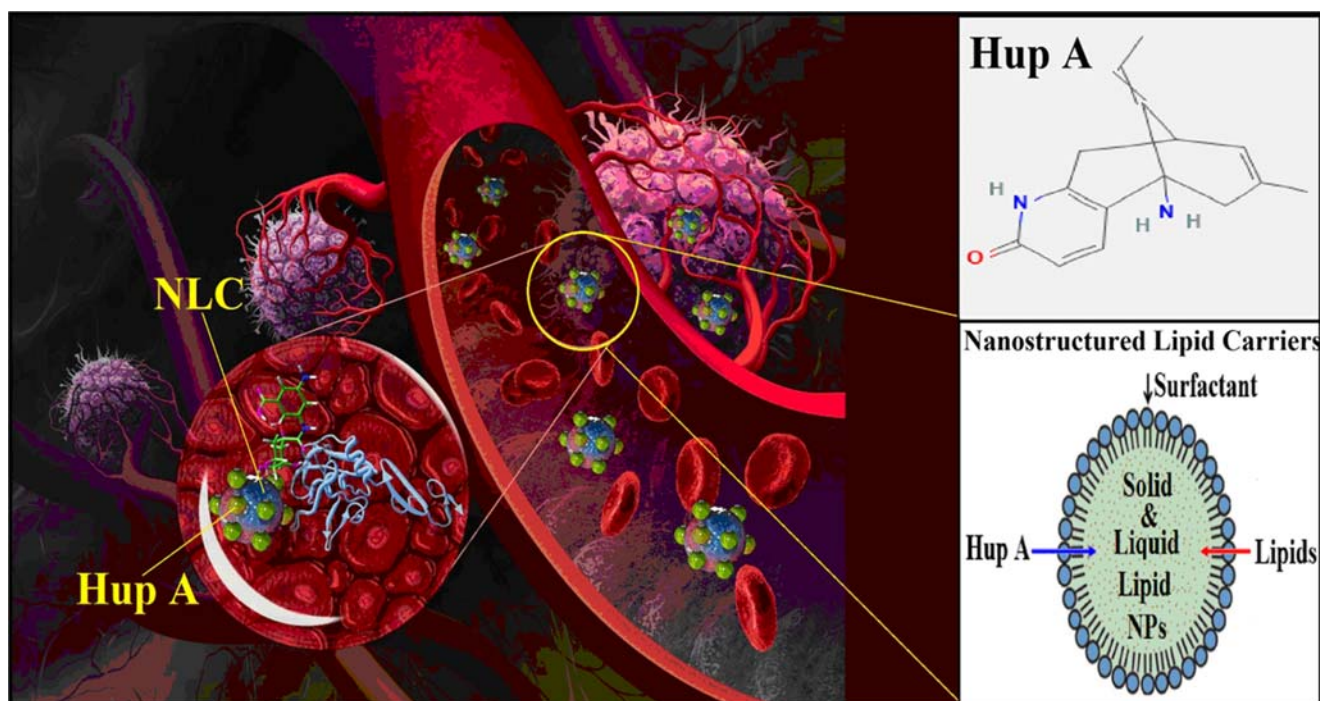
Fig. 4 HupA-loaded nano-structured lipid carriers for a potential drug delivery system for nose-to-brain delivery

of fermentation medium have a significant impact on microbial growth and productivity. Additionally, for the production of valuable compounds, medium components such as nitrogen, carbon, and phosphate sources have a large impact on the cost of overall process and the feasibility of product separation (Singh et al. 2017).

Moreover, chemical elicitors that enhance the production process can greatly affect the process cost. The ideal method for designing the optimum fermentation medium could be started by studying the optimum physical fermentation conditions and may end with optimizing medium components. There are few reports explaining the optimization of medium components and fermentation condition for maximizing HupA yield from endophytes (Li et al. 2007; Zhao et al. 2013; Fangfang et al. 2015).

Currently, multi-factorial statistical designs including response surface methodology could be used for the optimization of fermentation medium components. Recently, Zaki et al. (2019) used both Plackett–Burman and central composite models for optimizing the fermentation conditions and medium composition resulting in 40.8% enhancement of HupA production by the endophytic fungus *Alternaria brassicae* AGF041.

Related studies that employed factorial designs for optimizing the production of valuable microbial secondary metabolites were also reported. Abdel-Fattah et al. (2007) used this strategy for determining the optimum fermentation conditions required for maximum fungal cyclosporin A production.

Additionally, Xu et al. (2006), Garyali et al. (2014), and El-Sayed et al. (2019d) applied response surface methodology for maximizing the production of the anticancer drug taxol by endophytic fungi.

Strain improvement through irradiation mutagenesis for hyper-production of industrial products is of critical importance as the improved mutants can show more desirable properties, and give relatively higher productivity with lower cost (Parekh et al. 2000).

Subjecting the microbial cells to radiation leads to physiological, chemical, and metabolic differences as radiation imposes an extra stress to cells disturbing their organization (Unluturk 2017). Microbial strain mutation can be achieved by exposing the microbial cells to chemical or physical agents (such as UV. and gamma-rays) which is called mutagens. As a result, the genetic materials of the subjected cells would be affected resulting in a strain modification. UV. radiation is a non-ionizing radiation of a moderate effect, and considered as a first choice mutagenic agent (Irum and Anjum 2012).

Irradiation of microbial cell by UV. rays results in pyrimidine dimerization and DNA cross linking (Parekh et al. 2000), while irradiation by the highly energetic ionizing radiation such as gamma-rays causes mutations through DNA breakage in the single or double strands, nucleotide deletion, or structural modification and cross linking in the DNA-protein (Cadet et al. 1999).

Xiao-qion et al. (2015) applied UV irradiation mutagenesis of protoplast of HupA-producing endophytic fungal strain

Table 6 Comparative and survey studies regarding the incorporation of HupA with different nanostructure compounds

Source of HupA	Nanostructure compounds	Methods of incorporation	Significance	References
Provided by Ningbo Traditional Chinese Pharmaceutical Co., Ltd. (China).	Nano-structured Lipid Carriers (NLC)	Melt ultrasonication followed by high pressure homogenization method.	In vitro release studies showed a burst release at the initial stage followed by a prolonged release of HupA from NLC up to 96 h. The results suggested that the presented HupA-loaded NLC system is a potential delivery system for improving drug loading capacity and controlled drug release.	Yang et al. 2010
Supplied by WEPON (Zhejiang, People's Republic of China).	Lactoferrin (LF)-conjugated N-trimethylated chitosan (TMC) surface-modified polylactide-co-glycoside (PLGA) nano-particles (NPs).	Emulsion-solvent evaporation method.	HupALf-TMC NPs have good sustained-release effect, adhesion and targeting ability, and have a broad application prospect as a nasal drug delivery carrier.	Meng et al. 2018
Purchased from Huangpu Chemical Co. (Guangzhou, China)	Aprotinin-conjugated poly (ethyleneglycol)-poly (L-lactic-co-glycolic acid) nanoparticles (Apr-NPs) and huperzine A (HupA) loaded nanoparticles	Emulsion-solvent evaporation method.	Co-incubation with borneol could increase the uptake of nanoparticles by brain capillary endothelial cells (BCECs). Nanoparticles delivered into the rat brain were significantly enhanced by the co-administration of borneol. The pharmacological effects of HupA-loaded nanoparticles on improving the memory impairment of Alzheimer's disease (AD) in rats were greatly improved when combined with borneol.	Zhang et al. 2013
Provided by Traditional Pharmaceutical Co., Ltd.	HupA-loaded poly (lactide-co-glycolide) nanoparticles (HupA-PLGA-NP).	Emulsion-solvent evaporation method.	In vitro drug release studies showed that HupA-PLGA-Ns had a sustained-release behavior in phosphate buffer solution. The accumulated amount of HupA was about 72.1% at 48 h with a low burst release within 30 min. The LD_{50} values of HupA and HupA-PLGA-NP were 1.40 and 4.85 mg/kg respectively, showing that the toxicity of HupA was reduced by 3.5 times.	Zhang et al. 2015c

NX9 resulting in 16.7 times production increase. In literature, UV. and gamma-rays were employed for improvement of fungal strains and overproduction of different pharmaceutical products such as mycophenolic acid by irradiated *Penicillium roqueforti* AG101 and LG109 strains (Ismaiel et al. 2014, 2015; El-Sayed et al. 2019c), paclitaxel by UV-irradiated *Fusarium mairie* (Xu et al. 2006), taxol by UV. and gamma-irradiated *Aspergillus fumigates* and *Alternaria tenuissima* (El-Sayed et al. 2019d), and the cardiac glycoside digoxin by gamma-irradiated *Epicoccum nigrum* (El-Sayed et al. 2019a).

Nanotechnology and AChE-inhibitor HupA drug delivery

Various nano-materials possess exceptional physical, chemical, and physiological characteristics allowing them to be perfect candidates for many applications (Maksoud et al. 2018, 2019; Pal et al. 2018; Thirugnanasambandan et al. 2018;

Asiya et al. 2020; Elkodous et al. 2019c; Govindasamy et al. 2019; Pal et al. 2019; Jeevanandam et al. 2020). Biomedical applications of nano-materials have gained significant consideration from numerous researchers across the past decade because of the important role they can perform in improving the public health (Elkodous et al. 2019a, b, d, e; El-Batal et al. 2019; Wong et al. 2019; Abdelhakim et al. 2020; Elkhenany et al. 2020; El-Sayed et al. 2020a, b).

Nanotechnology advances through nano-material-based drug delivery systems have opened the way for better treatment of AD (Fonseca-Santos et al. 2015; Ansari et al. 2017; Wen et al. 2017). Designed nano-carriers can effectively cross the blood–brain barrier (Costantino et al. 2005; Mahajan et al. 2010; Guarnieri et al. 2014; Gao 2016). Thus, concentration of drugs in the target neural cells will be significantly increased with respect to the traditional methods of treatment without nano-carriers (Wen et al. 2017). This superior activity of nano-carriers can be attributed to their tiny size and their ability to be bounded with specific moieties for better

selectivity and permeability (Peer et al. 2007;Arruebo et al. 2009;Tiwari et al. 2011).

HupA can be delivered effectively to the brain through many nano-carriers such as polymeric nanoparticles (PNPs), lipid nanoparticles (LNPs), liposomes, nano-emulsions, and micro-emulsions as presented in Fig. 3.

Polymeric nanoparticles

Polymeric nanoparticles (PNPs) possess a lot of attractive characteristics allowing them to be well-suited for the delivery of HupA such as their biocompatibility, good stability, lower toxicity, controllable drug release, ease in production, and their lower immunogenic responses (Khalil and Mainardes 2009).

PNPs are colloidal NPs with higher surface area, and drugs can be linked to them through physical adsorption or by chemical bonds through surface modifications after grafting with other polymers such as polyethylene glycol (PEG) and poloxamers (Cismaru and Popa 2010; Elsabahy and Wooley 2012). However, the rapid clearance of PNPs from blood circulation as a result of their interaction with reticulo-endothelial system is a challenging issue that needs further investigation (Reis et al. 2006; Jawahar and Meyyanathan 2012).

Lipid nanoparticles

Unlike PNPs, LNPs are dispersions possessing the same advantages of PNPs with relatively smaller size and better control of drug release, targeting, and higher stability against drug degradation (Patel et al. 2013). LNPs comprise two main types: solid-lipid NPs (SLNPs) and nano-structured lipid carriers (NLCs) (Wissing et al. 2004; Naseri et al. 2015). The main difference between the two types is that SLNPs have lipid core which stay solid at body temperature while NLCs contain a mixture of solid and liquid lipids (Gastaldi et al. 2014).

SLNPs were developed in 1991 providing a promising drug delivery potential with respect to other colloidal vectors like PNPs, liposomes, and emulsions; they were particularly designed for the transfer of a lipophilic composite (Freitas and Müller 1999). The principal advantages of SLNPs are their higher selectivity, biocompatibility, absence of organic solvents during preparation, and improved drug release. However, they exhibited some restrictions such as fixed drug packing space and drug removal capacity throughout the storage process (poor drug loading efficiency) (Mukherjee et al. 2009). To overcome limitations of SLNPs, NLCs were developed and received considerable investigations (Müller et al. 2002). NLCs were extensively employed for HupA delivery due to their unique solid-liquid lipid matrix as displayed in Fig. 4.

NLCs are generated by mixing solid lipids with spatially inconsistent liquid lipids under controlled conditions which

are directed to nanostructures with increased drug conjugation and discharge features (Souto et al. 2004). HupA-loaded NLCs were effectively synthesized by a tailored method of melt ultrasonication followed by high pressure homogenization (Yang et al. 2010).

The DSC results revealed that HupA matrix that overloaded with NLCs possessed poor crystal arrangement. As a result, a lot of imperfections can be generated leading to gaps hindering drug loading. In vitro study showed an early rupture followed by an extended release (Yang et al. 2010). In addition, Patel et al. (2013) performed in vitro and in vivo comparative study to test the delivery of HupA; they used micro-emulsion template method to prepare SLNPs, NLCs, and micro-emulsions for the treatment of AD. Their results showed the significant ability of micro-emulsions followed by SLNPs and then NLCs to deliver HupA after 24 h of treatment. Moreover, mice groups treated with these HupA-loaded nano-carriers showed significant developments in cognitive abilities with respect to the control group.

Recently, a complex of HupA-loaded muco-adhesive and polylactide-co-glycoside (PLGA-NPs) with external adjustment by lactoferrin-conjugated N-trimethylated chitosan (Lf-TMC NPs) was generated for effective intranasal distribution of HupA to the brain for potential Alzheimer's disorder therapy (Sheng et al. 2015; Meng et al. 2018).

Unfortunately, NLCs have some limitations as the drug loading into them is controlled by many parameters such as lipid type, lipid character, production method, and surfactants used in production (Qian et al. 2013; Kathe et al. 2014; Lauterbach and Müller-Goymann 2015).

Liposomes

Liposomes are 50–100 μm spherical vesicles, composed of phospholipid-bilayer enclosing inner core (Wright and Huang 1989; Schroeder et al. 2009). They are highly biocompatible and non-toxic (He et al. 2019). In addition, they have the ability to encapsulate both hydrophilic and hydrophobic drugs and they exhibit superior protection ability of encapsulated drugs against enzymatic degradation (Bruch et al. 2019;Mirab et al. 2019). However, they showed phospholipid-type dependent stability; the charge of phospholipids affects their stability (Wen et al. 2017).

Nano-emulsion and micro-emulsions

Oil-in-water nano-emulsions are cloudy and surfactant-stabilized heterogeneous drug delivery systems; they are composed of oil droplets (10–100 nm) dispersed in water or other aqueous solutions (Hort et al. 2019; Karami et al. 2019). On the other hand, nano-emulsions can be

effectively used to encapsulate HupA with high protection against degradation and can be produced at the large scale (Jiang et al. 2019). Jiang et al. (2019) reported a new nano-emulsion-based system loaded with Lactoferrin for enhanced targeted drug delivery via intranasal administration. Their results showed the successful targeting of hCMEC/D3 cells, and their system is non-toxic and was highly stable up to 6 months (Jiang et al. 2019).

However, nano-emulsions have some limitations such as instability phase separation and non-controlled release (Basha et al. 2019). On the other hand, micro-emulsions are clear and transparent systems composed of water, oil, and amphiphile which is a stable liquid solution (Poh et al. 2019). The advantages of micro-emulsions over nano-emulsions are their thermodynamic stability and cost-effectiveness as they do not need too much energy (Kumar and Pravallika 2019). But, micro-emulsions need large amounts of emulsifiers (Doering 2019).

All and above, the chemical structure of HupA (as a secondary metabolite of microbial synthesis), with its important functional groups like C=O and N–H (Jiang et al. 2003), both O and N contain lone pair of electrons suggesting its capability for metal ions reduction, subsequent fabrication of different NPs that would be incorporated and/or conjugated with the reducing agent, and as a stabilizing agent that could prevent the precipitation and aggregation of metal NPs (Mosallam et al. 2018; El-Sayyad et al. 2019). Finally, some comparative and survey studies reporting the incorporation of HupA with different nanostructured compounds are listed in Table 6.

Conclusion and future approaching

The wide applications of the AChE inhibitors especially in AD treatment as there is a continuous increase in the number of AD patients, and the feasibility of AChE inhibitors production by microbial fermentation approach make it necessary to do excessive research efforts to discover more novel microbial AChE inhibitors. In addition, more microbial strain enhancement protocols are needed to be applied. As a recommendation, besides the conventional submerged fermentation (SMF), solid-state fermentation (SSF) may be a promising alternative for the production of AChE inhibitors. Over the years, SSF proved to be a promising system for the production of many valuable products. SSF has many advantages over the conventional SMF including resistance of microorganisms to catabolic repression, low-cost production process, production of metabolites at higher ,yields and the potentiality of applying agro-industrial wastes as nutritional rich substrates (El-Sayed et al. 2019c, 2020c).

Additionally, the technique of microbial immobilization would be very potential and promising for enhancing the microbial manufacturing of AChE inhibitors, and achieving a continual or a semi continual manufacturing process. This technique is effective for providing a time-consuming production process, and successive fermentation cycles with product extracting and purifying feasibility (Ismaiel et al. 2015; El-Sayed et al. 2019b, 2020d). After the prospective achieving of these steps, it could be possible to transfer the microbial production of AChE inhibitors from an improved lab scale to the promising large-scale industrial production.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This review does not contain any deal with humans or animals.

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