DEEP EUTECTIC SOLVENT–MICROWAVE-ASSISTED EXTRACTION OF TUBIFEX TUBIFEX PROTEIN FOLLOWED BY PRODUCTION AND CHARACTERIZATION OF DIPEPTIDYL PEPTIDASE-IV AND ANGIOTENSIN-CONVERTING ENZYME INHIBITORY PEPTIDES

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by

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LIST OF SYMBOLS

%	percentage
β	beta
α	alpha
°C	degree celsius
P	density
η	viscosity
ε′	solvent's dielectric constant
tan δ	loss tangent
mmHg	unit of pressure
μ	micro
<	less than
=	equals
>	more than
≥	more than or equal to
Å	angstrom
LogP	octanol-water partition coefficient
G'	storage modulus
G''	loss modulus
η0x	zero-shear rate viscosity
η∞	infinite shear rate viscosity
a	relaxation time
γ̇	shear rate
p	exponent
°	angle

g	relative centrifugal force
+1	high level
0	central point
-1	low level
P	protein concentration of the precipitate under various pH levels
T	total protein concentration of the sample
N	equivalents of solute per litre of solution
cm^{-1}	wavenumber

LIST OF ABBREVIATIONS

AA	Amino acids
AABA	2-aminobutyric acid
Ac-SDKP	N-acetyl-seryl-aspartyl-lysyl-proline
ACE	Angiotensin-converting enzyme
$A_{Control}$	the absorbance of system containing water, DPP-IV and Gp-pNA
$A_{control}$	the absorbance of the SWP
$A_{Control}$	the absorbance of system containing water ACE and HHL
Ala(A)	Alanine
Arg (R)	Arginine
A_{sample}	the absorbance of the SWPH
A_{Sample}	the absorbance of system containing SWPH, ACE and HHL
A_{Sample}	the absorbance of system containing SWPH, DPP-IV and Gp-pNA
Asn (N)	Asparagine
Asp (D)	Aspartic acid
ATP	aqueous two-phase system
ATR	attenuated total reflectance
b	Y-intercept
BBD	Box–Behnken design
BF_4^-	Tetrafluoroborate
BK	Bradykinin
BP	bioactive peptide
Br^-	Bromide ion
BSA	Bovine Serum Albumin
C	Carbon atom
C-H	single bond between Carbon and Hydrogen
C-O	single bond between Carbon and Oxygen
C-O-C	two Carbon atoms that are each bonded to an Oxygen atom
C=O	Carbonyl Oxygen

C18	18-carbon-long hydrocarbon chain
cACE	C-terminal of ACE
CAS	Chemical Abstracts Service
CAT	Catalog Number
CH2	Methylene group
CH3	Methyl group
ChAc	Choline acetate
ChCl	Chlorine Chloride
Cl^-	Chloride Ion
cm	centimetre
Cys (C)	Cysteine
Da	dalton
DES	Deep Eutectic Solvent
DH	Degree of hydrolysis
DHA	Docosahexaenoic acid
DMSO	dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase IV
e.g.	example
E/S	enzyme-to-protein ratio
EA	Emulsifying activity
EAA	Essential amino acids
EAI	Emulsifying activity index
EC	Enzyme Commission number
Eco	ecology
EG	Ethylene Glycol
ES	Emulsion stability
ESI	Emulsion stability index
ESI	electrospray ionisation
F^-	fluoride ion
FC	Foaming capacity
FS	Foaming stability
FTIR	Fourier Transform Infrared Spectroscopy
g	Change in mass of reactant

g	gram
$g \cdot cm^{-3}$	grams per cubic centimetre
g/mL	grams per millilitre
g/mol	grams per mole
g/s	Rate of reaction
gHz	gigahertz
GI	Gastrointestinal tract
GIP	Gastric inhibitory polypeptide
Gln (Q)	Glutamine
GLP-1	Glucagon-like peptide-1
Glu (E)	Glutamic acid
Gly	Glycerol
Gly (G)	Glycine
GMO	Genetically Modified Organism
Gp-pNA	Gly-Pro-*p*-nitroanilide
h	hour
H	Hydrogen
HA	Hippuric acid
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HCl	Hydrochloric acid
HEK	Human Embryonic Kidney
HHL	Hippuric-histidyl-leucine
His (H)	Histidine
HPLC	High-Performance Liquid Chromatography
HPO_4^{2-}	Hydrogen Phosphate anion
HSO_4^-	Hydrogen Sulfate
IC_{50}	half-maximal inhibitory concentration
Ile (I)	Isoleucine
IR	Infrared
$J \cdot mol^{-1}$	Joules per mole
K	Kelvin
K^+	Potassium ion

K_2CO_3	Potassium Carbonate
K_2HPO_4	Dipotassium Hydrogen Phosphate
K_a^+ and K_b^+	positively charged Potassium ions
Kcal	kilocalorie
kcal/mol	kilocalories per mole
kDa	kilodalton
kHz	kilohertz
kV	kilovolt
kWh	consumption
K_m	the Michaelis–Menten constant
L	liter
La	Lactic acid
LC-MS	Liquid chromatography coupled with mass spectrometry
Leu (L)	Leucine
LGC	Least gelling capacity
LMW	low molecular weight
LVR	linear viscoelastic range
Lys (K)	Lysine
M	molar
m	the slope of the line
M	mass of the initial weight sample
m/z	mass-to-charge ratio
Ma	Malic acid
MAE	Microwave-assisted extraction
Met (M)	Methionine
mg	milligram
mg/g	the amount of milligrams of a substance per gram of a sample
mg/mL	milligram per millilitre
min	minutes
mL	milliliter
mm	millimetre
mM	millimolar

MP	Myofibrillar Protein
mPa·s	millipascal-second
MS/MS	Tandem Mass Spectrometry
mU	milliunit
MW-DES	Microwave-synthesis DES
N-H	Nitrogen-Hydrogen bond
nACE	*N*-terminal of ACE
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	nanogram
nM	nano molar
nm	nanometer
NO_3^-	Nitrate Ion
O	Oxygen atom
O_a^- and O_b^-	negatively charged Oxygen
OH	Hydroxyl group
OH_1, OH_2 and OH_3	Hydroxyl functional groups
OHC	Oil holding capacity
OVAT	one variable at a time
P	*probability value*
P	Phosphorus
P-O	single bond between Phosphorus and Oxygen
P=O	Phosphorus-Oxygen double bond
P1	1st position on peptide sequence
PD	Propanediol
PEF	Pulsed Electric Field
Phe (F)	Phenylalanine
pI	Isoelectric point
PLE	Pressurized Liquid Extraction
Pro (P)	Proline
Psig	pounds per square inch gauge
PTFE	Polytetrafluoroethylene

Py	Pyrogallol
Q-TOF	Quadrupole Time-of-Flight
r	Pearson correlation coefficient
R^2	R-squared
R_{30}	the emulsified layer volume that heated at 80 °C for 30 min
Ri	the initial emulsified layer volume
Rpm	revolutions per minute
RSM	Response surface methodology
[S]	substrate concentration
s	second
S1	active site
S1’	S1 prime, adjacent to S1 pocket
S2	second binding pocket
S2’	S2 prime, adjacent to S2 pocket
SBP	Systolic Blood Pressure
SD	standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
Ser (S)	Serine
SFE	Supercritical Fluid Extraction
SHR	Spontaneous Hypertensive Rats
SP	Sarcoplasmic proteins
Sp.	species
SWP	sludge worm protein
SWPH	sludge worm protein hydrolysate
T2DM	Type 2 diabetes mellitus
Ta	Tartaric acid
TBAB	Tetrabutylammonium bromide
TBAC	Tetrabutylammonium chloride
TBAHS	Tetrabutylammonium hydrogen sulfate;
TBAX	Tetra-n-butylammonium salt
TEAB	Tetraethylammonium bromide

TEAB	Tetraethylammonium bromide
TEAC	Tetraethylammonium chloride
TEG	Triethylene glycol
Thr (T)	Threonine
Tm	melting point
TPAB	Tetrapropylammonium bromide
TPAC	Tetrapropylammonium chloride
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
UAE	Ultrasound-Assisted Extraction
UF	Ultrafiltration
µg/mL	microgram per millilitre
µL	microliter
µm	micrometer
µM	micromolar
µmol L^{-1}	micromoles per liter
µU/mL	micro-units per millilitre
UV	Ultraviolet
V	reaction velocity
V	volume
V	voltage
V	the initial volume of the sample before homogenization
V/Da	volts per dalton
v/v	volume by volume
V_0	the volume of the sample after homogenization
Val (V)	Valine
Ve	the emulsified layer volume
V_{max}	the maximum initial velocity
V_t	the volume of the sample after leave for specific time intervals
Vt	the total suspension volume
W	watt
w/v	weight by volume

w/w	weight by weight
W_0	the weight of the sample (g)
W_1	the weight of the sample and tube (g)
W_2	the weight of the pellet and tube (g)
W_A	the amount of selected amino acid (mg)
WHC	Water holding capacity
W_{TA}	the total amount of amino acid (mg)
X_1	microwave power
X_2	microwave exposure time
X_3	solvent-to-solid ratio
Y	protein yield
Zn^{2+}	Zinc (II) ion
3D	three dimensional

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PENGEKSTRAKAN PROTEIN *TUBIFEX TUBIFEX* DENGAN BANTUAN GELOMBANG MIKRO-PELARUT EUTETIK DALAM, DIIKUTI DENGAN PENGHASILAN DAN PENCIRIAN PEPTIDA PERENCAT DIPEPTIDIL PEPTIDASE-IV DAN ENZIM PENUKAR ANGIOTENSIN

ABSTRAK

Tubifex sp., sejenis oligochaete yang kaya protein, masih kurang diterokai dalam aplikasi yang mempromosikan kesihatan disebabkan tumpuan yang terhad terhadap strategi pengekstrakan protein yang cekap dan mampan. Terutamanya, penggunaan pelarut eutektik dalam (DES) beralkali bagi pengekstrakan protein daripada organisma ini belum diterokai secara menyeluruh. Untuk menangani jurang ini, sebuah DES beralkali baharu yang terdiri daripada gliserol (HBD) dan kalium hidrogen fosfat (HBA) telah disintesis menggunakan kaedah bantuan gelombang mikro. Microwave-sintesis DES (MW-DES) membentuk campuran eutektik dalam tempoh 3 minit pada nisbah molar 5:1 (HBD:HBA) dengan kuasa 270 W. FTIR dan docking molekul mengesahkan kewujudan interaksi ikatan hidrogen yang kukuh. Analisis fizikokimia menunjukkan sifat hidrofilik, pH beralkali (8.71–8.80), ketumpatan 1.41 g/cm³, kelikatan 229.9 mPa·s, dan suhu penurunan 143 °C. Proses sintesis ini mesra alam (skor EcoScale: 94.6), dengan hasil DES tinggi (97.2%) dan kadar tindak balas pantas (8.94×10^{-4} g/s), serta pemulihan protein cacing lumpur (SWP) yang kukuh (~33%). Walaupun teknik pengekstrakan bantuan gelombang mikro (MAE) sudah terbukti berkesan dalam sistem lain, penggunaannya dengan DES untuk Tubifex sp. adalah inovatif. Pengoptimuman menggunakan Kaedah Permukaan Tindak Balas (RSM) berjaya mencapai hasil protein maksimum SWP sebanyak

39.89% dalam keadaan optimum: kuasa 90 W, nisbah pelarut-ke-pepejal 50:1, dan masa ekstraksi 2 minit. SWP yang diekstrak kaya dengan protein myofibril β-sheet, mengandungi 45% asid amino perlu (w/w) dengan nisbah hidrofilik kepada hidrofobik 1.49, serta menunjukkan kapasiti buih cemerlang (81.43%) dan kestabilan tinggi (77.17%), menjadikannya berpotensi bagi aplikasi fungsi. Untuk menerokai potensi terapeutik peptida bioaktif (BP), SWP telah dihidrolisis secara enzimatik menggunakan tripsin, kimotripsin, dan pepsin, mencapai >75% hidrolisis dalam masa 3 jam. Penapisan in silico (BIOPEP) dan ujian enzimatik mengenal pasti dua peptida menjanjikan: KCFGMGVF dan EWSPF, dengan skor PeptideRanker tinggi (>0.9), serta profil tidak toksik dan tidak alergik. KCFGMGVF menunjukkan perencatan sederhana terhadap ACE (IC_{50} = 103.8 μM) melalui interaksi campuran pada tapak alosterik, manakala EWSPF memperlihatkan perencatan kuat terhadap DPP-IV (IC_{50} = 11.1 μM) di tapak sekunder. Secara keseluruhannya, kajian ini memperkenalkan kaedah pengekstrakan hijau dan cekap (MAE–DES) untuk *Tubifex* sp., menyerlahkan potensinya sebagai sumber protein bervisi mampan. Ia turut menekankan kepentingan integrasi strategi *in silico* dan *in vitro* dalam penemuan peptida bioaktif baharu. Penemuan ini membuka jalan ke arah penyertaan protein alternatif dalam sistem makanan dan kesihatan utama, menawarkan pendekatan mampan untuk menghadapi kekurangan protein global serta menghasilkan peptida dengan manfaat anti-hipertensi dan antidiabetes bagi pelbagai populasi.

DEEP EUTECTIC SOLVENT-MICROWAVE ASSISTED EXTRACTION OF *TUBIFEX TUBIFEX* PROTEIN FOLLOWED BY PRODUCTION AND CHARACTERIZATION OF DIPEPTIDYL PEPTIDASE-IV AND ANGIOTENSIN-CONVERTING ENZYME INHIBITORY PEPTIDES

ABSTRACT

Tubifex sp., a protein-rich oligochaete, remains largely unexplored in health-promoting applications, with limited focus on efficient and sustainable protein extraction strategies. In particular, the use of alkaline deep eutectic solvents (DES) for protein extraction from this organism has not been thoroughly explored. To address this gap, a novel alkaline DES composed of glycerol (HBD) and dipotassium hydrogen phosphate (HBA) was synthesized using a microwave-assisted method. The microwave-synthesised DES (MW-DES) formed a eutectic mixture within 3 minutes at a 5:1 molar ratio under 270 W microwave power. FTIR and molecular docking confirmed the presence of strong hydrogen bonding interactions. Physicochemical analysis revealed a hydrophilic nature, alkaline pH (8.71–8.80), density of 1.41 g/cm³, viscosity of 229.9 mPa·s, and a dissociation temperature of 143 °C. The synthesis process was environmentally friendly (EcoScale score: 94.6), with a high DES yield (97.2%), fast reaction rate (8.94×10^{-4} g/s), and strong protein recovery (~33%). Although the microwave-assisted extraction (MAE) technique has demonstrated efficiency in other systems, its application with DES for *Tubifex* sp. is novel. Optimization using Response Surface Methodology (RSM) achieved a maximum protein yield of 39.89% under optimal conditions: 90 W power, 50:1 solvent-to-solid ratio, and 2 min of extraction. The extracted sludge worm protein (SWP) contained

abundant β-sheet myofibrillar proteins and 45% w/w essential amino acids, with a hydrophilic-to-hydrophobic ratio of 1.49. It also showed excellent foaming capacity (81.43%) and stability (77.17%), indicating good functional properties. To explore the therapeutic potential of bioactive peptides (BP), SWP was hydrolyzed enzymatically using trypsin, chymotrypsin, and pepsin. More than 75% hydrolysis was achieved within 3 h. *In silico* screening (BIOPEP) and enzymatic assays identified two promising peptides: KCFGMGVF and EWSPF, with high PeptideRanker scores (>0.9), non-toxic and non-allergenic profiles. KCFGMGVF exhibited moderate Angiotensin Converting Enzyme (ACE) inhibition (IC_{50} = 103.8 μM) via mixed-mode interaction with ACE allosteric sites, while EWSPF demonstrated potent Dipeptidyl Peptidase-IV inhibition (IC_{50} = 11.1 μM), interacting at secondary binding sites. This study presents a green and efficient microwave-assisted extraction (MAE) method using deep eutectic solvents (DES) for *Tubifex* sp., confirming its potential as a sustainable protein source. It underscores the importance of integrating *in silico* and *in vitro* strategies to identify novel bioactive peptides. These findings pave the way for incorporating alternative proteins into mainstream food and health systems, offering a sustainable approach to tackle global protein shortages and unlock new bioactive peptides with anti-hypertensive and anti-diabetic benefits for diverse populations.

CHAPTER 1 INTRODUCTION

1.1 Background of study

With growing health awareness, consumer demand for functional food ingredients is increasing. The food industry is actively exploring cost-effective protein sources to accommodate this growing demand. Blue foods, found in both freshwater (e.g. lake, mangrove, river, wetland) and marine ecosystems (e.g. oceans) (Colombo et al., 2023), offering high protein content (Fu et al., 2021) and a relatively lower environmental impact, compared to terrestrial food sources foods, are gaining interest as a source of alternative protein (Bank et al., 2022). They supply valuable nutrients, such as omega-3 fatty acids (e.g. DHA), vitamins and minerals, which are essential for healthcare and development (Costello et al., 2020; Ucak et al., 2021). Among aquatic organisms, *Tubifex tubifex* (*Tubifex* sp.), commonly known as the sludge worm, presents notable potential for its protein functionality. This oligochaete worm (Christer, 2022), commonly found in temperate regions' sludge-fed water channels, (Haque et al., 2020), is known for its high abundance of protein (59.13%) and energy content (57.42 Kcal per 100 grams) (Alam et al., 2021; Chittapun et al., 2013; Mollah et al., 2012). They are resilient to harsh environments (Bouché et al., 2000; Chapman, 2001; Smith, 2001; Zhang et al., 2012) making it suitable as an environmental pollution indicator and a toxicity test model (Lagauzère et al., 2009; Moreno-Ocio et al., 2024; Şimşek et al., 2023). Its high reproductive rate, ease of cultivation (Rodrigues & Alves, 2018), minimalize the use of land, water, and feed inputs, which in turn reduces greenhouse gas emissions compared to the conventional livestock. Economically, their growth on low-cost organic waste makes this species a cost-effective and scalable protein source for supporting sustainable food systems. In line with the United Nations' urge for sustainability, green biotechnology is gaining

traction. However, the conventional extraction solvents used for invertebrates, such as Tris buffer, trichloroacetic acid, as well as distilled water (Cheng et al., 2005; Wang et al., 2010; Yoshii et al., 2018), often pose environmental, efficiency and safety concerns. Deep eutectic solvents (DES), formed by a hydrogen bond donor and acceptor, are emerging as eco-friendly alternatives with tunable physicochemical properties (e.g. pH, polarity, and solubility), biodegradability, low toxicity, and high extraction efficiency (Abbott et al., 2004; Suthar et al., 2023). DES has been successfully used for extracting proteins from various biomasses (e.g. oat protein, soy, sardine by-products, brewer's grain, cod skins, oilseed cakes, wheat germ and other biomass (Lin et al., 2022; Ling & Hadinoto, 2022; Olalere & Gan, 2023).

The traditional extraction method is often time-consuming, solvent-intensive, and low in protein recovery as a consequence of inefficiencies in extraction (Alara et al., 2018; Azwanida, 2015; Chemat et al., 2012; Chin et al., 2013; Cittan et al., 2018). Microwave-assisted extraction (MAE), on the other hand, works by generating rapid and uniform heating, resulting in a reduction of both time and solvent usage (Bedin et al., 2020; Kapoore et al., 2018), thereby minimizing environmental impact (Das et al., 2022). It appears as a sustainable, energy-efficient extraction method for improving protein yield and quality (Jiang et al., 2021; Motlagh et al., 2021; Olalere & Gan, 2023; Phuangjit et al., 2023; Sharma & Zalpouri, 2022; Shi et al., 2024; Wen et al., 2021). Additionally, combining DES with MAE has shown superior performance in protein extraction across multiple studies (Akter et al., 2020; Kimsanto et al., 2025; Liu et al., 2021; Olalere & Gan, 2023; Vo et al., 2024; Zhu et al., 2006; Zin et al., 2025). Furthermore, aquatic protein hydrolysate has been well documented to exert various bioactivities, such as anti-hypertensive (Amado et al., 2013; Darewicz et al., 2014; Theodore & Kristinsson, 2007; Vo et al., 2025; Zheng et al., 2022), anti-diabetic (Ben

Salem et al., 2018; Bui et al., 2023; Harnedy-Rothwell et al., 2021; Islam et al., 2021; Ji et al., 2022), antioxidant (Golpaigani et al., 2023; Shahosseini et al., 2022; Tkaczewska et al., 2020) and anti-microbial activities (Bi et al., 2020; Hasani et al., 2022). These properties are often attributed to unique cyclic or unusual amino acid residues that evolved under extreme aquatic conditions (Cheung et al., 2015; DiCosmo & Polese, 2013; Giordano, 2020; Kouno et al., 2005; Lee et al., 2017; Pangestuti & Kim, 2017; Phyo et al., 2018; Sable et al., 2017).

1.2 Problem statement

The proteomic research on *Tubifex* sp., a protein-rich aquatic invertebrate, is limited despite its promising potential. The bioactive properties of proteins and peptides derived from Tubifex remain largely unexplored due to scarce proteomic and peptidomic data (Macedo et al., 2021) and the lack of sustainable protein extraction methods. Although green solvents such as deep eutectic solvents (DES) have been widely utilized, it remains largely unexplored in this context. The use of alkaline DES formulated from GRAS-certified components, glycerol and dipotassium hydrogen phosphate (K_2HPO_4) has yet to be investigated for protein extraction from *Tubifex* sp., and their physicochemical suitability remains uncharacterized.

Moreover, while microwave-assisted extraction (MAE) has demonstrated improved extraction efficiency in other biomaterial systems, its synergistic application with novel DES for aquatic invertebrate protein extraction has not been systematically studied. There is also a gap in understanding the effects of process parameters, microwave power, extraction time, solvent-to-solid ratio, and DES concentration on influencing the protein extraction outcomes. Furthermore, the structural and functional food properties of proteins derived from *Tubifex* sp. has not been fully characterized.

Limited peptidomic database on *Tubifex* sp. makes the bioactive peptide (BP) discovery challenging. The potential of BP production through *in vitro* simulation digestion has not been studied. Conventional approaches are time-consuming and labor-intensive, highlighting the need for targeted bioinformatics-guided screening and biochemical validation to identify peptides with antihypertensive and antidiabetic potential.

To unlock the potential of *Tubifex* sp. as a sustainable source of functional food and nutraceutical ingredients, it is critical establish a green, robust and targeted approach for protein and peptide discovery.

1.3 Significance of study

This study addresses the niche to explore the unconventional aquatic species, *Tubifex* sp., as a sustainable alternative protein source. Development of novel DES using glycerol and K_2HPO_4 offers a new choice as a safe, sustainable and alkaline alternative solvent for protein extraction. By employing green extraction technologies—DES and MAE, it is proposed that the environmental and efficiency limitations of conventional methods would be overcome, while investigating the effects of operational parameters, which would provide an insight into the designing framework for extracting proteins that is adaptable for other aquatic organisms. By integrating *in silico* and *in vitro* approaches, the study enhances the proteomic and peptidomic database for *Tubifex*. It provides critical insight into the accuracy and limitations of *in silico* predictions when compared to real-world enzyme–peptide interactions. Importantly, the integrated approach could effectively streamline the discovery pipeline for novel bioactive peptides without excessive trial-and-error experiments, which aligns with the goals of sustainable innovation of functional food.

Bioactive peptides from natural sources, in this context, *Tubifex* sp.—a highly resilient aquatic species with essential biological and functional properties for stress adaptation. It is a promising candidate with significant potential for diverse applications in the food and pharmaceutical industries.

1.4 Main objective

To develop and evaluate a green and efficient protein extraction method from *Tubifex* sp. using novel alkaline deep eutectic solvents (DES) and microwave-assisted extraction (MAE), and to identify and characterize potential bioactive peptides through targeted peptidomic analysis.

1.4.1 Specific objectives

The research project is systematically divided into distinct phases, which are phase 1, 2 and 3, each corresponding to specific objectives to ensure focused and efficient progress.

Phase 1

1. To synthesis an alkaline DES composed of glycerol and dipotassium hydrogen phosphate (K_2HPO_4) for protein extraction from *Tubifex* sp. and characterize DES based on physicochemical and rheological properties.

2. To evaluate the efficiency of DES extraction and its preparation method.

Phase 2

3. To optimize the key parameter of MAE-DES approach by investigating the effects of microwave power, extraction time, solvent-to-solid ratio, and percentage of DES for maximum protein yield.

4. To evaluate the structural and functional food properties of the extracted protein and amino acid profiling.

Phase 3

5. To screen for the bioactivities of peptides and select the most promising hits of BP generated from enzymatic hydrolysis using digestive proteases (e.g. pepsin, trypsin and chymotrypsin) through bioinformatics and molecular docking.

6. To validate the selected BP with its identified bioactivity (e.g. anti-hypertensive and anti-diabetic) using biochemical assays and mechanistic studies.

CHAPTER 2 LITERATURE REVIEW

2.1 *Tubifex Tubifex*

Tubifex Tubifex (*Tubifex sp. or T. Tubifex*) is an aquatic invertebrate in the Tubificidae family, classified as Oligochaeta under the phylum Annelida (Müller, 1774), also known as sludge worms (Schoch et al., 2020). It is small, reddish in colour, with a maximum length of 2 cm as shown in **Figure 2.1** (Patekar et al., 2022), and inhabits broad freshwater settings such as streams or ditches, usually submerged in sediment layers rich in organic matter, regardless of the organic content and size (Lagauzère, et al., 2009; Şimşek et al., 2023). The full life cycle of *Tubifex* worms occurs entirely within the freshwater sediments, they feed on sediment particles, with sizes smaller than 63 μm, and selectively towards bacteria-rich components (Méndez-Fernández et al., 2013; Rodriguez et al., 2001). They are the food chain's primary consumer, serving as linkers between sediment microbes and higher organisms.

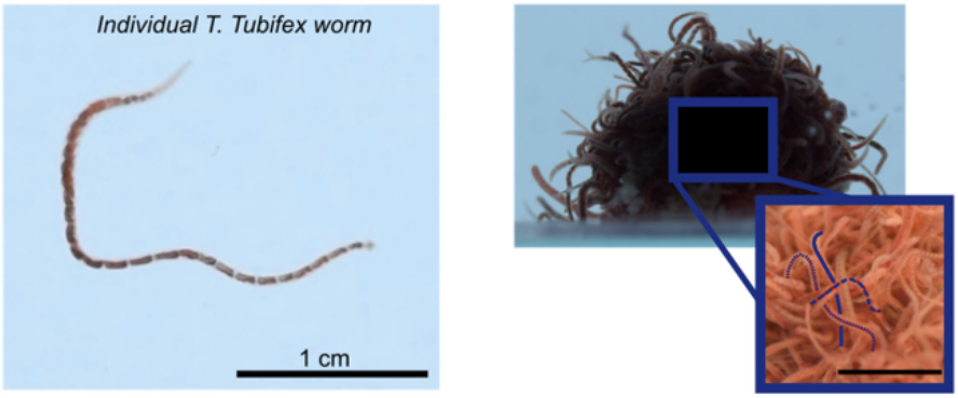

Figure 2.1 Active living *Tubifex* worm (a) A top-down view of a single T. Tubifex worm (b) A cluster of these worms naturally gathers into a large and entangled mass, which is their typical configuration in their natural habitat. The inset's scale bar measures at 2 cm (Deblais et al., 2022).

Tubifex act as natural decomposers in polluted aquatic environments, feeding on and breaking down raw sewage and organic waste. A reduction in their burrowing depth in heavily polluted sediments can be useful bioindicators of sediment quality (Chapman, 2001; Lagauzère, R. Terrail, et al., 2009; Mermillod-Blondin et al., 2005). In addition, they tend to accumulate heavy metals (uranium, cadmium, copper) (Bouché et al., 2000; Lagauzère et al., 2009; Lagauzère & Bonzom, 2009; Mosleh et al., 2006). *Tubifex* is considered the ideal model among other tubificid species for large-scale assessments due to its high availability, i.e. wide distribution and ease of maintenance in laboratory settings (ASTM, 2010). Thus, they can be valuable as ecotoxicological and bioaccumulation test models (Lobo et al., 2016; Lobo et al., 2021; Rodríguez & Reynoldson, 2011).

Furthermore, *Tubifex* worms are rich sources of essential nutrients, with the highest crude protein (11%), which made them a good protein source, followed by fatty acids (7.28 mg/100 mg) with an abundance of omega-3 and omega-6, relatively low 2.14% of lipid and 1.83% ash, carotenoid (15.02 mg/kg), caloric content of 5.57 kcal/g in dry weight and moderate moisture content (18.78%). They are comprised of high essential amino acid compositions of Lysine (K), Leucine (L), Valine (V) and non-essential amino acid Arginine (R), which are prominent in protein synthesis, muscle metabolism, wound healing and overall physiological functions (Mahmut et al., 2003). They have long been widely utilised as cost-effective aquaculture feed due to their rapid reproduction, ease of cultivation, and strong adaptability to diverse and extreme environmental conditions and rich nutrient content, which is essential for fish development (Mandal et al., 2016).

Despite their ecological, toxicological and nutritional significance in environment and aquaculture. Currently, there is very limited proteomics and

peptidomics information available on this aquatic oligochaetes species; most of the available data is about earthworms (e.g. (Ding et al., 2019; Fan et al., 2024; Lin et al., 2023; Wasunan et al., 2022) and other aquatic organisms, such as invertebrates (e.g. (Dong et al., 2024; Guryanova et al., 2023; Jayaprakash & Perera, 2020; Lee et al., 2012; Suárez-Jiménez et al., 2012) or fish species (e.g. (Ketnawa et al., 2018; Zhang & Cheng, 2019; Zhang et al., 2019). This makes it challenging to explore the potential beneficial effects on human physiology. Exploring the peptidomics and bioactivity of this widely distributed *Tubifex* sp. could unlock new value as an eco-friendly and sustainable alternative protein source for the human diet.

2.2 Protein from Aquatic Sources

The blue food is gaining interest as a source of alternative protein. Blue food refers to aquatic species that can be found in both freshwater (e.g. lake, mangrove, river, wetland) and marine ecosystems (e.g. oceans) (Colombo et al., 2023). There are over 2,500 of these species have been documented as part of the human diet (Bennett et al., 2021; Golden et al., 2021), including the commonly consumed freshwater and marine fish, crustaceans (e.g. shrimp and crabs), invertebrates (e.g. octopus and squid), molluscs (e.g. clams, sea snails and cockles) and aquatic plants (e.g. seaweed and water spinach). The benefit of blue food is the vast protein source in aquatic ecosystems, ensuring its availability and accessibility to meet the needs (Kidane & Brækkan, 2021). Additionally, blue foods serve as sustainable protein alternatives (Gephart et al., 2021), offering a lower environmental impact, particularly in terms of carbon output, compared to other animal-based foods (Bank et al., 2022). A review from Venugopal and Shahidi (1996) revealed the variation in protein content across aquatic species. Finfish in general contain an average of 18-22% protein, with cooked tuna reaching as

high as 35%. The crustacean protein ranges between 12% to 22%, whereas molluscs showed an average lower level of protein, ranging from 8% to 16%. Microalgae, *Spirulina Chlorella* and the green algae *Chlorella Vulgaris* have more than 50% crude protein in dry weight (Abdel-Latif et al., 2022), and fishmeal from fishery by-product has an average of 65% crude protein (Cho & Kim, 2010). These aquatic organisms exhibited excellent protein content compared to soybeans (30-40%) (Fu et al., 2021). Aquatic sources outperform terrestrial food sources as they provide an uncommon nutrient supply, such as fish oil (omega-3 fatty acids) (Costello et al., 2020), in particular, the DHA from omega-3 aids in cardiovascular health, autoimmune disease and mental health (Shahidi & Ambigaipalan, 2015). Besides, their high nutritional value was evidenced (Ucak et al., 2021) by numerous nutraceuticals made by macro- and micronutrients such as shark liver oil, chitin, vitamins A, D and E, vitamin B12, zinc, iron and functional enzymes and peptides, which are essential for healthcare and development. Based on literature, different types of protein from aquatic species are summarized in **Table 2.1**.

Table 2.1 ***Protein composition, origin from various aquatic species and their in situ function.***

Protein	Solubility	*In situ* Function	Origin source	Reference
Myofibrillar proteins				
myosin, actin, tropomyosin, troponins, m-protein, c protein, alpha-actinin, and beta-actinin	Water insoluble	Muscle contraction	Fish and shellfish muscle	(Vareltzis, 2000; Venugopal, 2009)
Sarcoplasmic proteins				
Heme proteins: hemoglobin and myoglobin	Water soluble	Oxygen transport and storage	Red sea bream, Pacific mackerel and carp	(Vieira et al., 2018; Yongsawatdigul et al., 2005)
Enzyme for energy metabolism (e.g. creatine kinase, glycogen phosphorylase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase B, phosphoglycerate kinase, phosphoglucomutase, beta-enolase, phosphorylase), protein modification (e.g. proteinase inhibitors, transglutaminase fructose-bisphosphate aldolase A, proteases A and C), lipid and oxidative metabolism (phospholipase and peroxidase) and calcium-binding proteins)		Metabolic and regulatory		
Stroma proteins				
Collagen and elastin	Water soluble	Tissue support, healing, supplements	Jellyfish, octopus, squid, sea urchins, sea anemones and starfish, shark, ray, skate	(Ahmed et al., 2020; Venugopal, 2009)

Gelatin			Skin of blacktip shark, skate, tilapia and Pacific cod Bones of Atlantic mackerel, blue whiting and pangasius catfish.	(Gomez-Guillen et al., 2002; Himaya et al., 2012; Khiari et al., 2013; Kittiphattanabawon et al., 2012; Mahmoodani et al., 2014; Ngo et al., 2014; Tacon & Metian, 2013; Duan, et al., 2012)
Albumin	Water soluble	Metabolism	*Snakehead fish,* common carp and pangas catfish.	(Asikin & Kusumaningrum, 2018; Nurfaidah et al., 2021)
Globulin (e.g. immunoglobulin)	Water soluble	Defense mechanism	Eel, tilapia, Indian major carp, pike, sea bream, Atlantic salmon, perch, sea bass, turbot, grouper; zebrafish, rainbow trout, largemouth bass.	(Choudhury & Pani Prasad, 2011; Danilova et al., 2005; Hansen et al., 2005; Yang et al., 2022)
Glycoproteins				
Lectin	Water soluble	Carbohydrate-binding protein	Brown, green and red seaweed	(Raja, Kadirvel, & Subramaniyan, 2022)
Phycobiliprotein		Chromophore proteins		

The protein quality is also associated with its digestibility and essential amino acid compositions that meet human diet requirements (Food and Agricultural Organization, 2013). It can be used when comparing different protein sources. The digestibility of terrestrial proteins such as meat (80 to 97%) (Faber et al., 2010; Mendes et al., 2016), milk (95%) (Dupont & Tome, 2014); and eggs (90%) (Evenepoel et al., 1998), whereas aquatic organisms such as molluscs, shrimp, and fish were 79%, 94% and 93.7%, respectively (Dayal et al., 2013; Deng et al., 2016; Wang et al., 2019). The digestibility of animal proteins is remarkably higher than that of plant-derived proteins (Berrazaga et al., 2019; Herreman et al., 2020). The animal protein is distinct from plant proteins in terms of size and shape. Its amino acid composition is more aligned with human dietary needs as compared to the plant protein. For example, fish contain the highest amount of lysine. Fish gelatin contains an abundance of lysine and methionine, which are deficient in terrestrial meat proteins (Gomez-Guillen et al., 2002; Tacon & Metian, 2013) and cereal proteins (Sha et al., 2023). Notably, wild fish and aquacultured fish shared similar amino acid compositions, implying some of the fish proteins are highly conserved and stable regardless of habitat and diet, as they are fundamental to fish physiology. However, variations in the molecular and functional properties are still present across different species and biological environments (Zheng et al., 2024). Pork is rich in histidine and threonine, and other meats, eggs and milk have similar amino acid profiles (USDA, 2021). Other amino acid compositions are similar in aquatic animal proteins and terrestrial meat, milk and egg, suggesting the quality of aquatic animal protein is comparable to the terrestrial. Some underexploited wild seafood fish and shellfish may serve as high-quality animal protein sources for highly dense populations with minimal total protein intake (Cropotova et al., 2023). Furthermore, aquatic animals such as freshwater and marine finfish, tilapia, catfish,

milkfish and carp, became reliable and sustainable diets in third-world countries as they are affordable with high nutritional value (FAO, 2020). Besides, aquatic animal protein has proven health benefits. A diet rich in sardine protein showed beneficial effects in rats with metabolic syndrome showed improvement in glucose metabolism by lowering insulin resistance and levels of leptin and TNFa and reducing oxidative stress in adipose tissue (Madani et al., 2012). The marine enzyme, LMW serine-protease derived from Sardinelle, was discovered with collagenolytic activity, which is crucial for wound healing, bone and tissue development process (Hayet et al., 2011). Zhu et al. (2010) revealed that both diabetic and hypertensive patients have improved lipid and glucose metabolism after being treated with Salmon skin protein hydrolysate. This possibly contributed to the histidine, which converts into histamine in the brain to lower the desire for food consumption. These showcase the value of aquatic animals to be further explored as alternative protein sources.

2.2.1 Functional properties of aquatic animal protein

Aside from high nutritional values, the aquatic animal protein also possesses outstanding techno-functional properties that can be used as food ingredients, such as emulsifying capacity, foaming, gelling properties and fat absorption capacity. The summary of functional food properties for different proteins derived from aquatic species. These values assess their potential for use in food formulations (**Table 2.2**). Fish protein hydrolysates possess desirable interfacial surface activity as emulsifiers in margarine and sauce dressing and foaming agents in mousse and whipped cream (Kristinsson, 2007). Gelatin is a hydrolysed protein from collagen. Marine gelatin is sourced from cold fish species, fish skins, squid and octopus. It formed weak gels at low temperatures as it had a relatively lower melting point (Choi & Regenstein, 2000). Its distinct gelling properties compared to terrestrial gelatin can be attributed to its

lower proline and hydroxyproline amino acid compositions. Nevertheless, marine gelatins have excellent performance in emulsifying properties. Silver carp scale gelatins exhibit linear effects of concentration to emulsion stability, with higher emulsion stability observed at a higher concentration (10 mg/mL). Besides, gelatin from the fish swim bladders was used as an industrial clarifying agent in fermented beverages to aggregate yeast and other insoluble particles (Hickman et al., 2000). Gelling agents, such as chitin and chitosan, are commonly present in crustaceans and lactic acid bacteria. Protein from arrow tooth flounder and herring possess better fat adsorption and emulsifying properties than soy protein (Sathivel et al., 2004). Collagen is a structural protein found in skin, cartilage and bone; it is widely utilised as edible casing in processed meat. Fish-derived collagen from tuna and silver-line grunt has a comparatively lower denaturation temperature than porcine collagen (Senaratne et al., 2006) and is beneficial in low-temperature gelation and casing processes.

Table 2.2 Functional properties of proteins include water- and oil-binding capacity, foaming, emulsifying, and gelation properties.

Aquatic Species	Source of protein	Functional food properties							Reference
		WHC	OHC	FC(%)	FS (%)	EAI (m^2/g)	ESI (min)	LGC (%)	
Clam (*Mactra veneriformis)*	MP	-	-	70	58	8	14	-	(Shi et al., 2019)
Oyster (*Magallana gigas*)	MP	-	-	-	-	9	12	-	(Wu et al., 2019)
Mussel (*Mytilus edulis*)	MP	-	-	17.3	29.7	15	4.3	-	(Yu et al., 2018)
Threadfin bream. (*Nemipterus hexodon)*	SP	-	-	277	57	210	197	-	(Krasaechol et al., 2008)
Jumbo squid mantle (*Dosidicus gigas*)	SP	-	-	88	98	83	73	-	(Lopez-Enriquez et al., 2015)
Mussel (*Mytilus edulis)*	SP	-	-	42	33	55	150	-	(Zou et al., 2020)
Roe	protein isolate	3.70–4 g/g	-	142	73.1	10	63	-	(Yoon et al., 2019)
Mixed Fish-By product (skins, heads, and skeletons)	Collagen	-	-	78	60	130	42	-	(Zamorano-Apodaca et al., 2020)
Cod (*Gadus morhua*)	Frame	-	3.16–4.49 g/g	-	-	-	-	-	(Bautista et al., 2023)

Striped catfish (*Pangasianodon hypophthalmus*)	Viscera	0.71–0.89 g/g	1.03–1.39 g/g	-	-	-	-	-	(Steinsholm et al., 2020)
Cobia (*Rachycentron canadum*)	Skin gelatin	97%	164.01%	175-200	75-149	8.53-15.71	80.77	2	(Amiza et al., 2015)
Silver carp (*Hypophthalmichthys molitrix*)	Skin gelatin	-	-	-	-	55-60	25-80	0.8-0.95	(J. Zhang, R. Duan, et al., 2012)
Cuttlefish (*Sepia pharaonis*)	Skin gelatin	-	-	198-200	86-90	18.17-24.30	17.84-26.40	-	(Aewsiri et al., 2009)
Marine snail (*H. trunculus*)	Meat gelatin	120%	-	75.44	61.86	32.77	38.12	-	(Zarai et al., 2012)
Unicorn leatherjacket (*Aluterus monoceros*)	Skin gelatin	-	-	110-152	98-139	13.49-39.17	8.58-39.63	-	(Ahmad & Benjakul, 2011)
Smooth Hound (*mustelus mustelus*)	Skin gelatin	-	-	50-63.33	40-49.83	11.74-32.39	25.2-43.42	-	(Bougatef et al., 2012)

Water holding capacity (WHC); Oil holding capacity (OHC); foaming capacity (FC); foaming stability (FS), emulsifying activity index (EAI); emulsion stability index (ESI); gelation indicator, least gelling capacity (LGC); Myofibrillar Protein (MP); Sarcoplasmic proteins (SP)

Besides, protein from various aquatic sources enhances sensory properties in food products (Khalili Tilami & Sampels, 2018). The formulation of protein hydrolysate of common barb at 50% concentration with other ingredients was successfully formulated into a natural flavour enhancer, evidenced by chemical tests (Witono et al., 2019). Apart from that, they are highly functional in other applications: food processing and medical use. For example, carotene proteins from shellfish can be used as a supplement. The gastric enzyme protease from fish stomachs can substitute rennet to form curd in cheese production (Shahidi & Ambigaipalan, 2015). The alkaline phosphatase found in frozen shrimp waste could help diagnose vitamin D deficiency, bone dysfunction and liver diseases. The addition of Capelin protein (0.5 to 3%) to minced pork effectively reduced 17% to 60% of the thiobarbituric acid reactive substances (TBARS) from secondary oxidation and improved the taste by removing the bitterness (Shahidi & Ambigaipalan, 2015). Similarly, squid protein hydrolysate in the sardine minced reduced TBARS by 42% and showed equivalent efficiency with ascorbic acids (41%), highlighting their strong antioxidant properties as natural preservatives to prolong the shelf life of processed meat and seafood (Sivaraman et al., 2016).

On top of that, it is important to note that the functional properties of proteins are greatly influenced by extraction conditions. Silver carp scale gelatin showed the presence of different structural modifications, i.e. β-chain and β-sheet content in acetic acid-, hot water- and pepsin enzyme extraction methods. Acetic acid-extracted gelatin had the best water-, oil-holding capacity and emulsifying stability. Kang et al. (2019) revealed that the functional properties of green carp are pH-dependent. Under different pH extract conditions, the protein of invasive green crabs exhibited distinct functional properties. Protein extracted at pH 2 enhanced emulsifying properties, whereas pH 10

improved gelling properties. This suggests extraction conditions can be optimised based on the desired food applications. Kang et al. (2019) revealed that the functional properties of invasive green crabs are pH-dependent. Under different pH extract conditions, the protein of invasive green crabs exhibited distinct functional properties. Protein extracted at pH 2 enhanced emulsifying properties, whereas pH 10 improved gelling properties. This suggests extraction conditions can be optimised based on the desired food applications.

2.2.2 Protein hydrolysate of aquatic animals

Protein hydrolysate is the digested product of protein. It usually consists of an abundance of peptides with 3-20 amino acid residues. These peptides can be active after being released from a precursor protein, known as a bioactive peptide (BP), which is associated with specific bioactive properties based on their sequence, amino acid composition, and structure (Kim & Wijesekara, 2010). Protein hydrolysate contains of di and tripeptides, which give a better availability than intact protein as it is more readily absorbed by the body (Di Pasquale, 1997). Aquatic protein hydrolysate has been well documented to exert a wide spectrum of potential bioactivities, such as anti-hypertensive (Amado et al., 2013; Darewicz et al., 2014; Theodore & Kristinsson, 2007; Vo et al., 2025; Zheng et al., 2022), anti-diabetic (Ben Salem et al., 2018; Bui et al., 2023; Harnedy-Rothwell et al., 2021; Islam et al., 2021; Ji et al., 2022), antioxidant (Golpaigani et al., 2023; Shahosseini et al., 2022b; Tkaczewska et al., 2020) and anti-microbial activities (Bi et al., 2020; Hasani et al., 2022; Shahosseini et al., 2022a). Some of the aquatic protein hydrolysate exhibited bioactivities were presented in **Table 2.3**.

Table 2.3 Protein hydrolysate with bioactivities, produced by the various enzymes from various aquatic species.

Aquatic species protein hydrolysate	Enzyme employed for hydrolysate production	Bioactivity	Reference
Hoki skin gelatin (*Johnius belengerii*)	Trypsin	Antioxidant	(Mendis et al., 2005)
Tuna (*Thunnus tonggol*)	Protease XXIII (PR) and orientase (OR)	DPP-IV inhibition	(Huang et al., 2012)
Tilapia skin gelatin (*Hypophthalmichthys molitrix*)	Multifect: Neutral and properase E	Antioxidant	(Zhang et al., 2012)
Sea squirt *(Styela clava)*	Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase, pepsin, tyrpsin and papain)	Anti-inflammatory	(Ko & Jeon, 2015)
Sardine (*Sardina pilchardus*)	Alcalase	ACE inhibition	(Carvalho et al., 2018)
Lizardfish (*Synodus macrops*)	Bromelain Alcalase Chymotrypsin Papain Acidic protease Trypsin	ACE inhibition	(Chen et al., 2018)
Rainbow trout (*Oncorhynchus mykiss*) by-products	Alcalase	ACE and DPP-IV inhibition	(Ketnawa et al., 2018)
Yellowfin tuna (*Thunnus albacores*)	Protamex	Antioxidant and antimicrobial	(Pezeshk et al., 2019)

Grenadier (*Macrourus sp.*), Megrim (*Lepidorhombus boscii*), European hake (*Merluccius merluccius*), Boarfish (*Capros aper*), Atlantic horse mackerel (*Trachurus trachurus*)	Alcalase	ACE inhibition and antioxidant	(Vázquez et al., 2019)
Croaker (*Johnius sp.*)	Protease from Rohu (*Labeo rohita*) Catla (*Catla catla*)	ACE inhibition	(Dara et al., 2020)
Mesopelagic fish (*Maurolicus muelleri*) and Northern Krill (*Meganyctiphanes norvegica*)	Alcalase, endocut-01, endogenous M/K enzymes and FoodPro PNL	ACE and DPP-IV inhibition	(Naik et al., 2021)
Rainbow trout (*Oncorhynchus mykiss*), Sole (*Dover sole*)		Antibacterial	(Wang et al., 2021)
Skipjack tuna (*Katsuwonus pelamis*)	Alcalase	ACE inhibition, antioxidant and antimicrobial	(Naghdi et al., 2023)
Salmon (*Salmo salar*)	non-GMO protease	DPP-IV inhibition	(Bjerknes et al., 2024)
White Jellyfish (*Lobonema smithii*)	Flavourzyme, Alcalase and Papain	Antioxidant and immunomodulatory activity	(Prommasith et al., 2024)
Baltic herring (*Clupea harengus membras*)	Alcalase and Flavourzyme	DPP-IV inhibition	(Wang et al., 2024)

2.3 Green Biotechnology

Extraction is a fundamental step in retrieving valuable compounds (Chemat et al., 2012), i.e. proteins, carbohydrates, lipids, amino acids, polysaccharides, alkaloids, phenolic compounds, etc., from various natural sources, including plants (Stéphane et al., 2022), food by-products (Gullón & Yáñez, 2022), and animals (Ghosh et al., 2022;

Lizárraga-Velázquez et al., 2025). Sustainable extraction techniques are increasingly given importance, following the urge of the United Nations to emphasis a sustainable global future. Guided by the 12 principles of green chemistry and green engineering (Anastas & Warner, 1998) to serve as the basis for environmentally friendly processes. Taken together, a framework for developing green extraction techniques that enhance sustainability while maintaining efficiency was established. The ideal green extraction technique surpasses conventional methods by minimizing the use of toxic solvents and reducing energy consumption. This enhances process efficiency, achieving the ultimate goal of cost-effective, environmentally sustainable production of safe and high-quality extracts or products (Chemat et al., 2012). In this context, our focus is on two key aspects: green solvents and green extraction techniques.

2.3.1 Green alternative solvents

The use of petrochemical solvents and volatile organic compounds (VOCs) (García et al., 2014) has been strictly regulated as these chemicals are typically toxic, volatile, and flammable, which raises health and pollution risks (Chemat et al., 2012; Jessop, 2011; Li et al., 2016). The strict regulations, in turn, raise the overall expenses associated with the chemical storage and disposal procedures (Li et al., 2016). The safety, environmental and economic concerns drive the public, i.e. industry and academic interest, to adopt alternative green solvents (Vovers et al., 2017). The growing research interest over the past decade was demonstrated by the significant increase publication trend from 2016 to 2025 from 72 in 2016 to 1,240 in 2024, and the publications (545) remained high to date (**Figure 2.2**). This reflects the increasing popularity of using DES as an alternative green extraction solvent due to its environmental friendliness, low toxicity, cost effectiveness and tunability.

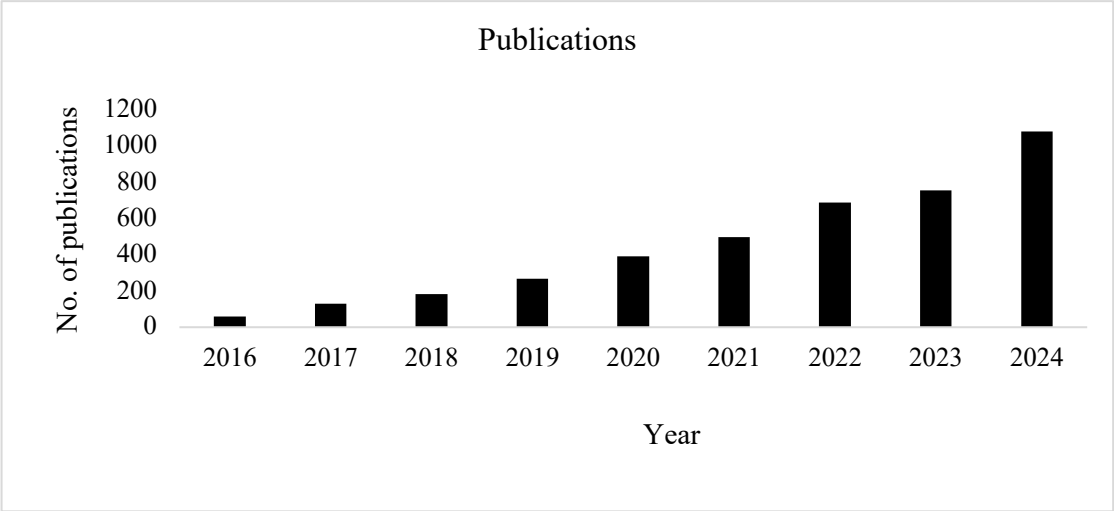

Figure 2.2 Publications trend using DES for extraction in the Scopus database from 2016 to 2024.

GlaxoSmithKline's solvent selection guides allow the assessment and selection of solvents based on environmental impact, safety, and waste (Curzons et al., 1999). Some commonly used bio-solvents, such as glycerol (Haynes & Lyde, 2010), ethanol (Virot et al., 2008), and methyl esters (Clark & Deswarte, 2012), demonstrated major drawbacks of high viscosity and elevated boiling points. Over time, as the guidelines redefined, the renewable nature of bio-based solvents alone does not have promising sustainability. Other green alternative solvents also showed some significant limitations, for example, supercritical fluids required additional use of organic co-solvent (Herrero et al., 2006), costly (Mohamed et al., 2002) and high consumption of energy (Płotka-Wasylka et al., 2017); Subcritical water showed limited extraction power, a challenging recovery process and needed high energy (Płotka-Wasylka et al., 2017); Ionic liquids exhibited moderate toxicity (Nikinmaa, 2014), high-cost (Kudłak et al., 2015) and complicated synthetic processes (Clough et al., 2015; Yang et al., 2011). Among these solvents, deep eutectic solvents (DESs) (Abbott et al., 2004) showed superiority in a few aspects, including minimal toxicity, cost-effectiveness,

high biodegradability, ease of preparation, low energy consumption, and extraction efficiency (Kudłak et al., 2015). DESs' established broad applications, such as extraction, act as carriers in pharmaceuticals, biotransformation, nanomaterial synthesis, and biotransformation fields (Ferreira & Sarraguça, 2024; Ling & Hadinoto, 2022), highlighting their effectiveness and versatility across scientific and industrial fields, being a better alternative to conventional solvents.

2.3.1(a) Deep Eutectic Solvent (DES)

DES is an eutectic mixture, formed by two components: hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) at the specific molar ratio through hydrogen-bonding interactions (Abbott et al., 2003). The eutectic mixture has characteristics of a lower melting point than its components and is in a liquid state at room temperature (Suthar et al., 2023). The lowered melting point is contributed by the hydrogen bonding network formed between HBD and HBA. Besides, the characteristics of DES, including pH, polarity, and solubility, can be tailored according to the nature of HBA and HBD. The capability to customize the solvent for various applications has been favoured by researchers (Kovács et al., 2020).

DES is classified into four types: Type I consists of an organic salt and a metal; Type II comprises an organic salt and a metal salt hydrate; Type III includes an organic salt as HBA and a HBD; and Type IV consists of an HBD combined with aluminium or zinc chloride (Khalid et al., 2024). Type III DES is of particular interest as it fits our research objectives. It has shown remarkable efficiency in the extraction of various bioactive compounds (Gullón et al., 2020), making it a central focus of this review. Type III DES is frequently employed as it is metal-free, easy to prepare with readily available components and is cost-effective (Gurkan et al., 2019; Zhang et al., 2021).