# QUATERNARY AQUEOUS BIPHASIC MIXTURE FOR PROTEIN EXTRACTION FROM PAPAYA

**LOH LI SIN**

**UNIVERSITI SAINS MALAYSIA**

**2022**

# QUATERNARY AQUEOUS BIPHASIC MIXTURE FOR PROTEIN EXTRACTION FROM PAPAYA

**By**

**LOH LI SIN**

**Thesis submitted in partial fulfilment of the requirement for degree of Bachelor of Chemical Engineering**

**July 2022**

## ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest sense of gratitude to my supervisor, Dr. Fadzil Noor Gonawan, for his precious encouragement, guidance, assistance, and suggestions to keep me on the correct path in completing my Final Year Project.

My appreciation and gratitude are also extended to everyone who assisted me directly or indirectly, as well as all of the lecturers, laboratory technicians, and librarians at USM Engineering for their politeness, collaboration, and assistance in guiding us step-by-step on how to conduct experiments in the proper way, how to write a good thesis template, and teaching us how to use Mendeley. Their willingness to share and provide professional knowledge, advice, and skills is deeply appreciated.

Last but not least, I would like to take this opportunity to express gratitude to my family, especially my parents, for their mental support, which enabled me to overcome all of the difficulties and problems. Thank you very much.

Loh Li Sin

July 2022

## TABLE OF CONTENTS

## LISTS OF TABLES

## LISTS OF FIGURES

## LISTS OF SYMBOLS

| Symbol | Descriptions | Units |
|---|---|---|
| $\Delta G_{hyd}$ | Free energy of hydration | kJ/mol |
| $K_p/K$ | Partition coefficient of total protein | Dimensionless |
| $K_e$ | Partition coefficient of enzyme | Dimensionless |
| P | Phase volume ratio | Dimensionless |
| $V_t$ | Volume of top phase of ABS | µL |
| $V_b$ | Volume of bottom phase of ABS | µL |
| $C_t$ | Protein concentration in ABS top phase | M |
| $C_b$ | Protein concentration in ABS bottom phase | M |
| E | Extraction efficiency | % |
| $\Delta H$ | Enthalpy | J/g |
| $T_g$ | Glass transition temperature | °C |

## LISTS OF ABBREVIATIONS

| | |
|---|---|
| ATPS | Aqueous two-phase system |
| ABS | Aqueous biphasic system |
| IL | Ionic liquid |
| DES | Deep eutectic solvent |
| NADES | Natural deep eutectic solvent |
| PEG | Polyethylene glycol |
| CALA | *Candida antarctica* lipase A |
| CALB | *Candida antarctica* lipase B |
| HBDs | Hydrogen bond donors |
| HBAs | Hydrogen bond acceptors |
| DSC | Differential Scanning Calorimetry |
| BSA | Bovin serum albumin |

**CAMPURAN BIPHASIC AIR KUANTARI UNTUK EKSTRAKSI PROTEIN DARI BETIK**


**ABSTRAK**

Sistem biphasic akueus ialah kaedah pengasingan cecair-cecair yang telah mendapat minat yang besar untuk pengekstrakan enzim, asid nukleik, dan sebatian bioaktif. Ini kerana DES berasaskan ABS mempunyai sifat mudah ditingkatkan, mesra alam dan memberikan hasil pemulihan yang tinggi. Walau bagaimanapun, tingkah laku partition yang terlibat adalah kompleks dan sukar untuk diramalkan. Dalam penyelidikan ini, parameter yang berbeza telah disiasat untuk pembentukan DES berasaskan ABS. Empat jenis DES yang berbeza telah disintesis termasuk ChCl:U, ChCl:Gly, ChCl:Glu, ChCl:Ala, dan ChCl:Lac pada nisbah molar yang berbeza. ABS telah disintesis menggunakan pelbagai jenis garam, termasuk $Na_2SO_4$, $KH_2PO_4$, dan $(NH_4)_2SO_4$ dengan PEG2000. Eksperimen faktor tunggal menunjukkan bahawa pembentukan ABS dipengaruhi oleh jenis garam, kepekatan larutan garam, kepekatan PEG, jenis penderma ikatan hidrogen dalam DES yang disintesis dan isipadu DES. Keputusan yang diperolehi menunjukkan korelasi negatif antara kepekatan larutan garam dan nisbah isipadu fasa, manakala kepekatan PEG dan isipadu DES berkorelasi secara positif. Kepekatan larutan garam dan isipadu DES berkorelasi positif dengan kadar pembentukan ABS, manakala kepekatan PEG berkorelasi negatif. Hasil maksimum protein yang diekstrak (8456.5667g/mL) daripada mentah ekstrak betik dicapai dalam keadaan pengekstrakan optimum berikut: 0.3M PEG2000 dan 1.0M $Na_2SO_4$, di mana nisbah isipadu ialah 1:1, dan ChCl:Lac DES dengan nisbah molar 1:2, di mana nisbah isipadu ABS dan DES ialah 10:1. Kecekapan pengekstrakan protein maksimum yang diperolehi ialah 90.03%, dengan pekali sekatan tertinggi ialah 3.6119.

**QUATERNARY AQUEOUS BIPHASIC MIXTURE FOR PROTEIN EXTRACTION FROM PAPAYA**


## ABSTRACT

The aqueous biphasic system is a liquid-liquid separation method which has gained great interest for the extraction of enzymes, nucleic acids, and bioactive compounds. This is because ABS-based DES has the properties of being easy to scale up, environmentally friendly, and providing a high recovery yield. However, the partition behaviour involved is complex and difficult to predict. In this research, different parameters were investigated for the formation of ABS-based DES. Four different types of DES were synthesized including ChCl:U, ChCl:Gly, ChCl:Glu, ChCl:Ala, and ChCl:Lac at different molar ratios. ABS was synthesised using different types of salt, including $Na_2SO_4$, $KH_2PO_4$, and $(NH_4)_2SO_4$ with PEG2000. Single factor experiments demonstrated that the formation of ABS was affected by the types of salt, the concentration of salt solutions, concentration of PEG, types of hydrogen bond donors in DES synthesized and the volume of DES. The results obtained demonstrated a negative correlation between the concentration of salt solutions and the phase-volume ratio, whereas the concentration of PEG and the volume of DES were positively correlated. The concentration of salt solutions and volume of DES were positively correlated with the rate of formation of ABS, whereas the concentration of PEG was negatively correlated. The maximum yield of protein extracted (8456.5667g/mL) from papaya extract crude was attained under the following optimal extraction conditions: 0.3M of PEG2000 and 1.0M of $Na_2SO_4$, where the volume ratio was 1:1, and ChCl:Lac DES with a molar ratio of 1:2, where the volume ratio of ABS and DES was 10:1. The maximum protein extraction efficiency obtained was 90.03%, with the highest partition coefficient of 3.6119.

# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

Papaya originated in Mexico and is a tropical fruit abundant in antioxidants, minerals, fiber, and protein, especially enzymes (Schwimmer, 2016). The proteins from papaya have been used as dietary supplements due to their proteolytic activity. Enzymes are globular proteins that function to accelerate the rate of biological processes, including fruit ripening, and provide an alternate pathway to speed up chemical reactions. Indeed, more than 80% of the total protein in papaya is made up of proteolytic enzymes like papain, chymopapain, glycyl endopeptidase, and caricain (Chaiwut *et al.*, 2007). The extraction of enzymes from papaya is essential for commercial product development, such as toothpaste, medicine, cosmetics, and meat tenderizers. The conventional processes for enzyme liquid-liquid extraction are organic solvent precipitation and salting out processes using saturated salt solutions, also known as aqueous two-phase systems (ATPS) or aqueous biphasic systems (ABS). Currently, enzyme extraction using a binary, ternary or quaternary mixture consisting mainly of ionic liquids (IL), deep eutectic solvent (DES) or natural deep eutectic solvent (NADES) is gaining a lot of attention.

Proteolytic enzymes hydrolyze protein molecules into peptide linkages and further break them down into monomers (amino acids). Papain, from the cysteine proteinase family, was first investigated by Roy in 1873 and then named by Wurtz and Bouchut in the late 19$^{th}$ century (Hitesh *et al.*, 2012). Papain can be extracted from the "latex vessels" existing in the leaves, bark, and stems of papaya trees, as well as the peels of unripe papaya fruits (Chaiwut *et al.*, 2007; Ningrum *et al*., 2018). The biological activity of papain is due to its disulphide bridges and a reducing sulfhydryl

group. Papain, with a molecular weight of 21.0–23.0 g/mol, has maximum activity at its optimum pH of 6.0 to 7.0 and temperature of 37˚C (Ningrum *et al.*, 2018). Generally, commercial papain is widely used in different industrial applications like textile and food industries, such as beer chill-proofing (Chaiwut *et al.*, 2007). Recovery of highly active papain for various applications at a lower cost is desirable.

Papain can be extracted using biphasic binary, ternary, or quaternary mixtures, which involve precipitation and diffusion from one phase to another. These mixtures are composed of two, three, and four components respectively. These mixtures could form two phases when mixed at a specific component mass or mole ratio. The two distinct phases are formed either due to hydrophobic-hydrophilic interactions, hydrogen bonding interactions, dipole-dipole and dipole-induced dipole interactions, or transferring of charges between molecules (Ketnawa *et al.*, 2017). The most common biphasic solvents are those ternary or quaternary mixtures based on the salt-polymer system. This mixture includes polyethylene glycol (PEG)-sulphate, PEG-phosphate and PEG-citrate, which is commonly known as ABS. Ternary mixtures exist in living cells to mediate the release of primary metabolites. A ternary mixture has been developed and tailored for the recovery of highly active enzymes at a larger scale. Applying saturated salt solutions to the mixture may result in the precipitation of protein for ease of recovery in alternative phases such as PEG.

Protein enzymes can be precipitated using organic solvents such as tetrachloroethylene and propanol. The drawbacks of using organic solvents are the poor solubility of biological molecules, low extraction efficiency, and denaturation of enzymes (Marchel *et al.*, 2020). Thus, ionic liquid (IL), a liquid molten salt at temperatures below 100 °C, is introduced to explore its possibility in the recovery of enzymes. IL consists of organic cations such as imidazolium and inorganic or organic

anions such as tetrafluoroborate. It has been reported that the type of IL has a strong effect on the enzymes' partition and stability. Therefore, the selection of an appropriate IL according to the type of enzyme to be extracted is important. For example, the extraction efficiency of *Candida antarctica* lipase A (CALA) using a ternary mixture of ILs ($[C_2C1im]$ $[C_4SO_4]$) based ABS and inorganic salts $(NH_4)_2SO_4$ was more than 99% (Ventura *et al.*, 2017). However, the disadvantages of utilizing IL are high cost, complexity in synthesizing, difficulty in purifying, low selectivity, high toxicity, and non-biodegradable (Shikov *et al.*, 2020).

Abbott published the first research on DES as a potential alternative to IL in 2003 because DES has similar properties to IL (Shikov *et al.*, 2020). Generally, DES is prepared by mixtures of hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs), such as choline chloride (ChCl)-methylurea (Pang *et al.*, 2017). The chemical interaction between the components reduces the lattice energy of the system and lowers the melting point of each individual component (Paiva *et al.*, 2014). Recently, it has been used as a solvent for enzyme extraction. For example, bovine serum albumin (BSA) and papain were extracted using ternary DES-$Na_2CO_3$ ABS with a high efficiency of 95.16% and 90.95% respectively. The research showed no degeneration of enzymes (Pang *et al.*, 2017). The advantages of using DES in the extraction process are simple preparation, low material cost, non-flammability, good thermal and chemical stabilities, low volatility, strong dissolvability, adjustable polarity, and excellent biodegradability (Pang *et al.*, 2017; Marchel *et al.*, 2020; Kalyniukova *et al.*, 2021). However, DES has the potential to cause toxicity (Kalyniukova *et al.*, 2021).

NADESs were proposed in 2011 by a group of scientists led by Prof. R. Verpoorte of Leiden University (Shikov *et al.*, 2020). They postulated that all living

organisms have a third liquid phase that can dissolve polar and non-polar molecules as well as macromolecules. The formation of NADES is similar to that of DES. Except that it is made up of natural primary metabolites such as urea, sucrose, and organic acids, NADES has lower toxicity than DES. The example of NADES are ChCl-glycerol, sorbitol-betaine-water and quercetin-glucose-ChCl (Y. Liu *et al.*, 2018; Liu *et al.*, 2019). Research work on using ternary NADES, which are composed of lactic acid, ChCl, and water, showed high purity (>90%) of lignin extracted from lignocellulose biomass residue (Kumar *et al.*, 2016). The applications of NADES are the extraction of plant secondary metabolites and ribonucleic acid, enzymatic reaction media, and deacidification of crude palm oil. For instance, NADES, composed of betaine and glycerol, extracted 34% of palmitic acid while preserving 99% of antioxidants in the refined palm oil (Yang, 2018).

Purification techniques using advanced equipment such as size-exclusion, ion exchange chromatography and affinity chromatography are available for the recovery of highly pure enzymes. However, due to the disadvantages such as large pressure drops, high costs, and low yielding of these highly specific and multi-step batch processing purification techniques, it is impractical for large-scale enzyme extraction and purification (Manzoor *et al.*, 2016). Therefore, ABS is a promising alternative for the separation of biomolecules because of the beneficial characteristics that ABS possesses, such as lower cost of the raw materials, less time-consuming, simplicity of the technique, easy scalability, and higher recovery of biomolecules with minimal enzyme denaturation. Generally, polymers have stabilising effects on the biological activity and the quaternary structure of proteins and enzymes (Ketnawa *et al.*, 2017).

Because of the rising demand for greener and sustainable processes, NADES, an innovative non-toxic extraction technology with an improvement in extraction

efficiency has been introduced (Kalyniukova *et al.*, 2021). Further research on the toxicity, impacts on human health, recovery and recyclability of NADES is still in progress (Shikov *et al.*, 2020). Another direction of research in NADES is the preservation or stabilization (Vanda *et al.*, 2018). Concerning issues in the extraction and purification process are the selection of the most effective extraction methods with simple extraction routes, high extraction efficiency with high quality of desired product, all of which must be balanced with production costs for industry to be profitable. Moreover, the downstream separation, purification, and recovery of desired compounds and solvent recycling are the biggest challenges faced by the industry (Kalyniukova *et al.*, 2021). Although NADES based ABS is an uncomplicated, easy scalability, selective, and economical separation method, on a commercial basis, it has not been widely implemented. This is because of the lack of scientific understanding of the mechanisms associated with the partitioning process in the ABS, where it is difficult to estimate the phase equilibrium and the extent of the product partitioning. As a result, further research into the partition mechanism involved in ABS, as well as a better understanding of phase forming components, would enable more advanced applications in recovering high-value products (Iqbal *et al.*, 2016). In short, enzymes are vital precursors in many applications because of their beneficial properties, such as the removal of necrotic tissue in the wound healing process. The extraction process of enzymes must be simple, environmentally friendly, and profitable. The development of innovative, greener solvents is undoubtedly vital in enhancing analytical procedures for extraction. Consequently, high purity of products and recyclability of solvents will bring benefits to consumers and producers in terms of commercialization.

## 1.2 Problem Statement

Bioseparation is a process to purify biomolecules from different biological sources, such as precipitation in ABS and ion exchange chromatography. Undoubtedly, the extraction of protein-based compounds such as enzymes employing biphasic systems based on liquid-liquid extraction has attracted lots of interest in the 21$^{st}$ century. The types of components in the biphasic systems have a significant effect on the extraction efficiency of enzymes. The most common mixture of components utilized in the extraction process is water, salt, and polymer or alcohol. However, alcohol is a petroleum-based solvent, which has health concerns for humans. A moderate concentration of salt solution will cause dehydration of the enzyme surface, resulting in the enzyme's exclusion from the polymer rich phase. However, excessive dehydration may cause the alteration of enzyme structure, which leads to a reduction in the bioactivity of enzymes recovered.

The retention of maximum biological activity of enzymes is a top priority after the extraction process. The inter-and intra-hydrogen bond interaction between amino acid chains in the protein is the main interaction to stabilize the enzyme in its quaternary structure. Therefore, DES and NADES-based ABS were introduced as alternatives for enzyme separation. Enzyme solubility and separation in DES and NADES-based ABS are primarily determined by hydrogen bonding and dipole-dipole interactions via the salting out effect. A proper molar ratio of HBDs and HBAs creates DES where choline chloride (ChCl) is the typical HBA used while urea, glucose, lactic acid, and glycerol are used as HBDs. ABS is formed through the mixing of PEG and salt solutions above a critical concentration. The addition of DES to the ABS would increase the hydrophobicity of the top phase, promoting protein accumulation in the polymer-rich top phase. Protein partitioning is strongly influenced by the hydrogen

bonding interactions and salting out ability where it acts as the driving force to move the biomolecule from a salt-rich phase to a polymer-rich phase.

It is necessary to investigate the interaction and effect of the types of HBDs on the efficiency of enzyme extraction. Hydrogen bonding interactions between the components and enzymes not only enhanced the solubility of enzymes but also retained the stability and activity of the enzymes. Thus, it is vital to have a deep understanding of the role of HBDs, in terms of hydrophilicity and functional groups, in affecting the partitioning of enzymes. An experiment will be conducted using ABS to extract enzyme from papaya by varying the type and concentration of different components and salts respectively. A deeper knowledge of the function and interaction of components would contribute to the better development of high-efficiency protein-based biomolecule extraction processes.

## 1.3 Objectives

The major focus of this research is the utilization of a biphasic system in extracting the highly active enzymes. The objectives of this research are:

i. To study the formation of eutectic mixtures with different HBA/HBD molar ratios using DSC analysis, where choline chloride acts as HBA.
ii. To investigate the effect of salts, PEG2000, and HBDs on the formation of ABS through the rate of formation and phase volume ratio.
iii. To investigate the efficiency of DES-based ABS using different HBDs for protein separation using papaya extract.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Enzyme in Papaya

*Carica* papaya is a tropical plant that grows in all tropical countries. Papaya fruits are good for human health because they are rich in antioxidants and fiber. According to Lambri *et al*. in 2014, papaya has the highest content of potassium, carotenoids, ascorbic acid, and fiber per serving among fruits (USDA National Nutrient Database for Standard Reference). Papaya peels are often dumped away from residential areas, cafeterias, and industries. The accumulation of papaya peels has the potential to cause environmental problems such as soil pollution. According to Chaiwut *et al*. (2007), more than 1000 tonnes of papaya peels are discarded as waste by Thailand's pickle industries every year. Therefore, it is very important to transform papaya peels into valuable raw materials or intermediates such as latex proteases. Papaya latex proteases are made up of four cysteine proteases, such as less than 10% of papain, 26-30% of chymopapain, 23-28% of glycyl endopeptidase, and 14-26% of caricain, which contribute to 69-89% of the total protein (Chaiwut *et al.*, 2007). Papain is a natural monometric protein with maximum activity at a pH of 6.7 and a temperature of 37 °C. The stability of the papain enzyme is contributed by the folding along three disulfide bridges, generating strong interaction among the side chains (Lambri *et al.*, 2014).

Papain is beneficial in a variety of applications, including clotting dairy (cheese), meat tenderization, beer chill-proofing, cosmetics, detergents, and textiles. Medicinal uses include treating diarrhea, reviving dyspepsia, treating burns, wounds, and peeling, and treating bleeding haemorrhoids (Ningrum *et al.*, 2018). The dried *Carica* papaya latex proteases are commercially sold as crude papain in the global

market. Traditionally, the maximum yield of latex is obtained by tapping papaya latex from green, unripe papaya fruits. However, the collection of latex from unripe papaya fruits is time consuming and laborious. Therefore, alternative sources of latex collection have been discovered for crude papain production, such as green unripe papaya fruit peels, leaves, stems, and bark (Chaiwut *et al.*, 2007). Currently, the extraction of papain for commercial purposes is done through drying. Sun drying is the simplest and most traditional method, which produces the lowest grade of dark papain in crumb form. Oven drying is an improved drying method of latex on glass trays in a brick oven, which produces a moderate quality of white powder or crumb. Vacuum drying is a new and innovative method to produce high quality papain in white powder form at the atmospheric temperature while maintaining the higher level of papain activity. (Schwimmer, 2016)

Incisions are made on the peels of unripe papaya fruits using a sharp non-metallic instrument such as a stainless-steel razor blade. This is due to the enzymes in the latex being sensitive to metals such as iron, copper, or brass. A viscous, colourless latex flowing from the incisions is collected into a non-metallic container such as an earthenware or polythene-lined container with a close-fitting lid (Lambri *et al.*, 2014; Ningrum *et al.*, 2018). The storage of the container with a close-fitting lid in a shaded area is to prevent the reactions of enzymes with contaminants, which will cause the loss of papain activity and discolouration. This is due to the exposure of latex to the atmosphere, which will cause the oxidation of sulfhydryl groups into dithiol groups. Sulfhydryl groups act as activators which protect papain against inactivation (Shikov *et al.*, 2020). It is necessary to dry latex to under 5% moisture to have a long shelf life. The completion of the drying process is shown by the dry and crumbly texture of latex after approximately 4-5 hours at a drying temperature of 35°C–40°C (Anon, n.d).

## 2.2 Aqueous Biphasic System (ABS)

Due to the existence of disadvantages of industrial-based protein extraction technologies such as low solubility of aqueous solutions composed of organic solvents, low-purity products, and unstable enzyme activity, it is important to establish a new industry-desired technology for separation and purification of high-purity and stable papain. ATPS was pioneered by Albertson and is a liquid-liquid extraction method where two immiscible water-rich phases are formed based on polymer-polymer interactions for the partition or extraction of bioactive molecules such as enzymes, antibiotics, and phenols and non-biomolecules such as dyes, metallic ions, and nanoparticles (Pang *et al.*, 2017; Yu and Zhang, 2020). ABS is formed when the mixing of two incompatible hydrophilic polymers or polymer-salts occurs above a critical temperature or concentration. The utilization of aqueous two-phase polymer–polymer or polymer–salt systems in bioprocessing applications has been well-investigated. ABS possesses several advantages, such as low energy consumption, low costs, tunable composition, mild operating conditions, non-toxicity, inflammable, biocompatible, environmentally friendly (due to materials used being biodegradable), technological simplicity, and is easily scalable to industrial scale. ABS could be made up of PEG, ILs, DESs, and NADESs (Yu and Zhang, 2020). The components of ABS and their surface characteristics influence the protein partitioning in both phases. Protein will always accumulate in the top, hydrophobic, and less polar phase, which is normally PEG. The partition coefficient of protein partitioning will be affected by modifying the molecular weight of the polymer, ion type, or ionic strength in the salt-rich phase. The effect of the molecular weight of proteins on the partitioning is not very significant, which further confirms that hydrophobicity is a dominant factor in protein partitioning in ABS, particularly in polymer–salt systems (Iqbal *et al.*, 2016).

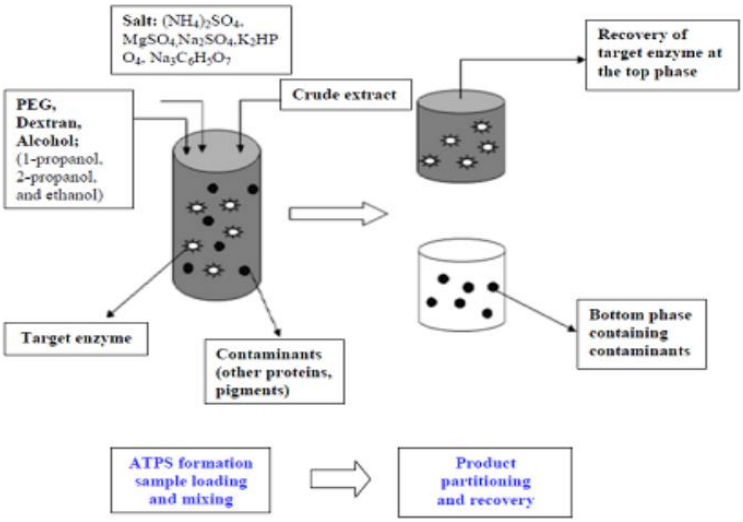


**Figure 2.1** Schematic diagram of the aqueous two-phase system of biomolecules (Ketnawa *et al.*, 2017)

### 2.2.1 Deep Eutectic Solvents (DESs)

The increased number of published research papers related to DESs in the extraction of biomolecules from the SCOPUS Database Platform (www.scopus.com) in the last ten years demonstrates the high level of interest in potential green-approach DESs in industrial applications. According to the SCOPUS Database Platform, with the words "deep eutectic solvents", "enzyme" and "extraction" either in the title, abstract or keywords, the first publication related to DES was in 2010 with the title "Deep eutectic solvents for *Candida antarctica* lipase B-catalyzed reactions". There are 673 articles and 140 review papers published from the years 2010 to 2022. Moreover, there are 548 patents published from the years 1983 to 2022, where the patents mostly come from the United States Patent & Trademark Office and Japan Patent Office. The research areas include application in drug discovery and delivery systems, desulfurization of fuel oil, application in chromatography, and synthesizing or extracting of organic materials (Ivanović *et al.*, 2020).

The use of DESs as an alternative to ionic liquids was pioneered by Abbott *et al*. in 2003. DES is an eutectic mixture composed of HBAs such as halide salts of

quaternary ammonium cations and HBDs such as amides, organic acids, and polyalcohol. The depression of the melting point of the eutectic mixtures relative to the melting point of each individual component is caused by the formation of Van der Waals forces and hydrogen bonds between HBAs and HBDs in the DES. Because of the beneficial properties of DES, such as low cost, biodegradability, non-toxicity, high thermal stability, and tenability, DES has gained a lot of attention as the potential solvent utilized in chemical synthesis, bio catalysis processing, electro-chemistry, and extraction (Lin *et al.*, 2020). For instance, ChCl based DES has developed as an attractive solvent to be used in ABSs due to its benificial properties such as negligible volatility, non-flammability, low toxicity, good chemical and thermal stability, and biodegradability (Pang *et al.*, 2017). The different combinations and proportions of HBA and HBD determine the physico-chemical properties such as viscosity, density, and pH and also affect the extraction efficiency of DES. The high viscosity of DES might limit its applications in large-scale industries. Thus, the addition of water could lower the viscosity and density of DES. However, an excess amount of water would cause the breaking of hydrogen bonds between components in DES, and the eutectic properties of DES would be lost (Ivanović *et al.*, 2020).

### 2.2.2 Natural Deep Eutectic Solvents (NADESs)

The great attention towards the potential green approach of NADES in industrial applications in different fields can be shown by the increase of published research papers related to NADES in the extraction of biomolecules, especially enzymes, in the last ten years from the SCOPUS Database Platform (www.scopus.com) because of its environmental and economic perspective. According to the SCOPUS Database Platform, with the words “natural deep eutectic solvents”, “enzyme” and

"extraction" either in the title, abstract or keywords, the first publication related to NADES was in 2011 with the title "Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology?". There are 152 articles and 42 review papers published from the years 2011 to 2022. Moreover, there are 363 patents published from 2012 to 2021, where the patents mostly come from the United States Patent & Trademark Office and Japan Patent Office. NADES is gaining popularity due to its favourable physicochemical properties, which include its ability to remain in liquid form over a wide temperature range, chemical and thermal stability, non-flammability, low volatility, biocompatibility, biodegradability, and re-usability. Furthermore, the extracts in NADES can be used directly without a purification process, which then reduces the overall production costs (Mehariya *et al.*, 2021).

NADES are made from natural raw materials such as organic acids, sugars, amino acids, and organic bases. Eco-friendly NADES has gained a lot of interest in food, nutraceutical, and cosmetic formulations due to its main characteristics of non-toxicity of constituting components and high availability in nature and in human diets (Mehariya *et al.*, 2021). For example, extraction of cynaropicrin from leaf extract of Cynara cardunculus L. is used as a phytochemical-based nutraceutical source. A high yield of cynaropicrin was extracted using decanoic acid-tetrabutylammonium chloride-based NADES at 25°C for 60 minutes and a solid-liquid ratio of 1:30 (Hikmawanti *et al.*, 2021). In addition, papain enzyme extracted from *Carica* papaya fruit latex is used in food industries for meat tenderization and pharmaceutical industries for wound defibrination. Highly purified papain, more than 96%, was obtained under the optimum conditions, which were 14.33–17.65% (w/w) PEG 6000, 14.27–14.42% (w/w) $NaH_2PO_4/K_2HPO_4$ ATPS at pH of 5.77–6.30 and a temperature of 20°C (Li *et al.*, 2010).

The ChCl:Pro NADES showed the shortest synthesis time, while Bet:Ca showed the longest time for synthesizing NADES. This could be explained by differences in viscosity, as the viscosity is directly related to the molecular interactions (hydrogen bonds, electrostatic forces, and Van der Waals forces) between HBAs and HBDs, which will eventually influence the molecular mobility. Carboxyl, hydroxyl, and amine functional groups are the common functional groups that are involved in these molecular interactions.

**Table 2.1** Composition, molar ratio and formation duration of the six NADESs (Benvenutti *et al.*, 2020)

| Code | HBA | HBD | Molar ratio | Duration for NADES formation |
|---|---|---|---|---|
| ChCl:Pro | choline chloride | propylene glycol | 1:2 | 21 minutes |
| ChCl:Ca | choline chloride | citric acid | 1:1 | 6 hours |
| ChCl:Ma | choline chloride | malic acid | 1:1 | 6 hours |
| Ca:Pro | citric acid | propylene glycol | 1:1 | 5 hours |
| Ca:Glu:Wa | citric acid | glucose, water | 1:1:3 | 50 minutes |
| Be:Ca | betaine | citric acid | 3:1 | 7 hours |

Among the different combinations of NADES, the Be:Ca NADES has the highest viscosity (36.922± 0.532 Pa.s), followed by ChCl:Ca NADES (14.480 Pa.s), while ChCl:Pro NADES has the lowest viscosity (0.070 ± 0.001Pa.s) at ambient temperature. Citric acid in NADES contributed to the high viscosity value due to its structure consisting of three carboxyl functional groups, which then leads to intensive molecular interactions between components when compared to glucose or malic acid. ChCl:Pro NADES has the lowest viscosity due to the weaker molecular interactions of hydroxyl functional groups in propylene glycol than carboxyl functional groups in

citric acid. For the Ca:Glu:Wa, the viscosity was considered low due to the presence of water in NADES (Benvenutti *et al.*, 2020).

**Table 2.2** Apparent viscosity of NADES at 298 K, 323 K and 353 K (Benvenutti *et al.*, 2020)

| Code (HBA:HBD) | Molar ratio | Apparent viscosity (Pa.s)at 298K | Apparent viscosity (Pa.s) at 323K | Apparent viscosity (Pa.s) at 353K |
|---|---|---|---|---|
| ChCl:Pro | 1:2 | 0.070 ± 0.001 | 0.019± 0.001 | 0.009± 0.001 |
| ChCl:Ca | 1:1 | 14.480 ± 0.055 | 2.242± 0.012 | 0.331± 0.004 |
| ChCl:Ma | 1:1 | 2.082 ± 0.030 | 0.610± 0.006 | 0.146± 0.001 |
| Ca:Glu:Wa | 1:1:3 | 0.159 ± 0.001 | 0.038± 0.006 | 0.014± 0.002 |
| Ca:Pro | 1:1 | 2.188 ± 0.032 | 0.571± 0.003 | 0.088± 0.001 |
| Be:Ca | 3:1 | 36.922 ± 0.532 | 2.775± 0.480 | 0.471± 0.002 |

### 2.2.3 Differential Scanning Calorimetry (DSC)

The most common used thermal analysis method is Differential Scanning Calorimetry (DSC) analysis, with the advantages of being quick, easy, and readily available. In DSC, the holders hold a sample and a reference together while the heaters hold the DSC isothermally or ramp the temperature linearly as a function of time. The device computes the heat flow differential between the sample and the reference, where the basic principle of this instrument is to measure the amount of heat absorbed or emitted during the phase transitions in order to retain the sample and reference at the same temperature when the sample undergoes physical transformation. The change in heat flow depends on the types of processes (exothermic or endothermic). To raise the temperature at the same rate as the reference during an endothermic transition, more heat must be transferred to the sample and vice versa. DSC might be used to examine additional physical property changes, such as melting points, enthalpies of

formation and fusion, glass transition temperatures, heat capacity values, and the thermal stability components or mixtures. DSC is especially helpful for identifying unusual behaviour in DES since it can record phase change occurrences (Hansen *et al.*, 2021).

According to Paiva *et al*. (2014), DSC was conducted to investigate the thermal stability of NADES by increasing the temperature up to 250°C. The NADES evaluated were ChCl:glucose (1:1), ChCl:citric acid (1:1). ChCl:sucrose (4:1 and 1:1), ChCl:tartaric acid (2:1) and ChCl:xylose (3:1 and 2:1). The decomposition temperatures of all NADES were above 120°C where a single degradation peak appeared after 120°C. Due to the occurrence of glass transition, a discontinuity in the heat flow is seen in the first scan at lower temperatures. It is equivalent to the structure of a material changing from a glass-like state to a rubber-like state, showing that there are changes in heat capacity. The glass transition temperature ($T_g$) was positively correlated with the number of scans until it became constant. This phenomenon was caused by the evaporation of water (Paiva *et al.*, 2014). An endothermic peak in pure choline chloride was observed at 65.11°C, showing a transition phase of the crystallographic configuration. A second peak was observed at 224.01°C, which can be ascribed to the melting point followed by thermal degradation. A broad exothermic peak started occurring at 125°C, followed by an endothermic peak beginning at 160°C for glycerol:citric Acid NADES (1.5:1.5). This area reflects a cold crystallisation process in which molecules liberate energy for the adoption of a more ordered structure. This phenomenon has typically been demonstrated in eutectic mixtures with high viscosity due to insufficient component mixing. There was no peak of pure choline chloride at 65.11°C observed in any ChCl-containing NADES mixtures, which

indicates that all the ChCl molecules were participating in the molecular interactions with the HBDs (Trivisiol *et al.*, 2020).

### 2.2.4 Application of Aqueous Biphasic System

Downstream processing is a crucial process in the purification and separation of bioactive compounds, which requires high operation costs due to its high complexity. ABS, which is made up of polymer/salt or DES, was introduced as a cost-effective and efficient downstream separation technology for purifying biomolecules on a large scale for industrial use. According to Yau *et al*. (2015), PEG/sulphate ABS was utilized to purify biopharmaceutical compounds including interleukin-18 binding protein (IL-18BP) from serumfree Chinese hamster ovary (CHO) cell supernatant, where IL-18BP is a vital immune system regulator. The recovery of IL-18BP in the PEG-rich top phase using PEG/sulphate ABS reached 98% with a final purity of 86%. Moreover, the purification of monoclonal antibodies (mAbs) acts as a therapeutic agent using ABS for the purpose of infectious diseases, autoimmune disorders, and cancer treatment (Iqbal *et al.*, 2016). Production of vaccines and delivery vectors for gene therapy through the recovery of viruses such that 90% of Adenovirus was extracted from a crude lysate of HEK293 with the utilization of PEG300-phosphate ABS. The separation of drug residues from animal-based food and herbal extracts using ABS is also gaining lots of interest in the food industry to ensure the safety of consumers. For instance, using ABS composed of Poly (ethylene glycol-ranpropylene glycol) EOPOL31 and $K_2HPO_4$ to extract Ciprofloxacin (a drug) from milk samples yielded an extraction efficiency of 97.7% for the first extraction procedure and 85.6% for the second extraction process. Also, the usage of ABS in the removal of toxic and carcinogenic dyes produced by the textile industry is important to reduce the exposure of these dyes to aquatic ecosystems and human health (Iqbal *et al.*, 2016).

## 2.3 Factor affecting the formation of biphasic systems and the yield of extracted enzyme from papaya

The extraction processes of hydrophilic or hydrophobic biomolecules using NADES are greatly influenced by the properties of solvents. For instance, the types of HBAs and HBDs in NADES would affect its polarity, viscosity, solubilisation capacity towards target compounds and extraction efficiency (Mehariya *et al.*, 2021).

**Table 2.3** Critical properties used to achieve and factor affecting phase separation of protein or enzymes (Ketnawa *et al.*, 2017)

| Partitioning properties | Description | Factor |
|---|---|---|
| Hydrophobicity | Separation according to the hydrophobicity of proteins | - Molecular weight of polymer<br>- Concentration of polymer<br>- Ionic strength of salt |
| Electrochemical | Separation according to the charges of molecules | - pH of the system<br>- Addition of NaCl<br>- Ionic strength of salt |
| Size-dependent | Separation according to the size and surface area of proteins | - Molecular weight of polymer |
| Conformation-dependent | Separation according to the conformation of the proteins | - Molecular weight of polymer<br>- Concentration of polymer<br>- pH of the system<br>- Addition of NaCl |

Hikmawanti *et al*. (2021) stated that the high solubility of solutes in NADES is mainly due to the dipole-dipole and hydrogen bonding interactions. An increase in the ratio of polyol constituents in NADES will improve the diffusion and mass transfer.

For example, citric acid with one hydroxyl tricarboxylic acid (three-COOH functional groups) has a high chance of forming more hydrogen bonds than malic acid with one hydroxyl dicarboxylic acid (two-COOH functional groups) and lactic acid with one hydroxyl monocarboxylic acid (Hikmawanti *et al.*, 2021). The salt concentration will also influence the enzyme extraction efficiency and the yield and activity of the extracted enzyme.

### 2.3.1 Effect of Type of Salt

Salabat (2001) presented the data on the equilibrium of aqueous systems composed of PEG6000 and $MgSO_4$, $Na_2SO_4$ and $(NH_4)_2SO_4$ and analyzed the impact of different salts on the binodal curve and the length of the tie line. The strength of salting-out ability of cations had been investigated using the binodal curve, where $MgSO_4$ had the greatest salting-out strength when compared to $Na_2SO_4$ and $(NH_4)_2SO_4$ because it depresses the binodal to lower polymer concentrations. The salting-out strength of cations where $Mg^{2+}$ takes precedence over $Na^+$ and $NH_4^+$. Iqbal *et al*. (2016) stated that the presence of additional NaCl in the PEG-phosphate based ABS had increased the separation power, which affected by the protein surface hydrophobicity. Protein surface hydrophobicity was twice as high when compared to the biphasic system without additional NaCl. On the other hand, the effect of anions is more significant than the cations in terms of salting-out ability. According to the free energy of hydration ($\Delta G_{hyd}$), the salting-out effect and the effectiveness of phosphate ($PO_4^{3-}$) in ABS formation is larger than that of the citrate ($C_6H_5O_7^{3-}$) as the $\Delta G_{hyd}$ value of phosphate and citrate are -2835 kJ/mol and -2793 kJ/mol respectively. The greater the negative value of $\Delta G_{hyd}$, the greater the salting-out ability and hence the ease with which ABS can be formed (Glyk *et al.*, 2014).

According to Silvério *et al*. (2013), the cations of $Na^+$ were more effective than $K^+$ in the ABS formation because $Na^+$ has a greater salting-out effect than the cations of $K^+$ and $Li^+$. The comparison can be proven by analysing the free energy of hydration ($\Delta G_{hyd}$) where the $\Delta G_{hyd}$ value for $K^+$ was -295 kJ/mol while the $\Delta G_{hyd}$ value for $Na^+$ was -365 kJ/mol. This is because the larger the negative value of $\Delta G_{hyd}$, the larger the hydration shell, which then contributes to the salting-out effect by reducing the chance for hydration of the polymer by water molecules. Moreover, the effectiveness of potassium salts for the formation of ABS can be observed in the following order: $HPO_4^{2-}$ > $C_6H_5O_7^{3-}$ > $HCO_2^-$. Also, sulphate anion was more effective than the monocarboxylic acid anion in the ABS formation in the following order: $SO_4^{2-}$ > $HCO_2^-$ with the explanation of the higher the valence of an anion, the better the salting-out capacity and thus the lower the concentration of anion required for the formation of ABS. This is because of the ability of higher valence anions to capture more water molecules and therefore decrease the chance for hydration of polymers by water molecules. (Silvério *et al.*, 2013).

### 2.3.2 Effect of Different Salt Concentration

Furthermore, the concentration of salt solution in ABS also plays a key role in affecting the enzyme extraction efficiency as the change in salt concentration influences the electrostatic and non-electrostatic interactions. Salts have the capability to strengthen the hydrophobic interactions between proteins. For example, an increase in the hydrophobicity difference between two phases could strengthen the hydrophobic association between PEG molecules and proteins, improving the effectiveness of the extraction process (Glyk *et al.*, 2014). The enzyme extraction efficiency increases with the concentration of salt solutions until a maximum level due

to the salting out effect, which decreases the solubility of protein in the bottom phase as the bottom phase becomes more hydrophobic. Thus, protein is salted out from the bottom phase and will move into the top phase. Increased salt concentration encourages the movement of more protein from the bottom to the top phase. However, the further increase in the salt concentration will result in a decrease in extraction efficiency because of the huge decrease in water content in the top phase, and thus the proteins will move into the bottom phase (Li *et al.*, 2016).

According to Yu and Zhang (2020), the papain activity recovery and protein recovery rose with the salt concentration until maximum and declined with further increases in salt concentration. The highest papain activity recovery and protein recovery were observed at 20 wt% of salt concentration, $(NH_4)_2SO_4$, where the papain activity recovery is 93.44% and the protein recovery is 86.21%. However, when the salt concentration exceeds 20 wt%, the results obtained decreased from 93.44% and 86.21% to 89.45% and 83.89% respectively. This is because the inorganic salt solution will affect the accessibility of the active site of the enzyme (Yu and Zhang, 2020). In addition, Foda *et al*. (2016) stated that the effectiveness of salt solutions was related to their concentration. The pectinesterase activity increased with sodium chloride concentration until 0.3 M, where the activity is 15.6 units/mL and the specific activity is 32.50 U/mg protein. Also, a further increase in NaCl concentration (0.35 M) leads to a reduction in activity (15.0 units/ml) and the specific activity (31.25 U/mg protein) of pectinesterase. In addition, the increment in the $(NH_4)_2SO_4$ concentration from 10% to 40% would lead to a reduction of the phase-volume ratio with the constant concentration of PEG1500 at 20%. This is because the salt could bind to more water molecules from the top phase into the bottom phase. The maximum recovery of flavonoids (96%) was obtained at a salt concentration of 20%. The further increase in

salt concentration would cause the partitioning of flavonoids into the salt-rich bottom phase, causing a reduction in the recovery percentage of flavonoids in the top phase (C. Liu *et al.*, 2018).

According to Glyk *et al.* (2014), the partition coefficient of total protein (Kp) increased from 0.48 to 3.33 when there is an increment of $Na_2SO_4$ concentrations from 10% to 12% respectively. This is because the increase in salt concentrations results in an increase in salting-out effect, which then increases the partitioning of protein into the top phase of ABS. The highest purification factor and recovery of invertase are 3.33 and 134% respectively, where the $Na_2SO_4$ concentration is 12% (w/w). The purification factor and the recovery percentage decline as salt concentrations continue to rise because of the enzyme precipitation at the interphase (Glyk *et al.*, 2014). Moreover, an increase in the salt concentration from 0.85 g/mL to 0.90 g/mL also leads to an increase in the phase volume ratio from 0.4 to 0.52 but a further increase in salt concentration from 0.90 g/mL to 1.10 g/mL results in a decrease in the phase volume ratio from 0.52 to 0.45. This is due to the fact that the hydrophilic capacity of DES was initially higher than salt but decreased as the salt concentration continued to rise. In addition, the extraction efficiency of BSA increased from 75% to 96% when the salt concentration was increased from 0.85 g/mL to 0.90 g/mL. However, the further increase of salt concentration from 0.90 g/mL to 1.10 g/mL results in a decrease in extraction efficiency from 96% to 83.5%. The increasing salt concentration would directly enhance the hydrophobicity of the bottom phase. This can be justified by the salting-out effect of the BSA, where the solubility of BSA in the bottom phase decreases dramatically because of BSA and a significant number of salt molecules competing for water molecules. As further increases in salt concentration would result in the reduction of water content in the DES-rich top phase, the protein tended to move

to the salt-rich bottom phase because the structure and activity of protein are preserved by the hydrogen bonding interactions between protein and water (Xu *et al.*, 2015).

### 2.3.3 Effect of Different PEG Concentrations

The concentration of PEG plays a vital role in the partitioning of protein in ATPS. According to C. Liu *et al*. (2018), when there was an increment in the concentration of PEG1500 from 13% to 35% with the constant concentration of $(NH_4)_2SO_4$ at 15%, the phase-volume ratio also increased, and the flavonoids partitioned more to the PEG-rich top phase. In addition, Glyk *et al.* (2014) conducted the experiment by varying the PEG concentrations from 10% to 17.5% where the pH value is 5.0 and the $Na_2SO_4$ concentration is constant at 12.5% w/w. The partition coefficient of enzyme ($K_e$) reached the greatest value of 3.54 with a purification fold of 2.7 and 70 % recovery of invertase when the PEG3000 concentration was increased to 15% (w/w). However, the further increase of PEG3000 concentrations until 17.5% (w/w) led to the decrease in partition coefficient to 1.83. This is because the increase in the PEG concentrations would directly increase the viscosity of the solution and hence increase the interfacial tension between molecules in the top phase of the ABS. As a result, the effectiveness of partitioning invertase into the top phase of ABS decreased due to the mass transfer resistance of invertase into the top PEG phase increasing (Glyk *et al.*, 2014).

### 2.3.4 Effect of Different Type of Hydrogen Bond Donors in DES

Moreover, the type of HBDs, such as salt solutions in NADES, has a substantial effect on the enzyme extraction efficiency. For instance, the extraction efficiency of BSA in ABS, with 0.64g/ml of ChCl-based DES concentration, 0.12 g/ml

of salt solution concentration, and 0.03g/L protein concentration at 25°C, decreased in the order of $Na_2CO_3$ (81%), $Na_3C_6H_5O_7$ (78%), and $NaH_2PO4$ (76%). This is because pH has played a significant role in extraction efficiency as pH values of NADES are mainly related to the HBD chemical structures, such that ChCl-based DES–$Na_2CO_3$ ABS has a pH value of 12.83, ChCl-based DES–$Na_3C_6H_5O_7$ ABS has a pH value of 8.74, and ChCl-based DES–$NaH_2PO_4$ ABS has a pH value of 3.44 (Pang *et al.*, 2017). pH values will affect the charged properties of the amphoteric protein and thus influence the distribution behavior of protein between two phases (Yu and Zhang, 2020). Furthermore, Pang *et al*. (2017) explained that the electrostatic interaction between the charged groups of protein and the solvent was the key influence in protein extraction. In addition, the betaine-glycerin (1064±3 mPa.s) has a lower viscosity than betaine-xylitol (11504±5 mPa.s) while the choline chloride-glycerin (336±2 mPa.s) has a lower viscosity than choline chloride-xylitol (7541±5 mPa.s). This can conclude that the viscosity of glycerin-containing NADES was lower than xylitol-containing NADES regardless of the type of hydrogen bond acceptor. This phenomenon might be explained by the intensive hydrogen bonding between NADES components, which contributes to the increment in viscosity (Nian *et al.*, 2020).

Generally, the enzyme extracted has higher activity and is more stable in the low viscosity extraction media. *Candida antarctica* lipase B (CALB) showed higher activity in choline chloride-glycerin based ABS (8690.98±249.26 U/mL) than in the choline chloride-xylitol based ABS (5004.77±144.23 U/mL) (Nian *et al.*, 2020). Furthermore, different combinations of components in NADES would affect the density of NADES and thus influence the extraction efficiency. For instance, Babu Balaraman *et al*. (2021) reported that choline chloride-dodecanoate (CC:DD) based ABS and choline chloride- decanoate (CC:DA) based ABS have higher density but