ENZYME EXTRACTION FROM PINEAPPLE VIA NATURAL BASED AQUEOUS QUATERNARY BIPHASIC SYSTEM

By

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LIST OF ABBREVIATIONS

PEG	Polyethylene glycol
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
UV-VIS	Ultraviolet-visible
DES	Deep eutectic solvent
AQBS	Aqueous biphasic quaternary system

PENGEKSTRAKAN ENZIM DARIPADA BUAH NENAS MENGUNAKAN CAMPURAN CECAIR TERNER SEMULAJADI

ABSTRAK

Pengekstrakan enzim menggunakan pelarut eutektik dalam telah mendapat minat kerana mudah dan kos aplikasi yang rendah. Pembentukan 4 jenis DES iaitu ChCl : glukosa, ChCl : asid laktik, ChCl : glicerol dan ChCl : urea dijalankan dengan nisbah molar yang berbeza. Dalam kajian ini didapati DES akan terbentuk cecair lutsinar dalam suhu bilik dengan nisbah tertentu. Nisbah molar untuk ChCl : glukosa, ChCl : asid laktik, ChCl : gliserol dan ChCl : urea masing-masing ialah 2:1, 1:2, 1:2 dan 1:2. Dengan pembentukan DES, pembentukan ATPS dilakukan untuk membentuk pelarut DES-ATPS. Pembentukan ATPS boleh dicapai antara 0.3M polietilena glikol dan 1.0M natrium sulfat. Nisbah antara fasa atas ke fasa bawah ialah 1.3 dengan masa pembentukan ialah 41 minit. Nisbah isipadu antara PEG:Na_2SO_4:DES ialah 1:1:0.1. isipadu ditambah dengan cara ini untuk membentuk pelarut DES-ATPS. Grif penentuukuran dicipta menggunakan reagen BSA dengan bacaan spektrometer uv vis mempunyai penentuan hampir sama dengan satu. Ini adalah untuk semua DES kecuali glukosa kerana bacaannya sesuai dengan graf data. Pengekstrakan bromelain daripada nanas adalah berjaya dengan berada di fasa atas yang mempunyai kepekatan protein masing-masing 1124.98μg/L, 1042.699μg/L dan 1841.649μg/L untuk DES ChCl : urea, ChCl : gliserol dan ChCl : asid laktik. .

ENZYME EXTRACTION OF PINEAPPLE VIA NATURAL BASED TERNARY LIQUID MIXTURE

ABSTRACT

Enzyme extraction using the deep eutectic solvents has gain interest because of its simplicity and low cost of application. The formation of 4 type of DES in which is ChCl : glucose, ChCl : lactic acid, ChCl : glycerol and ChCl : urea is carried out with different molar ratio. In this study it has been found that the DES formed transparent liquid in room temperature with specific ratio. The molar ratio for ChCl : glucose, ChCl : lactic acid, ChCl : glycerol and ChCl : urea is 2:1, 1:2, 1:2 and 1:2 respectively. With the formation of DES, the formation of ATPS is carried out in order to form the DES-ATPS solvent. The formation ATPS can be achieve between 0.3M of polyethylene glycol and 1.0M of sodium sulfate. The ratio between top phase to bottom phase is 1.3 with the formation time is 41 minutes. The ratio of volume between PEG:Na_2SO_4:DES is 1:1:0.1. the volume is added in this way to form the DES-ATPS solvent. The calibration curve is created using BSA reagent with uv vis spectrometer reading has the coefficient of determination nearly equal to one. This is for all DES except for glucose since the reading does fit the regression curve. The extraction of bromelain from pineapple is a successful with the top phase has the concentration of protein of 1124.98$\mu g/L$, 1042.699$\mu g/L$ and 1841.649$\mu g/L$ for the DES of ChCl : urea, ChCl : glycerol and ChCl : lactic acid respectively.

CHAPTER 1

INTRODUCTION

1.1 Research Background

One of the most consume fruits in the world is pineapple. Pineapple is mostly planted in tropical environment. There is variety of products can be made from pineapple as it rich in minerals and nutrition (Bakar et al., 2021). In pineapple there is an enzyme called bromelain that mostly contain digesting enzyme (Gautam *et al*, 2017). In addition, bromelain has a wide range of therapeutic benefits such as sinusitis, bronc

hitis and thrombophlebitis. The extraction of bromelain through liquid-liquid extraction one of the most employed operation in the chemical industry. This is due to its high efficiency, low energy consumption and environmental impact (Madeira and Amaral, 2019).

Pineapple *(Ananas comosus)* is ranked third in production of tropical fruit after banana and citrus. This fruit is known with flavourful fruit with unique taste and sweet aroma. Pineapple come in with different size, shape, colour and flavours. The maturity of the fruit is evaluated based on the physical, physiochemical and chemical attributes of the fruits with acceptable flavour and morphological characteristics. However, the quality and shell life of pineapple is strongly being influenced by postharvest handling and management(Mohd Ali et al., 2020). The top producer of pineapple in the world is Costa Rica followed by Philippines and Brazil (Bakar et al., 2021).

Table 1 Top 5 countries producing pineapple(Bakar et al., 2021)

Country	Volume (tonnes)	Rank
Costa Rica	3,328,100	1
Philippines	2,747,860	2
Brazil	2,426,530	3
Indonesia	2,196,460	4
China	1,727,610	5

The proteolytic enzyme that is called bromelain that can be found in pineapple is what make the fruit different from the other. Proteolytic enzyme is a group of enzyme that can break a long chain protein(polypeptide) into shorter chain protein(peptide). Bromelain has the potential to be use as an anti-inflammatory, anti-cancer activity, cardio-protective and antioxidant. Furthermore, bromelain is beneficial for woman that are pregnancy or menstruation as it can reduce the excessive water in the body (Mohd Ali et al., 2020) . Apart from being used in medication, bromelain is also used in the food processing. For example, bromelain is used in the meat tenderization because it is quite efficient in hydrolysing myofibril proteins present in the meat like nebulin, actomysin and titin. (Gautam *et al*, 2017).

As people began to realize the important of bromelain application, the global demand of the product will also increase. Thus it is important that fully extraction of bromelain from pineapple stem, crown, fruit, peel and core. There are a few methods that has been apply to extract bromelain from pineapple. Ultrafiltration, salt precipitation, aqueous two phase system, reverse micelle extraction and column chromatography are the method that is widely use to extract bromelain. Ultrafiltration that is used widely use in large scale processes utilise membrane that can separate protein with molecular weight ranging from 3 kDa to 100 kDa. Ultrafiltration process is considering to be long separation process and there is risk of membrane clogging. Next method is salt precipitation, that use high concentration of precipitating agent like ammonium sulphate to reduce the solubility of proteins in solution. Reducing the solubility of protein will cause it to precipitates. This mechanism is called salting out. The following method called is aqueous two phase system where the process use of two immiscible phases that formed by mixing polymer and salt. Two layer will be form with protein at the top and bottom with contaminants. The protein can be further separated with centrifugation. Next, reverse micelle extraction method that involves liquid-liquid extraction for separation of biomolecules. The system consists of an organic phase that separated from

droplets of aqueous phase by surfactants present in the interface of two phases. Among all the method that is listed, aqueous two phase system and reverse micelle extraction stand out to be better in the business. The two aqueous phase system have the disadvantage in complete recovery of the protein(Gautam *et al*, 2017).

Ternary liquid mixture is a mixture that have three different components in on mixture. The equilibrium behaviour of the three component liquid is usually described using a ternary phase diagram. Ternary phase diagram consists of equilateral triangle which each side is divided into equal parts. The side of the triangle represent the binary mixtures and the vertices of the triangle represent the pure component (Madeira, 2019). Deep eutectic solvents (DES) is one of the new solvent that can maintain ionic liquid IL. This solvent can be prepared easily through mixing and heating. Additionally, it is cost effective. Natural deep eutectic solvents (NADES) is considered to be less toxic than IL. Deep eutectic solvents can be used as solvents when extracting bromelain from pineapple. This is done protein precipitation. Two phase will be formed during the extraction.(Das et al., 2021)

In conclusion, bromelain has proved to be important in food, medical and dietary. Utilization of ternary liquid mixture for extraction has high efficiency, low energy consumption and environmental impact. Further research should be done on the extraction of bromelain via natural based ternary liquid mixture in order to improve the extraction.

1.2 Problem Statement

The extraction of enzyme bromelain from pineapple is usually complex that involved ultrafiltration, salt precipitation, ultracentrifugation, aqueous two phase system and size exclusion chromatography. The aqueous two phase system will make use two immiscible phases formed by mixing a polymer and salts or two incompatible polymers like polyethylene

glycol(PEG) and dextran in an aqueous system. The enzyme next will be separated by centrifugation.(Gautam *et al*, 2017)

In order to have high amount bromelain recovery the concentration of PEG and salt must be high. However, by Increasing the concentration of PEG and salt in the mixture, the partitioning of bromelain in top phase which is PEG will be decrease. This due to high molecular weight PEG. Moreover, in high concentration, the ternary structure of the enzyme which cause the biological activity of the enzyme will be reduce. (Gautam *et al*, 2017)

The ternary structure of the enzyme which has many interactions that hold them together. These interactions make sure the enzyme activity site is stabilizing and able to carry out its function. The interaction in enzyme is hydrogen bond, hydrophobic interaction, disulfide bridge and ionic bond. In salt system, water in the protein will be hydrated causing it to become hydrophobic site forming two phase because of the hydrophilic-hydrophobic interaction.

PEG which is chemically derived, is consider to be toxic that can cause health effects. By using naturally solvent like natural deep eutectic solvent would eliminate this concerns. Deep eutectic solvents which its physical properties can be tuned. Studies have shown that DES as a medium can maintain the structure and activity of enzyme or even increased. Even though, DES able show great results as solvents, there is still less understanding on why the enzyme are stable and able to retain their activities in DES (Das et al., 2021). Understanding on how the deep eutectic solvents effects the structure and active site of protein will develop a better extraction of protein-based compounds.

1.3 Research Objectives

The main purpose of this research is to extract bromelain enzyme by using a salt system with natural deep eutectic solvent. The objective is including the following detailed studies.

1. To investigate the formation of deep eutectic solvents by using natural substances.
2. To investigate the effect of deep eutectic solvents on formation of aqueous quaternary biphasic system.
3. To investigate the feasibility of bromelain extraction using aqueous quaternary biphasic system with added deep eutectic solvents.

CHAPTER 2

LITERATURE REVIEW

2.1 Deep eutectic solvent

The use of deep eutectic solvents in extraction has been widely study and research. From Scopus the journal publishes using deep eutectic solvents in extraction show an increasing trend from 2013 with 14 published journals to 2021 with 559 published journals that year. The number of patents that has been published is 100 plus per year starting from 2019. This show that deep eutectic solvents in extraction has given the researcher attention to further discover this solvent. The area that most of journal targeted is on the chemistry. The research published mostly address the deep eutectic solvent use in extraction mainly in the protein-based. DES comprised with the mixture of a hydrogen donor(HBD) and a hydrogen bond acceptor(HBA). HBA is often a quaternary salt while HBD can include carboxylic acids, alcohols or carbohydrates.(Anderson and Jared, 2017)

The most common HBA for DES is choline chloride. To further increase the application of this solvent advance research of alternatives of choline chloride is a must for easier accessible hydrogen bond acceptor. One of the natural cheap resources of HBA is betaine. Betaine which is extracted from sugar beets is biodegradable and non-toxic.(Li et al., 2016) The reason the number of DES is increased considerably is because DES is easy to prepare, inexpensive and biodegradable. DES is also known as functional medium that can be modified with polymers or silica becoming DESs-modified materials. DES has been applied in extraction because of their unique interaction with the target compound, such as anion exchange, hydrogen bonding or $\pi - \pi$, etc. It has been reported that more than 100 DES from plants has been discovered and it is reported that the solvation ability of DES is higher than water. Many common HBD and HBA counterpart will form viscous DESs. One of the most popular DES combination is between urea as HBD and choline chloride as HBA.(Tang et al., 2015)

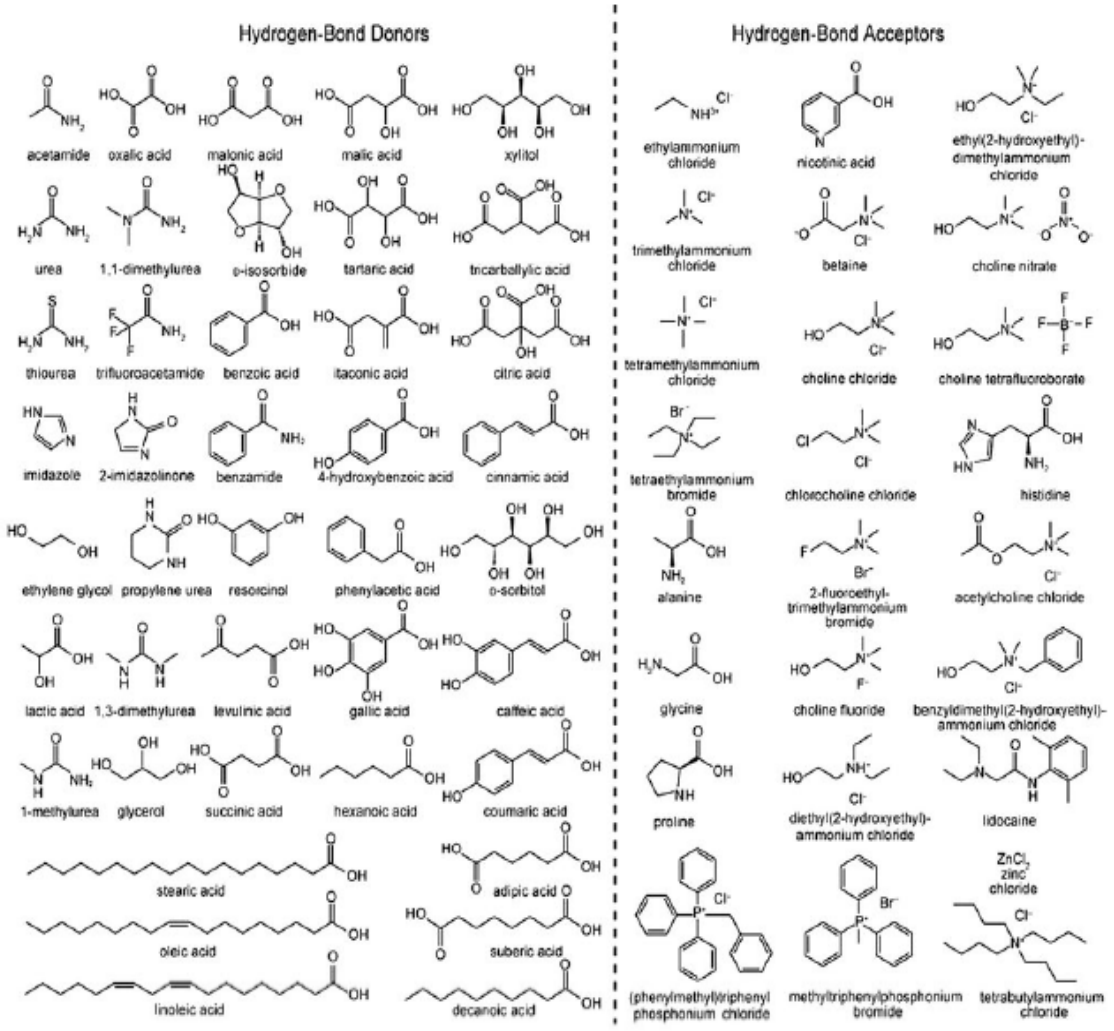


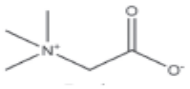
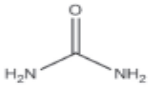
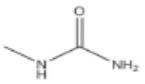
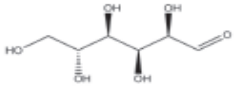
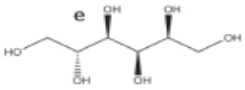
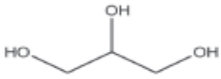
Figure 1 Some of HBD and HBA counterparts that can be combined to form DES(Tang et al., 2015)

There are two ways to prepare DESs, in which by simply mixing a HBA and HBD at a suitable temperature. The first way, is by lower melting point component is melted first, then higher melting point component will be added and the mixtures will melted together. The other way, if both of the component have high melting point, the two components will be mixed and melted together. Choi *et al.* has reported that many abundance of primary metabolites changes their state from solid to liquid when they were mixed at proper ratio.(Choi et al., 2011) It has been hypothesized that NADES as alternative media to water in living organisms.(Tang et al., 2015)

With the increase of NADES and DES, the properties of this solvent must have attracted considerable attention of researcher. The physiochemical properties of DES is mostly attributed from their compositions. The freezing point, density, conductivity and viscosity can be designed according to their structure. The freezing point of DES is lower than their HBD and HBA. This is because of the reduction of coloumb forces of DESs with the large volume and the molecular ions asymmetric charge distribution. Tang *et al* , has reported that DES of ChCL/Urea has lower melting point of 12℃ from it own component ChCL with 302℃ and urea with 133℃. Up to this date, most of DESs is in liquid state and can be prepared in room temperature. It also has been reported by some researcher that hydrogen bond donor as complexing agents will interacts with anionic group, which will increase the molecular size of DES, thus reducing interaction with cation groups causing decrease in the melting point (Abbot et al., 2004). Abbot *et al* reported that with the decreasing symmetry of ammonium cations, the melting points of DES will decrease. Ratio of HBD in DES influences the melting point. (Tang et al., 2015)

DES density is an important property that is usually determined by gravimetrically. Most of DESs have density higher than water and it is higher than pure HBD. The DES density is greatly influence by the ratio of HBD/HBA. Viscosity also an important factor to DES and most of DES have high viscosity higher than 100cP at room temperature. The viscosity of DES will increase with the decreasing of temperature. Another property that is worth mentioning is DES has excellent solubility of metal oxides, carbon dioxide and drugs. In DES the solubility will increase with increasing in pressure it work also work the other way around. (Tang et al., 2015) Li et al. (2016) has reported 6 DES can be formed by using heating method with HBA (Betaine) and HBD (Urea, Methylurea, Glucos, Sorbitol, Ethylene glycol, Glycerol) with an accurate molar ratio and stirred evenly at 100℃ until evenly, colorless liquid is formed. Table 2 show the information molar ratio needed to form DES solvent.

Table 2 Structures and composition of the six betaine-based deep eutectic solvent.(Li et al., 2016)

Hydrogen-bond acceptor	Hydrogen-bond donors	H_2O	Molar ratio
	 Urea		1:2:1
	 Methylurea		
	 Glucos		1:3:1
	 Sorbitol		1:1:2
	 Glycerol		
	 Ethylene glycol		1:2:1

2.2 Differential scanning calorimeter

This technique is primarily for measuring the thermal properties of material to establish a connection between temperature and specific physical properties of substances. Calorimeters are frequently employed in the fields of chemistry, biochemistry, cell biology, biotechnology, and pharmacology to study the thermodynamic properties of biomolecules and nanoscale materials. Differential scanning calorimeters (DSC) are one of many calorimeter varieties. A thermal analysis tool called DSC measures changes in a sample's physical characteristics as well as its temperature over time. In other words, the instrument is a tool for thermal analysis that calculates the temperature and heat flow related to material transitions as a function of temperature and time. DSC measures a heat quantity that is excessively radiated or absorbed by the sample during a temperature shift based on the temperature difference between the sample and the reference material. The evaluation of variables that are crucial to protein

stability can be done using a variety of DSC applications. As a result, the best circumstances for stabilising protein liquid formulations may be found. The macromolecule stability in DSC experiments is represented by the temperature (Tm) at the Cp curve's maximum point. (Gill et al., 2010). From figure 2, the type of graph formed can be characterised as the melting point, thermal degradation, oxidation and cold crystallization.

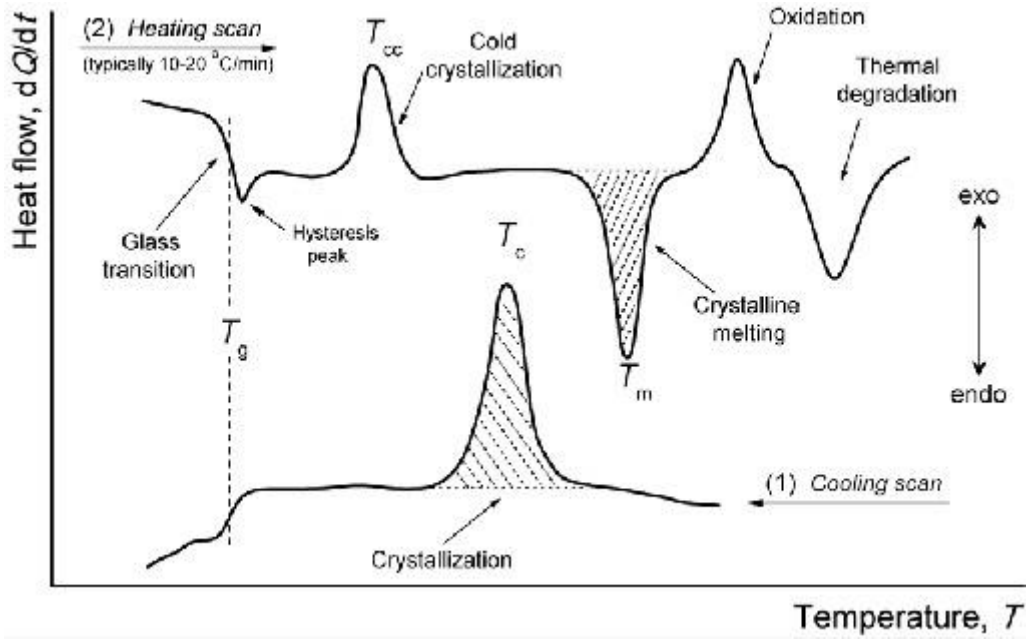


Figure 2 Differential scanning calorimeter type of graph

In DES it is important to identified the eutectic mixture thermal behaviour so that the application of a certain DES can be more widely use. Understanding the material transition would allow us to know at what temperature the DES working at optimum level.

2.3 Aqueous Two phase system

Many type of ATPS is consist of two incompatible polymers. For example, polymer and salt, a cationic and an anionic surfactant, an ionic liquid and salt or a molecular weight alcohol and salt. ILs- based ATPS has been applied in many separations because it can from transparent solution without emulsifying phenomenon. However, the cost of IL is expensive and difficult to prepare. DES has emerged as a suitable replacement for IL in the new

generation of solvent. DES-based is primarily used as enrichment in compound such as protein, DNA, RNA and anthraquinones.(Rong-tao et al., 2020)

The main component of DES-based ATPS is inorganic salt that play an important role in phase equilibrium. Li et al. (2016) has reported in their studies, using three kind of salt (Na_2HPO_4, K_2HPO_4 and KH_2PO_4) to form ATPS with DES. K_2HPO_4 has high solubility that allow it to form DES-based ATPS. Phosphate based salt has is the most commonly used because of their ability to form liquid-liquid biphasic system. The salting-out ability of inorganic salt have a great impact on the formation of ATPS. The salting-out ability is primarily affected by the anion species of inorganic salt, thus it is important to choose inorganic salt small ionic radius and highly charged ion that can induce phase separation.(Rong-tao et al., 2020)

Another important factor that affect the phase equilibrium is the concentration of inorganic salt. Wang et al. (2017) has reported that by increase the concentration of salts will facilitate ATPS construction and enhances the salt-out effect caused by the hydration effect os salt ions with water. The water rich in DES phase will move towards salt-rich phase, which cause the hydrophobicity of salt-rich phase. To further understand the concept of effect of salt concentration and salt effect on ATPS formation based on DES, usually phase diagram will be constructed. (T. Wang et al., 2017)

DES chosen also have effect on the phase equilibrium that is formed. This is because DES that is from HBA and HBD has different hydrophilic property. Li et al (2016) has reported from the formation of ATPS with K_2HPO_4 and DES (K2HPO4 and DES (ChCl/1,4-putylene glycol, ChCl/ethylene glycol, ChCl/glycerine, ChCl/glucose) and found that the ability of forming phase follows this order: ChCl/1,4-putylene glycol > ChCl/ethylene glycol> ChCl/glycerine > ChCl/glucose. It has been found that ChCL/urea has a great phase separation

ability. HBD plays an important role in determining the difference in DES hydrophobicity. (Rong-tao et al., 2020)

Zhang et al. (2017) stated that the average molecular weight of DES effect the formation capacity of DES-based ATPS. The formation of phase-forming will increase with the increase of molecular weight. This is because of the electrostatic repulsion that caused by the mutual miscibility between hydrated ions and DES to gradually decrease with increasing of molecular weight. This process will increase the hydrophobicity of DES.(Rong-tao et al., 2020)

For a better understanding mechanism of separation DES-based ATPS, the microscopic structure must be studied. The top phase will include DES, water and small amount of salt while the bottom phase will include salt, water and small amount of DES. At top phase, in high DES concentration, HBA and HBD will have a stable association because of their hydrogen bonding. Strong solubilizing capability of DES is due to the electrostatic interaction between the target analytes.(Rong-tao et al., 2020)

2.3 Extraction of protein

Multiple factor effects the extraction of protein using DES-based ATPS such as ATPS, pH, extraction time and temperature. In the solution of salt K_2HPO_4 with DES betaine/urea the temperature effect with the increasing of temperature from 20℃-30℃ will increase the extraction efficiency. The protein extraction is inherently endothermic. The extraction efficiency will continue to decline if the temperature is increase up to 50℃. This is due to high temperature that will destroy the hydrogen bonding in DES and protein, resulting a decline in extraction efficiency. (Rong-tao et al., 2020)

The pH has always be known to affect protein in great extent. The case is the same in DES-based ATPS protein extraction. Research has recoded that the extraction efficiency reach it climax at isoelectric point. Protein will represent in cation when pH<pI and vice versa. This

effect the electrostatic interaction between the ionic group of DES and charged group of protein. However, this factor does not improve the extraction efficiency. This is due to pH only effects the surface charges of the protein. (Rong-tao et al., 2020)

In term of concentration of inorganic salts, in high concentration of salt in protein solution it will increase the hydrophobic interaction, increase distribution of proteins in DES and reduce solubility of proteins in water. However, the extraction efficiency will start to decrease when the concentration of salt is too high. This is due to the molecular structure of protein. There is a driving forces resulting from hydrogen bonding interaction between water molecule on surface of protein and amino acid residue that is transfer the protein into the salt-rich bottom phase when the bottom phase is hydrophilic.(Rong-tao et al., 2020)

The effect of DES on the activity of protein also has been reported by Pang et al. (2017) that the proteins detected before and after extraction via UV-vis spectroscopy. The spectrum of proteins in the pure water is the same as the proteins in DES-rich phase after extraction. This results indicate that there is no chemical interaction occurred between protein and DES during extraction. (Rong-tao et al., 2020)

CHAPTER 3

METHODOLOGY

3.1 Research flowcharts

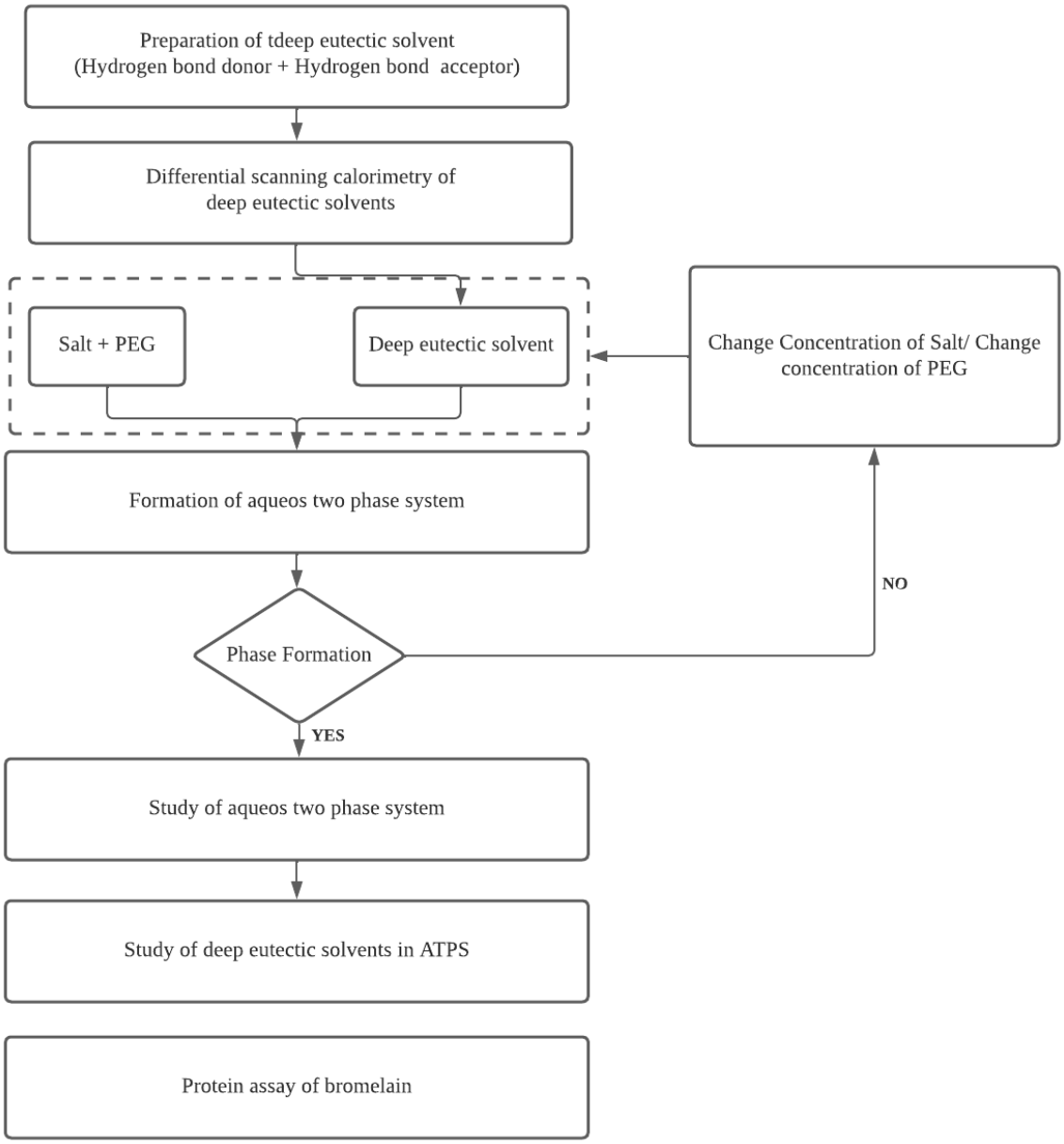


Figure 3 Research flowsheet

3.2 Materials and chemicals

Pineapple fruit from local store. Deionized water from laboratory. Choline Chloride (ChCl), urea (U), lactic acid (LAC), glucose (Glu), glycerol, Water, potassium phosphate (K_2SO_4), sodium sulfate (Na_2SO_4), ammonium sulfate (NH_4SO_4), Polyethylene Glycol 2000 (PEG) are supplied by Sigma-Aldrich, Co. Novagen BCA kit is supplied by Fisher Scientific

and contain BCA solution, Bovin serum albumin, 4% cupric sulfate and standard protein with concentration 2g/ml. BCA solution contain bicinchionic acid, sodium carbonate, sodium tartrate, sodium bicarbonate in 0.1M NaOH, pH 11.25.

3.3 Process of the experiment

3.3.1 Preparation of deep eutectic solvent

DES was synthesized by mixing hydrogen bond donor urea with hydrogen bond acceptor choline chloride. The preparation was done by using heating method. HBA and HBD is mix according to molar ratio either with 1:1 ,1:2 or 2:1. Only one type of HBA is use in which choline chloride while there is four HBD which is urea, lactic acid, glycerol and glucose. HBA and HBD was weighted on the analytical balance before mixing to ensure there molar ratio is accurate. After both of them is mix in a beaker, the mixture is heated at 70℃ while being stir at 400rpm. The mixture is mix until the become clear homogenous liquid. After that the mixture is being let cooled to the room temperature.

3.3.2 Formation of aqueous quaternary biphasic system

The PEG was prepared in 0.3M as a phase former is mix with four type of salt which is sodium sulfate, ammonium sulfate and potassium phosphate. The PEG is mix with each of the salt for every concentration (0.5M, 1.0M, 1.5M, 2.0M, 2.5M and 3.0M). The PEG and salt is mix 1:1 in volume. The height of top and bottom phase of the mixture is measured if the there two phase form. If there is no formation of two phase the salt concentration will be change. The time take for the formation of two phase for each of the mixture is also taken. Time taken will be the main factor in determining the best salt.

After obtaining the fastest salt form the two phase, the PEG concentration will be manipulated. The concentration of PEG will be 0.2M and 0.3M. The same analysis was done towards the mixture from the manipulated of salt and concentration.