

PRODUCTION OF GAMMA-CYCLODEXTRIN BY
Bacillus cereus CYCLODEXTRIN GLYCOSYLTRANSFERASE
USING EXTRACTIVE BIOCONVERSION IN AQUEOUS TWO-
PHASE SYSTEM

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PHASE SYSTEM**

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ABSTRACT

The first aim of the study was to introduce a practical approach to the recovery of CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTase) from *Bacillus cereus* using polyethylene glycol (PEG)/potassium phosphates aqueous two-phase flotation (ATPF) system. The optimal condition for purification of CGTase was attained at 18.0 % (w/w) PEG 8000, 7.0 % (w/w) potassium phosphates, V_R of 3.0, 20 % (w/w) crude load at pH 7, and 80 min nitrogen flotation time at a flow rate of 5 L/min. With this optimal condition, purification factor (P_{FT}) of 21.8 and a yield (Y_T) of 97.1 % were attained. CGTase was successfully purified in a single step using the ATPF. Another aim of the study was on the extractive bioconversion of CGTase using ATPS. Aqueous two-phase system (ATPS) extractive bioconversion provides a platform to integrate bioconversion and purification into a single step process. Thus, the investigation focused on the design of different ATPSs to reduce the steps in bioconversion and purification, thereby increasing the yield and reducing the cost and time of production. Each of the ATPS was evaluated independently. Extractive bioconversion of gamma-cyclodextrin (γ -CD) was evaluated using polyethylene glycol (PEG)/potassium phosphates based ATPS with ATPF-purified CGTase enzyme and soluble starch. The optimum condition was attained in the ATPS constituted of 30.0 % (w/w) PEG 3000 and 7.0 % (w/w) potassium phosphate. A γ -CD concentration of 1.60 mg/mL with a 19 % concentration ratio was recovered after 1 hour bioconversion process. The γ -CD was primarily partitioned to the top phase ($Y_T = 81.88\%$), with CGTase partitioning in the salt-rich bottom phase ($K_{CGTase} = 0.51$). Alcohol/salt based ATPS for the extractive bioconversion of γ -CD was studied after the PEG/potassium phosphate-based ATPS experimentation. The effects using alcohols (ethanol, 1-propanol, and 2-propanol) on CGTase bioactivity were

investigated. The results showed that the optimum condition for extractive bioconversion of γ -CD was achieved in ATPS of ethanol 24 % (w/w) with potassium phosphates 20 % (w/w) system in the presence of 3.0 % (w/w) sodium chloride (NaCl). The 1 hour extractive bioconversion process recorded a concentration of 0.23 mg/mL of γ -CD harvested. Finally, extractive bioconversion of γ -CD from soluble starch with CGTase enzyme was studied using ethylene oxide-propylene oxide (EOPO)/potassium phosphates ATPS. Different molecular weights of EOPO with potassium phosphates were tested to study their partitioning effect on γ -CD and CGTase. The results showed that the optimum top phase γ -CD yield condition (74.4 %) was achieved in 35.0 % (w/w) EOPO 970 and 10.0 % (w/w) potassium phosphate with 2.0 % (w/w) NaCl. A total of 0.87 mg/mL concentration of γ -CD was produced in EOPO/phosphates ATPS top phase after two hours. The PEG/potassium phosphates ATPS was found to be a better system for extractive bioconversion of γ -CD. The main conclusion of this study was ATPS has a good potential to be applied industrially for the recovery of CGTase and γ -CD production.

ABSTRAK

Tujuan pertama kajian ini adalah untuk memperkenalkan strategi untuk penulenan CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTase) dari *Bacillus cereus* dengan menggunakan sistem pengapungan berair dua fasa (ATPF) polietilena glikol (PEG)/kalium fosfat. Keadaan optimum penulenan CGTase telah dicapai pada 18.0% (w/w) PEG 8000, 7.0 % (w/w) kalium fosfat, V_R 3.0, 20 % (w/w) enzim mentah pada pH 7, dan 80 min masa pengapungan gas nitrogen dalam kadar 5 L/min. Dengan keadaan optimum, faktor penulenan (P_{FT}) 21.8 dan penghasilan (Y_T) 97.1 % telah dicapai. CGTase telah berjaya ditulen dengan menggunakan ATPF dalam satu langkah. Tujuan selain itu adalah mengaji konversi bio ekstratif CGTase dengan menggunakan akueus sistem dua fasa (ATPS). Konversi bio ekstratif ATPS memberikan pentas untuk mengabungkan proses konversi bio dengan proses penulenan. Oleh itu, projek eksperimen ini mengaji pelbagai ATPSs untuk mengurangkan langkah-langkah dalam penukaran bio dan penulenan, bagi meningkatkan penghasilan produk, mengurangkan kos dan masa proses, setiap ATPS telah diuji secara bersendirian. Konversi bio ekstratif cyclodextrin gamma (γ -CD) diuji oleh ATPS PEG/kalium fosfat menggunakan enzim CGTase yang ditulen oleh ATPF dan kanji larut. Keadaan optimum telah dicapai dalam ATPS yang mengandungi 30.0 % (w/w) PEG 3000 dan 7.0 % (w/w) kalium fosfat. Kepekatan γ -CD 1.60 mg/mL dengan nisbah kepekatan 19% telah dihasilkan selepas 1 jam proses konversi bio ekstratif. Kebanyakan γ -CD melarut dalam fasa bahagian atas ($Y_T = 81.88$ %), manakala kebanyakan CGTase melarut dalam fasa bahagian bawah ($K_{CGTase} = 0.51$). Konversi bio ekstraktif γ -CD dikaji guna alkohol/garam ATPS selepas ATPS PEG/kalium fosfat. Pengaruh alkohol (etanol, 1-propanol dan 2-propanol) dalam aktiviti bio CGTase telah dikaji. Hasil kajian menunjukkan bahawa keadaan

optimum untuk konversi bio ekstratif γ -CD telah dicapai dalam ATPS mengandung etanol 24 % (w/w), kalium fosfat 20 % (w/w) sistem dan 3.0 % (w/w) natrium klorida (NaCl). Kepekatan sebanyak 0.23 mg/mL γ -CD direkod selepas 1 jam proses konversi bio ekstratif. Akhir sekali, konversi bio ekstraktif γ -CD daripada kanji larut dengan enzim CGTase telah dikaji menggunakan oksida etilena oksida propilena (EOPO)/kalium fosfat ATPS. Pengaruh berat molekul EOPO telah dikaji untuk melihat kesan pelarutan fasa γ -CD dan CGTase. Kajian menunjukkan bahawa keadaan optimum Y_T γ -CD (74.4%) telah dicapai dalam ATPS mengandung 35.0 % (w/w) EOPO 970, 10.0 % (w/w) kalium fosfat dengan 2.0 % (w/w) natrium klorida. Kepekatan γ -CD sebanyak 0.87 mg/mL telah dihasilkan di fasa atas ATPS EOPO/fosfat selepas proses konversi bio 2 jam. ATPS PEG/kalium fosfat merupa sistem yang terbaik untuk konversi bio ekstraktif γ -CD. Kesimpulan kajian ini menyatakan bahawa ATPS mempunyai potensi untuk penghasilan dan penulenan CGTase dan γ -CD dalam skala industri.

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LIST OF SYMBOLS AND ABBREVIATIONS

ATPS	aqueous two-phase system
BCA	bicinchoninic acid
BSA	bovine serum albumin
CGTase	CYCLODEXTRIN GLYCOSYLTRANSFERASE
dH ₂ O	distilled water
<i>E</i>	efficiency
EO	ethylene oxide
h	hour
K	partition coefficient
kDa	kilodalton
KH ₂ PO ₄	potassium di-hydrogen phosphate
K ₂ HPO ₄	di-potassium hydrogen phosphate
LCST	lower critical solution temperature
M	molar concentration
min	minute
mL	milliliter
M _n	number-average molecular weight
mol. wt.	molecular weight (g mol ⁻¹ or kDa)
NaCl	sodium chloride
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
<i>P</i> _{FT}	purification fold in top phase
pH	puissance hydrogene

pI	isoelectric point
PO	propylene oxide
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
sp.	species
TLL	tie-line length
V_B	volume of bottom phase
V_R	volume ratio
V_T	volume of top phase
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
Y_T	yield in top phase

CHAPTER 1: INTRODUCTION

CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTase, E.C. 2.4.1.19) is an enzyme capable of synthesizing cyclodextrins (CDs) from starch and is in high demand in the enzyme market (Mazzer et al., 2008). To date, several purification strategies have been formulated for CGTase recovery from feedstocks with multiple steps of downstream processes are needed to purify the enzyme. CGTase conventional purification strategies often involve ammonium sulfate precipitation, starch adsorption followed by chromatographic purification process. This constituted a major cost drawback for the CGTase production. The problem is compounded by a low production yield (Gawande and Patkar, 2001; Martins and Hatti-Kaul, 2002; Cao et al., 2004). In this study, aqueous two phase floatation (ATPF) was applied for the purification of a bacterial CGTase.

The CDs are cyclic oligosaccharides produced by CGTase enzyme. CDs showed a structural feature that enables them to form inclusion complexes with a wide range of guest compounds. The CDs inclusion effects promote their applications in various industries such as food, pharmaceuticals, cosmetic, agricultural and chemical (Lofisson and Brewster, 1996; Del Valle, 2004). There are three major types of CDs, α -, β -and γ -CD which formed by 6, 7 and 8 glucopyranose units respectively (Singh et al., 2002). Among three types of γ -CD, gamma-Cyclodextrin (γ -CD) is the most expensive CD type in the market because it is generally least accessible (Thatai et al, 1999). Hence, ATPS was investigated in this work to potentially improve productivity and separation of γ -CD by newly isolated *Bacillus cereus* CGTase (Ng et al., 2011).

In this study, the aqueous two phase system (ATPS) was adopted for the extractive bioconversion of γ -CD, ATPS is formed by mixing two incompatible aqueous solutions at a concentration beyond the specific threshold concentration (Beijerinck, 1896). The application of ATPS in extractive bioconversion provides a technique which integrates bioconversion and purification into a single step process. Extractive bioconversion of ATPS is normally designed to make the biocatalyst partitioned in one phase and desired product in another phase. The biocatalyst retained in one phase can be reused. Thus, continuous extractive bioconversion process is achievable in this condition, with the reduction of time and cost as compared to the conventional method (Ziljistra, 1996; Ziljistra et al, 1998).

Conventional purification methods such as packed bed chromatography have been widely used in biopharmaceuticals purification processes due to their high resolving power and simplicity. However, its high media cost and long cycle time resulted in high production cost (Przybycien et al., 2004). It has also been reported that the high pressure in a packed column of conventional chromatography caused compaction of the bed and low flow rate (Safert and Etzel, 1997). These problems impose practical limitation for throughput scalability. Purification method through ATPS can overcome the limitations of packed-bed chromatography. The advantages of this method include shorter processing time, high capacity, high yields and high biocompatibility and ease of scaling up procedure (Albertsson, 1986). Moreover, the ATPS offers integration of the numerous conventional downstream processes such as clarification, concentrating, and partial purification into a single step operation (Madhusudhan et al., 2008). The ATPS provides a simple, low cost, and low pollution technique for protein purification. It has been proven to provide a biocompatible environment for the separation of proteins and enzymes from protein mixtures or cell extracts, since both phases consisted of

predominantly high water content (Albertsson, 1986; Agasoster, 1998; Gupta et al., 1999). ATPS is, therefore, a better purification technique for biomolecules as compared to other conventional purification methods.

The ATPS is formed by dissolving the water-soluble phase components beyond a critical concentration that allows the formation of two immiscible phases (Zaslavsky, 1995). It selectively partitions and concentrates target biomolecules into one of the phases, and the basis of partitioning depends upon surface properties of the particles and molecules, such as size, charge, and hydrophobicity (Walter and Johansson, 1994). It generates a gentle purification environment that can maintain the native structure of biomolecules since both phases of ATPSs are predominantly water-based (80-85 %) (Arun, 2008). High purification can be achieved in ATPS by manipulating the system properties that are involved in chemical and physical interactions of partitioning process (Rosa et al., 2010).

Reports from the literature have shown that ATPS has been successfully used for purification of various protein such as lipase and alcohol dehydrogenase (Ooi et al., 2011; Madhusudhan et al., 2008). However, there is no detailed information on the ATPF purification of microbial CGTase and ATPS extractive bioconversion of γ -CD has yet to be explored. Different ATPSs were investigated and optimized for the CGTase recovery and an integrated extractive bioconversion for γ -CD production was developed with the aim to purify CGTase and to achieve an efficient and high production yield of γ -CD.

OBJECTIVES:

- i. To recover and purify *Bacillus cereus* CGTase from crude feedstock using ATPS combined with solvent sublation;
- ii. To investigate extractive bioconversion of γ -CD using PEG/salt ATPS;
- iii. To develop an integrated soluble starch bioconversion and recovery of γ -CD using alcohol/salt ATPS;
- iv. To study the integrated bioconversion and recovery of γ -CD using thermo-separation ATPS;
- v. To compare and determine the most efficient ATPS for extractive bioconversion of γ -CD among ATPSs tested.

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CHAPTER 2: LITERATURE REVIEW

2.1 CYCLODEXTRIN GLYCOSYLTRANSFERASE

CGTase is an extracellular enzyme belonging to α -amylase family glycosyl hydrolases (Stam et al., 2006). CGTase is a hydrolytic enzyme resembling the structure of α -amylase with 30 % amino acid sequence similarity including its active site. Although the enzyme is capable of hydrolyzing starch into shorter starch chain or malto-oligosaccharides, CGTase catalyses mainly the transglycosylation reaction, resulting in cyclic malto-oligosaccharides in contrast to the α -amylase degrading starch into random linear malto-oligosaccharides (Kimura et al., 1987; Martin, 2004).

The CGTase catalytic mechanism, also known as the α -retaining double replacement mechanism, involves two catalytic residues: Glu257 as an acid/base catalyst and Asp229 as the nucleophile (Sinnott, 1990). In the presence of substrate, Glu257 performs the role of an acid donating a proton to the scissile glycosidic bond oxygen and the glycosidic bond is cleaved constructing an oxocarbenium ion-like transition state. The transition state will transform into a stable covalent intermediate that is glycosidically bonded to the nucleophile i.e. Asp229 after the bond cleavage reaction. Glu257 will then act as a base activating the guest compound (acceptor). The activated acceptor will attack the previous covalent intermediate forming the new glycosidic product bond (Sinnott, 1990; White and Rose, 1997). In all the CGTase catalysed-reactions, glycosidic exchange of a guest saccharide and substrate occurs at the part of the substrate that is not covalently bonded to the enzyme CGTase (Uitdehaag et al., 2002).

The CGTase has the unique capability to catalyze the conversion of linear starch oligosaccharide chains into cyclodextrins (CDs) *via* an intramolecular transglycosylation reaction (Paloheimo et al., 1992). In the cyclization reaction, cyclodextrins are formed in consequence of the addition of glycosidic C4 hydroxyl group non-reducing end across the scissile on β -(1,4) glycosidic bond by CGTase (Doukyu et al., 2003).

Apart from the capability to catalyze the intramolecular transglycosylation reaction (cyclization reaction), CGTase is a multifunctional enzyme which also catalyze the intermolecular transglycosylation (coupling reaction) and disproportionation. Coupling reaction is the reverse reaction of cyclization in which the γ -CD ring is cleaved at CGTase active site before transferring to the acceptor molecule to form linear products. Moreover, CGTase possesses weak hydrolytic activity where water acts as the glycosyl acceptor (Penninga et al., 1995). In brief, CGTase catalyzes four enzymatic reactions involving oligosaccharides *viz.* coupling, cyclization, hydrolysis and disproportionation (Kelly et al., 2008).

2.1.1 Sources and characteristics of bacterial CGTases

CGTases change starch into a mixture of α -, β -, and γ -CDs in a different ratio depending on the enzyme source and the conditions of reaction. CGTases are classified into α -, β -, and γ -CGTases based on the main CD synthesized (Gawande and Patkar, 2001). Most CGTases produce mainly α - and β -CDs with small amounts of γ -CDs. CGTases are found in a wide array of bacterial species like *Bacillus*, *Brevibacterium*, *Clostridium*, *Corynebacterium*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Thermoanaerobacter*, *Thermoanaerobacterium* and most recently in *Archaea* (Tachibana et al., 1999; Biwer et al., 2002; Tonkova, 1998). CGTases produced by the

genus *Bacillus* are among the most common and extensively studied CGTases because of the rapid enzyme expression time, stable over higher CGTases production and a broad pH range at optimal growth condition (Hatti-Kaul and Martins, 2002).

CGTases synthesis in *B. cereus* culture is advantageous in comparison with other bacterial CGTases synthesis since they produce a larger quantity of CGTases in a shorter time, normally after 16 to 20 hours of cultivation to reach enzyme activity peak (Jamuna et al., 1993). Most bacterial CGTases are extracellular enzymes in nature secreted into the fermentation broth during cultivation (Tonkova, 1998). Bacterial CGTases are readily produced by submerged fermentation (Patkar and Gawande, 1999; Gawande et al., 1998; Stefanova et al., 1999) at the optimum physio-environmental factors e.g. pH, temperature, aeration, incubation period and agitation (Ramakrishna et al., 1994) and the nutrient medium with varying composition (e.g. carbon, nitrogen source, and some micronutrients) (Tonkova, 1998). CGTase highest production was observed in the stationary phase (Savergave et al., 2008). Extracellular expression of CGTases in culture broth is greatly influenced by the presence of carbon sources ranging from variety of starches (e.g. soluble starch, soluble starch, corn starch and cassava starch) (Ibrahim et al., 2005) to sugars (e.g. sucrose, fructose, maltose and glucose) and some dextrans (e.g. maltodextrins) (Tonkova, 1998). Soluble starch seems to be an interesting alternative carbon source for γ -CD production since it is easily available in high quantity and low production cost as compared to other starches (Fasihuddin and Williams, 1996).

Maximum enzyme activities are exhibited by bacterial CGTases at a wide pH range from 4.5 to 10.0 and a wide thermal stability from 40°C to 85°C (Tonkova, 1998). Most of the reported CGTases from *Bacillus sp.* are monomeric and possess a molecular

weight of 33 to 103 kDa (Hatti-Kaul and Martins, 2002). *B. cereus* is a Gram-positive bacterium secreting extracellular CGTase at a neutral growth condition with carbon source (Jamuna et al., 1993). CGTases are produced by the bacteria to convert starch into compounds that can be readily utilized by themselves (Pocsi, 1999).

2.1.2 Specific CGTase activity

CGTases from different microbial sources often show varying cyclization levels, coupling, disproportionation and hydrolysis activities (Martins and Hatti-Kaul, 2003). There are some enzyme activity assays that are developed to quantify CGTase based on these catalytic properties. CGTase cyclization activity is normally determined through discoloration of phenolphthalein solution at 550 nm, occurring after an inclusion complex formation by β -CD in the presence of phenolphthalein (Miikeléi et al., 1988; Goel and Nene, 1995).

Coupling CGTase activity can be determined using commercial α -, β - and γ -CD as the donor molecules while methyl- α -D-glucopyranoside as the acceptor. The glucose produced as the result of the coupling activity was measured using the Glucose/GOD-Period method (Nakamura et al., 1993). To measure the disproportionation activity of CGTase, an assay using 4-nitrophenyl- α -D-maltoheptaoside-4-6-O-ethylidene (EPS) as a donor substrate and maltose as the acceptor has been developed. The glucose and *para*-nitrophenol liberated are evaluated through light absorption in this assay (Nakamura et al., 1994; van Der Veen et al., 2000). On the other hand, hydrolytic activities exhibited by CGTase can be determined through the number of reducing ends presence after the hydrolysis process using dinitrosalicylic acid (Penninga et al., 1996).

2.1.3 Applications of CGTases

CGTases are enzymes involved in the various industrial applications, and the most important CGTases application is the production of cyclodextrins. CGTases are also utilized in starch liquefaction due to their inability to cut through α -(1,6) linkages in gelatinized starches (Pedersen et al., 1995). Degradation of starch by CGTases leads to a decrease of viscosity whereas maintaining the high-molecular feature of starch. This unique feature of CGTase is well adapted in surface sizing or coating of paper, improving the writing quality by forming a glossy surface for printing (Uitdehaag et al., 2001). In addition, CGTases are employed in the synthesis of modified oligosaccharides (e.g. stevioside) where CGTases catalyze stevioside glycosylation to increase the water solubility and sweetness (Jung et al., 2007).

2.1.3.1 Cyclodextrins and their applications

The CDs are cyclic oligosaccharides of six (α -CD), seven (β -CD), eight (γ -CD) or more glucopyranose units linked by α -(1,4) glycosidic bonds (Szejtli, 1998, Li et al., 2007). The α -CD, β -CD and γ -CD are the main products of starch degradation by CGTase are also known as the cycloamyloses, cyclomaltoses, and Schardinger dextrin (Eastburn and Tao, 1994). The γ -CD is normally the least accessible and most expensive CD (Thatai et al., 1999).

The CD molecules possess the steric arrangement of glucose units forming a hollow truncated cone shape with hydrophilic outer surface, making CD water-soluble with a hydrophobic internal cavity (Lejeune et al., 1989; Moriwaki et al., 2007). This unique structure of CDs enables them to form inclusion complexes with a wide range of guest compounds by encapsulating the compounds partially into their hydrophobic cavity (Szejtli, 1990; Lofsson and Brewster, 1996). There are cleavage and no formation of

covalent bonds that are involved during the establishment of the inclusion complex (Stalcup and Schneiderman, 2000) where its formation between the guest compounds and the γ -CD are mainly driven by the release of enthalpy-rich water molecules from the γ -CD cavity. More hydrophobic guest compounds take the place of the water molecules leading to an apolar-apolar association and reduction of γ -CD ring strain. In consequence, a more stable lower energy state is attained (Szejtli, 1998).

The CD inclusion effects change the physiochemical properties of the guest compounds promoting the utilization of CDs in diverse industrial applications such as in food, pharmaceuticals, cosmetics, environment protection, bioconversion, packing and the textile industries (Gawande and Patkar, 2001; Del Valle, 2004). Several applications of CDs in industrial processes and biotechnology are summarized in Table 2.1.

Table 2.1: Overview of applications of CDs (from Del Valle, 2004).

Industry/Field	Application
Cosmetic	<ul style="list-style-type: none">• Volatility suppression• Stabilizer and odour controller• Flavour protection• Odours reducer and antimicrobial control
Foods and flavours	<ul style="list-style-type: none">• Used in process aids such as to remove cholesterol• Texture improver• Reduce bitterness, ill smell and to stabilize flavours• Complexation with sweetening agents to improve taste• Flavour enhancer• Preparation of antimicrobial food preservatives• Flavour retainer
Pharmaceuticals	<ul style="list-style-type: none">• Enhancing drug delivery across biological membranes• Reduction in volatility• Improved bioavailability of drugs• Stabilization of substance• Reducing skin damage• Reducing irritant tasting of drugs
Agricultural and chemical	<ul style="list-style-type: none">• Separation of isomers and enantiomers• Stabilization, encapsulation and adsorption of contaminants• Solubility enhancement• Delay of seed germination

2.2 Separation, purification and recovery strategies for bacterial CGTases and CDs

In spite of the various pharmaceutical and industrial utilizations of CDs and CGTases, they suffer from several drawbacks limiting the applicability of CDs and CGTases. CGTases destined for pharmaceutical administration often require higher purity level. Besides, the natural CGTases produce CDs in a mixture of (α -, β -and γ -CDs, and the purification of γ -CDs are mostly complemented through selective crystallization using hazardous and costly organic solvents. In addition, the immediate separation of CDs was required for product inhibition of CGTases during starch conversion from the production mixtures (Peninga et al., 1996).

2.3 Aqueous two-phase system

The phenomenon of two-phase separation in a liquid was first discovered by Dutch microbiologist M. Beijerinck, who reported that two phases will be formed from the gelatin and agar mixture beyond the specific threshold concentration (Beijerinck, 1896). However, the ATPS phase separation technique was first developed by a Swedish biochemist, P. A. Albertsson in 1986 (Albertsson, 1986). Since then, ATPS has become a powerful tool for separation of biomaterials. Various biological products such as proteins, nucleic acids, virus particles, microorganism, plant and animal cells have been successfully purified using ATPS method (Albertsson, 1986; Hatti-Kaul, 2001).

ATPS involves two operational steps, which are equilibration and phase separation. Equilibration can be done by shaking or agitation, which mixes the phase components to achieve equilibrium. The time requires for phase separation of ATPS under gravity may vary from a few minutes to a few hours, centrifugation is therefore generally applied in phase separation of ATPS in order to quicken the process (Hustedt et al.,

1985). Equilibration and phase separation can be performed by mixer-settler, column contactor or centrifugal separators in large-scale ATPS (Cunha and Aires-Barros, 2000).

The interfacial tension of ATPS is between 0.0001 and 0.1 dyne/cm. This extremely low interfacial tension creates high interfacial contact area of the dispersed phases, which in turn, enhances the efficiency of the mass transfer (Albertsson, 1986). The distribution of substance between two phases is usually dominated by the properties of partitioned substance such as size, net charge and surface properties (Albertsson, 1986). Partitioning of small molecules is usually more even, whereas partitioning of large molecules is relatively one-sided. The interactions such as hydrogen bond, van der Waals forces, electrostatic interactions, steric effects, hydrophobicity, biospecific affinity interactions and conformational effects between the phase components and the substance also contribute to the partitioning of the particular substance (Albertsson, 1986; Albertsson et al., 1990). Therefore, optimization of the ATPS purification process can be achieved by manipulating the variables such as the concentration of phase components, volume ratio (V_R), pH and the presence of additives (e.g. NaCl) (Rito-Palomares, 2004).

2.3.1 Advantages of ATPS

The ATPS provides a simple, low cost, and low pollution technique for protein purification. ATPS has been reported as an interesting alternative purification method since it combines several early processing steps (recovery, concentration and purification) into a single step process (Mazzola et al., 2008). The desired biological product can be concentrated by partitioning the desired substance into the smaller volume of the extraction phase of ATPS. The use of inexpensive phase components in ATPS helps to lower the investment cost since chemical cost is considered as the

dominant cost factor for large-scale protein purification. Additionally, ultrafiltration can be used to recycle both polymer and salt and thereby making the whole process more economical (Hustedt, 1985; Veide et al., 1989). Problem of downstream pollution may also be avoided by recycling the ATPS phase components (Hatti-Kaul, 2000).

ATPS is suitable for purification of protein that is difficult to be purified by other separation techniques. It has proven to provide a biocompatible environment for the separation of proteins and enzymes from protein mixtures or cell extracts, since both phases are consisted of predominantly high water content (Albertsson, 1986; Agasoster, 1998; Gupta et al., 1999). Moreover, polymers such as PEG in ATPSs have stabilizing effect on maintaining the biological activities and native structure of the protein (Albertsson, 1986).

Extraction of ATPS is relatively rapid and the processing capacity of ATPS is high. The extent of ATPS purification to large scale application is feasible due to the simplicity and reliability of scaling-up approach as compared to other over conventional purification methods (Hatti-Kaul, 2000).

2.3.2 Preparation of ATPS

The phase-forming components in each phase have to be selected in the first step of ATPS preparation. The published data may serve as general references and guidance in making the choices. If negative purification result occurred, other phase-forming components should be tested until acceptable purification result is achieved (Benavides and Rito-Palomares, 2008). After that, phase diagram should be constructed in order to create a series of ATPS for preliminary partitioning experiments. The system

parameters of the ATPS such as TLL, V_R and pH can be manipulated to achieve an optimum purification performance (Rito-Palomares, 2004; Rosa et al., 2010).

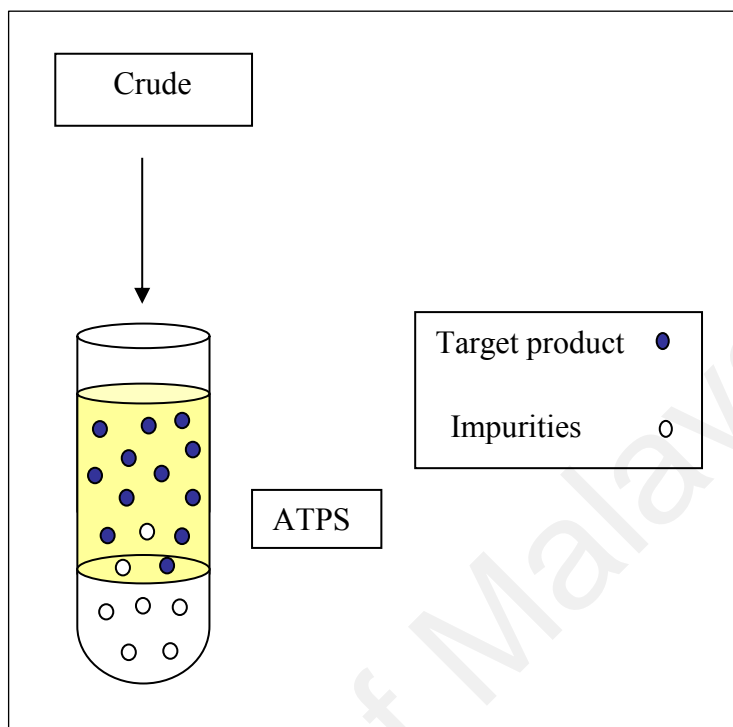


Figure 2.1: Illustration of ATPS.

2.3.3 Phase diagram

Phase diagram (Figure 2.2) is a tool to study the potential working area of particular ATPS. The phase diagram is able to provide information such as concentration of phase components that is required to form a two-phase system, concentration of phase components in top and bottom phase, and volume ratio (V_R) of two-phase system (Kaul, 2000). The region above the binodal curve in the phase diagram shows concentration of phase-forming components that form two immiscible phase system. Binodal curve defines the borderline between two-phase region and one-phase region. Turbidometric titration, cloud-point method, and node determination are the three common methods for the determination of binodal curve (Kaul, 2000).

Tie-line length (TLL) is used to describe the concentration of phase components in the ATPS. Increasing TLL corresponds to an increase in concentration of top and bottom phase compositions. The two nodes T and B (Figure 2.1) on binodal curve which connect the tie-line show the concentration of phase components in top and bottom phases, respectively (Kaul, 2000). The points along the same tie-line contain different V_R but they share the identical concentration of phase compositions in their respective phases. The A1, A2, and A3 plot (●) in phase diagram (Figure 2.1) represent the total compositions of three systems lying on the same tie-line with different volume ratios. The critical point, Cp (○) is determined by extrapolation (----) through the midpoints of a number of tie-lines. Length of the tie-line can be calculated by the analysis of the top and bottom phase compositions, using the equation:

$$TLL = (\Delta X^2 + \Delta Y^2)^{1/2} \quad (1)$$

where X and Y denote the concentration of top and bottom phase components, respectively.

Optical rotation, refractive index, dry weight and conductivity are the measurements which can be used to analyze the concentration of phase composition (Albertsson and Tjerneld, 1994).

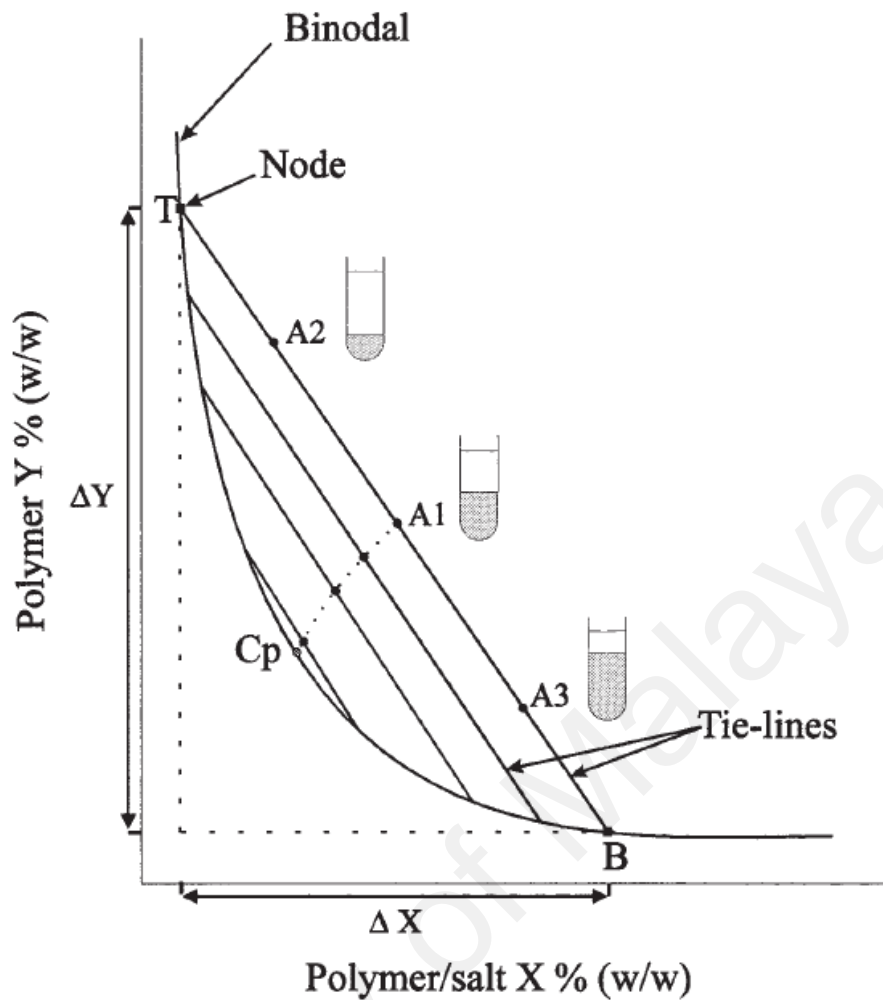


Figure 2.2: Illustration of phase diagram for an ATPS (Kaul, 2000).

2.4 Product recovery

ATPS has been widely exploited in the recovery of biological products due to its unique feature of high water content. The isolation of biological products from crude feedstock is often coupled with the laborious and time-consuming investigations on the effects of ATPS parameters (i.e. pH, concentrations of phase components, and etc) for efficient purification of the biological products. ATPS can be easily applied as the first step of purification process to remove cells, organelles and insoluble particles (Azevedo et al., 2009). In the recovery of therapeutic proteins and food products in industries, non-toxic ATPS such as PEG/dextran ATPS has been widely adapted for the industrial processes. The implementation in food and cosmetic industries using large-scale ATPS

for recovery of products such as aromatic compounds and natural products is feasible. The development of ATPS techniques in biotechnology has provided an alternative for the purification of therapeutic drugs such as monoclonal antibodies, inclusion bodies, viral or plasmid gene therapy vector and human serum albumin (Azevedo et al., 2009; Rito-Palomares, 2004). Besides, the potential of ATPS to be applied in the isolation of small molecular weight biological products such as amino acids has been demonstrated by other researches (Sikdar et al., 1991).

2.5 Extractive bioconversion

Extractive bioconversion by using ATPS has advantages on large-scale manufacturing. The ATPS has been designed in such a way that target product is concentrated in a specific phase and the enzyme is partitioned completely into the other phase for efficient recovery of product from extractive bioconversion. In this method, the enzyme is retained and is reusable for the continuous conversion of target product. In addition, the product concentration in the vicinity of enzyme is low where the inhibition and degradation of enzyme activity can be prohibited (Zijlstra, 1996; Zijlstra et al., 1998). The recycling of the enzyme can be practically achieved by the periodic fresh replacement of the product-extracting phase (Planas et al., 1996; Tjerneld et al., 1991).

Furthermore, other studies also reported continuous processes where the extracting phase being continuously replaced and the enzyme-containing phase is recycled employing an in-line settler (Taguchi et al., 1996; Park and Wang, 1991). ATPS has been adapted in several extractive bioconversions of products such as lactic acid (Kwon et al., 1996), cellulose (Taguchi et al., 1996), antibiotics (Paquet et al., 1994) and extracellular enzymes (Persson et al., 1991; Lee et al., 2017).

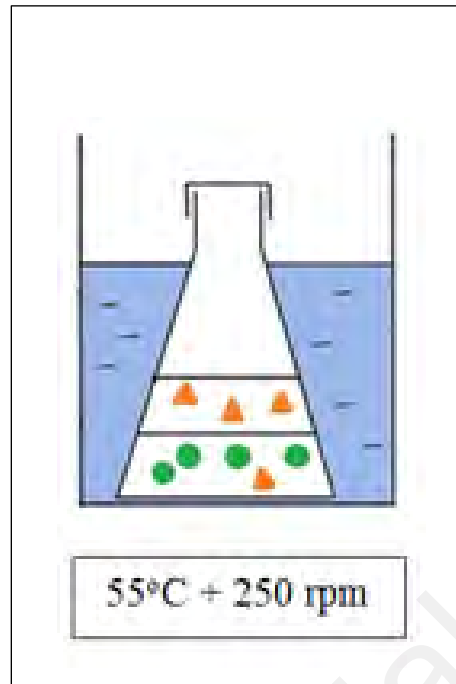


Figure 2.3: Illustration of extractive bioconversion using ATPS.

2.6 Applications of ATPS

With the advancement of ATPS techniques over the years, applications of ATPSs are not only restricted to proteins from microbial cells but also have been expanded to other applications such as recovery of viral or plasmid gene therapy vector (Garca-Perez et al., 1998; Phong et. al., 2017), inclusion bodies and viral coat protein for the protein vaccines (Rito-Palomares, 2004). Moreover, ATPSs have been applied for the isolation of membrane proteins which normally are rather difficult and time-consuming to be purified (Cole, 1991; Mathiazakan et. al., 2016). Additional applications of ATPSs are summarised in Table 2.2.

Table 2.2: Application of ATPSs.

ATPS	Application	Reference
PEG/dextran	Separation of polymerase chain reaction (PCR)-inhibitory substances from bacterial cells	Lantz et al., 1996
PEG/phosphate	Recovery of viral coat proteins from recombinant <i>E. coli</i>	Rito-Palomares and Middelberg, 2002
	Recovery of aroma compounds under product inhibition conditions	Rito-Palomares et al., 2000
	Preparation of highly purified fractions of small inclusion bodies	Walker and Lyddiatt, 1998
PEG/sulphate	Drowning-out crystallisation of sodium sulphate	Taboada et al., 2000
	Recovery of food coloring dyes from textile plant wastes	Huddleston et al., 1998
	Partition of small organic molecules	Rogers et al., 1998

Source: Dreyer (2008)

2.7 ATPF

2.7.1 Conventional methods

The majority of the bacterial CGTases are purified to homogeneity utilizing common purification procedures such as starch adsorption, ultrafiltration, chromatographic separation and ammonium sulphate precipitation. In general, centrifugation and filtration were executed in initial stage of the downstream processes so as to remove high molecular mass biomass and the insoluble particles from the fermentation broth (Moriwaki et al., 2009; Levison, 2001). There are plenty of reports regarding the purification of CGTases where column chromatography's multiple steps were applied

after the cell removal (Gawande and Patkar, 2001; Martins and Hatti-Kaul, 2002). The purification strategies of CGTases that is applied usually involved the starch adsorption of CGTases then complemented by gel filtration (Savergave et al., 2008). The major disadvantage of this purification procedure is that the starch attached to the starch column will react with the CGTases and causes CDs production during the elution process. To avoid product inhibition, additional steps are required to remove CDs which then resulting in low yields of the purified CGTases (Savergave et al., 2008). Besides, the gel filtration method was limited by the low capacity of loaded protein. As such, gel filtration is used as the final purification step (polishing) of the downstream processes to increase target protein purity.

There are several studies on the purification and recovery of CGTases using ion exchange chromatography and ultrafiltration (Hirano et al., 2006; Alves-Prado et al., 2007; Charoensakdi et al., 2007; Yim et al., 1997). The ion-exchangers that are utilized in the ion exchange chromatography of CGTases purifications are usually phenyl sepharose, anion exchange of diethylaminoethyl (DEAE) and cation exchange of carboxymethyl (CM). All these chromatographic separation methods are generally involved multi-step purification procedure with low recovery of the purified CGTases (Hirano et al., 2006; Doukyu et al., 2003).

In summary, all the conventional methods that have been applied in the recovery and CGTases purification showed low specificity, reproducibility and multistep, time-consuming, and difficult to be scaled up to industrial scale. Therefore, it is important to find a new purification method for recovery of CGTases that can produce CGTases in large quantity with improved yield at lower costs and processing time.

2.7.2 Practical implementation of ATPS for product recovery

The basic knowledge of ATPS is needed to facilitate the development of ATPS extraction processes for product recovery. Establishment of preliminary system conditions such as pH and TLLs are also required for the process development of ATPS. The process design of the ATPS begins with the general selection of the type of the ATPS (also termed as process economic consideration), which is followed by the selection of the types of phase-forming components. The development of the ATPS is associated with the knowledge of the physiochemical properties of the target product as well as the impurities, which is the main determining factor of the partition of the target product in an ATPS (Rito-Palomares et al., 2000a; Rito-Palomares et al., 2000b). Therefore, the phase diagram is required once the general phase components of ATPS are selected.

Phase diagram can be referred from literature that previously described by other researchers to define the system conditions by evaluating of the effects of various ATPS parameters (e. g. TLL, pH and V_R) on the partition behavior of the target product (Hustedt et al., 1985; Rito-Palomares and Lyddiatt, 1996). Further investigation is required if the selected ATPS resulted in low product recovery until an acceptable partition of the target product is achieved (Rito-Palomares, 2004). Once the acceptable product recovery is obtained, the prototype can be then further optimized for product recovery. Alternatively, upstream process (i.e. fermentation) and downstream recovery processes of the enzyme can be integrated into the optimized ATPS. The optimized ATPS can be scaled-up for commercial application.

Despite the outline of the general rules of partitioning in ATPS, the application of ATPS is still hampered by the poor understanding of the complex mechanism governing phase

equilibrium and partition behavior of target product (Hatti-Kaul, 2001).

Furthermore, the process design of ATPS is relatively empirical and time-consuming (Bensch et al., 2007), which restricts the application of ATPS in large-scale. Random screening procedure with less elaborated variables and low predictability on the impact of the process alterations on the efficacy of the separation process (Rosa et al., 2010) are claimed to have hampered the exploitation of ATPS at a larger scale. In view of these limitations of ATPS, complementary modeling using lattice-model, viral expansions, UNIQUAC, Flory-Huggins solution theory, thermodynamic approach and others have been attempted to predict the protein partition behavior and succeeded in revealing several molecular-level mechanisms influencing protein partitioning (Abbott et al., 1990; Baskir et al., 1989).

Therefore, the optimized conditions of ATPS for a particular product can be established in a short time by rapid evaluation of different ATPS parameters and interactions between the particles involved (Rosa et al., 2007; Bensch et al., 2007). However, the quantitative modeling of protein partitioning are inadequate for reliable prediction because they are dependent on a wide array of factors and the physical mechanism associated with the complex ATPS are not well understood (Baskir et al., 1989).

2.8 Polymer/salt ATPS

The polymer/salt ATPS is one of the widely studied ATPS. PEG is primarily used as polymer phase component in most of the polymer/salt ATPS studies since it is inexpensive and it has wide variety of polymer molecular weight. The PEG/salt ATPS also has the advantages such as lower viscosity and shorter time for phase separation as compared to polymer/polymer ATPS (Hatti-Kaul, 2000). Furthermore, recycling of

PEG can be achieved by back-extraction step, which transfers the extracted protein from PEG-rich top phase to the salt-rich bottom phase (Van Berlo et al., 1998).

The size and hydrophobic characteristic of the target compound must be taken into consideration in the selection of PEG molecular weight for ATPS purification, which is related to the free volume available for the top and bottom phase. The application of low molecular weight PEG (<4000 g/mol) favoured the purification of hydrophilic high molecular weight compounds (>10000 g/mol) while the application of high molecular weight PEG (>4000 g/mol) improved the low molecular weight (<10000 g/mol) hydrophilic compounds on the other way round (Cabezas, 1996).

Effect of salts on water and proteins has been reported by Franz Hofmeister more than a century ago, who classified ions according to their ability to change water structure and their effect on the stability of the secondary and tertiary structure of proteins (Hofmeister, 1888). The 'kosmotrope' ions which are usually small and highly charged exhibit strong interactions with water molecules and thereby increase the structuring of water, while 'chaotropes' ions which are usually large, with low charged which decrease the structuring of water (Zhao, 2006). However, the mechanism of the Hofmeister series is not entirely clear.

Apart from the ATPS phase-forming salt, addition of neutral salt such as NaCl into ATPS may diminish the amount of bound water in ATPS and cause an increase in hydrophobicity difference between the phases (Hachem et al., 1996). The hydrophobic interaction between the protein and the polymer was therefore promoted, which in turn enhanced the partitioning of proteins to the top phase.

2.9 Alcohol/salt ATPS

Greve and Kula were the first to study the ability of ethanol/phosphate mixture to form stable ATPS for purification (Greve and Kula, 1991). As compared to polymer/polymer or polymer/salt system, the alcohol/salt system possesses the following advantages: the extracted compounds from alcohol phase can be easily isolated by evaporation, rapid segregation into two phases, lower cost as well as lower environmental toxicity (Tianwei et al., 2002).

Most ATPS studies are either based on polymer/polymer system or polymer/salt system, the alcohol/salt system received less attention in prior studies. This is probably due to the irreversible denaturation effect of alcohol on certain biological products and the safety concern of alcohol/salt ATPS at industrial scale, since alcohol is highly flammable and has high volatility (Gutowski et al., 2003).

2.10 Thermo-separating polymers ATPS

Polymer/polymer ATPS is another type of ATPS that has been extensively studied and applied to date. Efficient and biocompatible PEG/dextran ATPS is the primary polymer/polymer ATPS that has been widely used for the separation and purification of diversified biomolecules such as cell organelles, proteins and nucleic acids (Albertsson, 1986; Walter and Johansson, 1994; Lu et al., 1996). However, the application of PEG/dextran ATPS in industrial scale has been hindered by high cost of the phase-forming chemicals (i.e. dextran) (Tjerneld et al., 1986). Various synthetic polymers such as PEG, polypropylene glycol (PPG), polyvinyl alcohol (PVA), dextran and random copolymer of ethylene oxide and propylene oxide (EO-PO) have been shown to be capable to form top phase (i.e. more hydrophobic) of polymer/polymer ATPS. Meanwhile, the most common polymer that constitutes the relatively hydrophilic

bottom phase of the polymer/polymer ATPS is dextran, a polyglucose. Due to high cost of the commercial dextran, less expensive polymeric sugar substitutes has been exploited as the bottom phase component in polymer/polymer ATPS. For instance, unfractionated dextran, Ficoll (polysucrose), methylcellulose, hydroxyethylcellulose (HEC) Repeal PES (hydroxypropyl starch) and so forth are used to replace dextran in polymer/polymer ATPS in other literatures (Harris et al., 1997; Persson et al., 1999a; Persson et al., 1998). In addition, several studies on the ATPS using thermo-separating polymers (eg. EOPO copolymers, Ucon-benzoyl dextran and etc.) that is recyclable have been investigated with the aim to reduce the cost of the ATPS (Persson et al., 2000; Lu et al., 1996).

Phase separation of polymers in ATPS is dependent on the molecular size and the thermodynamic properties of polymers in solution (Albertsson, 1986). The phase separation is driven by the enthalpy of hydration a result of the interactions between the polymers (Flory, 1953; Hatti-Kaul, 1999; Johansson et al., 1998). The selectivity of protein partitioning in polymer/polymer ATPS, particularly PEG/dextran system can be easily improved because both the polymers are adaptable to modification or derivation resulting more hydrophobicity-dependent protein selectively phase partitioning in an ATPS. Synthesis of the derivatives of polymers can be attained by addition of ion charge or covalently coupled hydrophobic ligand at their functional groups (Lu and Tjemeld, 1997). Examples of modified polymers are Ucon-benzoyl dextran, thionyl bromide-PEG (Bromo-PEG), positively charged trimethylamino-PEG (TMA-PEG), negatively charged sulfonate-PEG (S-PEG), DEAE-dextran and other hydrophobic PEG-ester with fatty or aromatic acids (Johansson et al., 1993; Lu et al., 1994; Lu et al., 1991).

Thermo-separating polymers including Ucon 50 HB-5100 (EO₅₀PO₅₀) (Tubio et al., 2009), Breox 50A 1000 (EO₅₀PO₅₀), EO₃₀PO₇₀ (Lu et al., 1996), poly(N-isopropylacrylamide) [poly(NIPAM)] have been used as an alternative for PEG to form top phase of the polymer/polymer ATPS. The solubility of these thermo-separating polymers will decrease and a secondary ATPS consisted of a polymer phase and a water phase is formed when they are heated above their lower critical solution temperature (LCST). This thermo-separation enables the rapid recovery of target product that separated into the water phase whereas the polymer in the other phase can be easily recovered and reused in subsequent ATPS extraction (Harris et al., 1991, Tubio et al., 2009). Even though PEG is considered to be a thermo-separating polymer, its LCST is too high (> 100 °C) for separation of labile biomolecules and is not economical to be recycled. The LCST of EO₅₀PO₅₀ and poly (NIPAM) are at 60 °C and 32 °C, respectively that makes them a suitable substitute for PEG in various polymer/salt and polymer/polymer ATPSs (Cabral, 2007).

CHAPTER 3:

DIRECT RECOVERY OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM *Bacillus cereus* USING AQUEOUS TWO-PHASE FLOATATION

3.1 Introduction

Cyclodextrin glycosyl transferase (CGTase, 1,4- α -D-glucopyranosyl transferase, cyclizing, E.C. 2.4.1.19) is an extracellular enzyme belonging to the glycosyl hydrolases of α -amylase family. This enzyme is capable of converting starch into cyclodextrins (CDs) through cyclization reactions involving bond cleavage, circularization and a new bond formation that produce the CDs product (Stam et al., 2006). In addition, due to the inability of CGTase to cut through α -(1, 6) linkages in gelatinized starches which decrease the viscosity while maintaining the high molecular features of the starches (Pedersen et al., 1995), the CGTase can be used in the liquefaction of starch.

CGTase purification methods such as ion exchange chromatography (Savergave et al., 2008) and column chromatography (Bharat et al., 1999) have been studied extensively in recent years. However, there are major problems with these methods. For instance, due to the large amount of CGTase lost after multiple steps of the purification process (Savergave et al., 2008), ion exchange chromatography was reported to generate low recovery yields of CGTase. To overcome this problem, the aqueous two-phase floatation (ATPF) system which combines aqueous two-phase system (ATPS) with solvent sublation was introduced (Show et al., 2011). This ATPF system employs water-soluble phase components which results in the formation of two immiscible phases. The surface-active compounds are adsorbed to the surfaces of the nitrogen bubbles which in turn dissolve in the polymer layer, resulting in a higher purity of the final product (Figure 1) (Show et al., 2011). Advantages of the ATPF technique include

ease of operation, economic feasibility and environmental friendliness (Tubio et al., 2009). Other applications of ATPF to separation studies include purification of lincomycin (Li et al., 2010), penicillin G (Bi et al., 2009), puerarin (Bi et al., 2010), lincomycin (Li et al., 2010), and lipase (Tan et al., 2014). In comparison to ATPS, ATPF (given its higher partition coefficient (Bi et al., 2010)) appears to be a more promising alternative for separating puerarin from a *Puerariae* extract.

Previous report has shown that ATPS have been successfully used for the purification of CGTase (Ng et al., 2011). However, the use of PEG-potassium phosphate ATPF for the purification of *Bacillus cereus* CGTase has yet to be explored. Motivated by this, this paper aims to study the feasibility of using PEG-potassium phosphate ATPF for the purification of CGTase. In this paper, the partition behavior of CGTase was determined and optimized in ATPF with the goal of achieving a high purification factor (P_{FT}) of CGTase.

3.2 Materials and methods

3.2.1 Materials

PEG with average molecular weights of 4000, 6000, 8000, 10000 and 20000 g/mol were sourced from Fluka Co. (St. Louis, MO, USA). CDs were sourced from Sigma (St. Louis, MO, USA). Protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Potassium di-hydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were purchased from Merck (Darmstadt, Germany).

3.3.2 CGTase production

The CGTase was produced using *B. cereus* culture. The *B. cereus* culture was grown at 37 °C with a continuous agitation at 250 rpm for 30 hour from 10 % (v/v) of inoculum

in a 250 mL baffled shake flasks. The crude enzyme supernatant was harvested after 15 min of 4000×*g* centrifugation.

3.2.3 CGTase activity assay

The CGTase activity (β -CD production rate) was assayed using the method of phenolphthalein modification described by Higuti et al. (Higuti et al., 2004). 50 μ L enzyme sample was added to 750 μ L substrate solutions (1 % (w/v) starch in 0.05 M Tris-HCl buffer pH 8.0) and incubated at 55 °C for 10 min. Enzymatic reaction of CGTase was terminated by adding 375 μ L of 0.15 M NaOH followed by the addition of 100 μ L 0.02 % (w/v) phenolphthalein (in 5 mM Na₂CO₃) for a spectrophotometric evaluation of CGTase (550 nm). The amount of β -CD produced from the starch hydrolysis was measured using the standard curve of β -CD.

3.2.4 Protein assay

The total protein concentration was analyzed in accordance with the instruction in the Bio-Rad (Hercules, CA, USA) protein assay kit manual. Samples were placed in a 96 wells plate and absorbance was determined at 595 nm using the Tecan sunrise (Salzburg, Austria) absorbance reader. Bio-Rad (Hercules, CA, USA) bovine serum albumin was used as a control.

3.2.5 ATPS and ATPF

Phase systems were prepared in 15 mL centrifugal tubes with distilled water by dissolving predetermined quantities of PEG and potassium phosphates. 2 grams of crude extract was then gradually added into the system until it reaches a total weight of 10 g. To speed up phase separation, systems were mixed thoroughly *via* gentle agitation with $4000 \times g$ centrifugation for 10 min. Then, the top and bottom phases were collected for analysis of both the total protein concentrations and CGTase activity. At the ATPF stage, a 100 ml aliquot of aqueous two-phase solution was transferred into the flotation cell and the CGTase was adsorbed to the nitrogen gas bubbles which were introduced at a rate of 5L/min (Figure 3.1). The CGTase was then collected in the top phase and the purification factor of the system was evaluated.

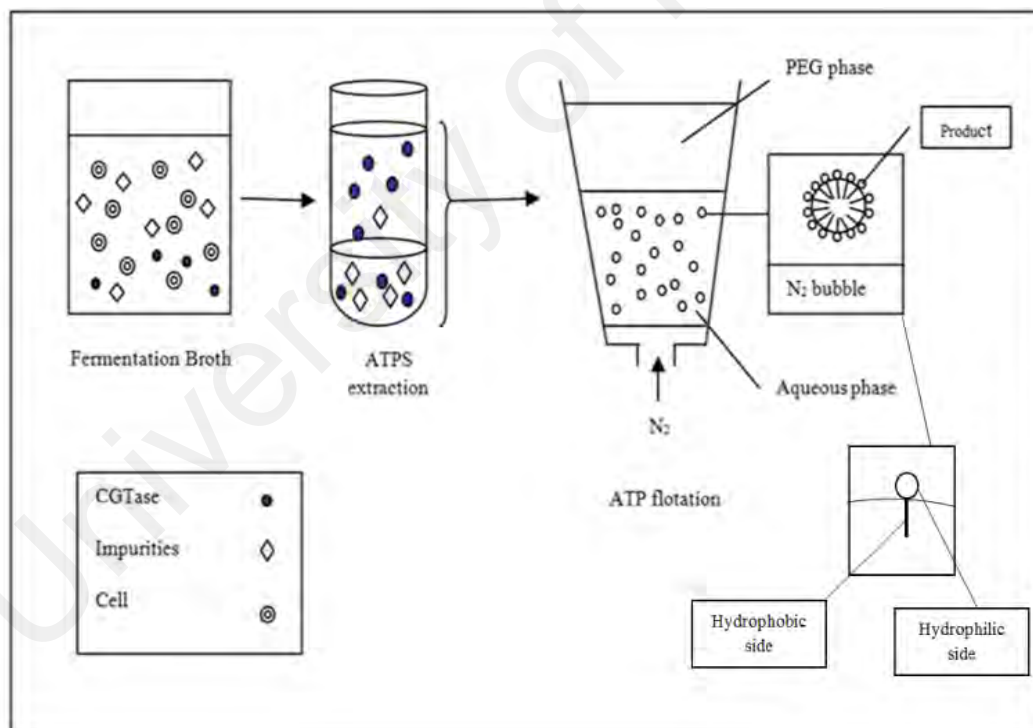


Figure 3.1: Schematic view of CGTase purification in ATPF.

3.2.6 Calculations

The V_R is defined as

$$V_R = \frac{V_T}{V_B} \quad (2)$$

where the V_T represents volume in the top phase and V_B represents volume in the bottom phase.

The specific activity (SA, in U/mg) of the sample was designated as the fraction of the enzyme activity (U) and the total protein concentration (mg):

$$SA = \frac{\text{Enzyme activity (U)}}{[\text{Protein}](\text{mg})}. \quad (3)$$

The P_{FT} was determined according to the relationship

$$P_{FT} = \frac{\text{SA of phase sample}}{\text{SA of crude extract}} \quad (4)$$

where the SA of phase sample is the CGTase specific activity in the top phase and SA of crude extract is the original CGTase specific activity in the crude extract.

The partition coefficient (K_{CGTase}) of the CGTase is defined as

$$K_{CGTase} = \frac{C_T}{C_B} \quad (5)$$

where C_T represents the CGTase activity (U) in the top phase and C_B represents the CGTase activity (U) in the bottom phase.

Yield of CGTase in top phase was calculated using the relationship

$$Y_T(\%) = \frac{100}{1 + [1/(V_R K)]} \quad (6)$$

where V_R is the volume ratio and K is the partition coefficient.

Purification efficiency (E) was calculated using Eq. (7)

$$E(\%) = \frac{C_T V_T}{C_0 V_0} \times 100\% \quad (7)$$

where C_T and V_T represent the CGTase activity in the top phase and volume of the bottom phase respectively. V_0 and C_0 are added volume and CGTase activity in prepared crude solution respectively.

3.2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE method (Ooi et al., 2009) was used to analyze the protein samples from crude extract and ATPF top phase by using 12 % acrylamide gel with a 4.5 % stacking gel.

3.3 Results and Discussion

3.3.1 The effects of PEG Molecular Weight and tie-line length (TLL) on P_{FT}

The CGTase purification performance was investigated in two-phase systems consisting of PEG with different molecular weights (PEG 4000, 6000, 8000, 10000 and 20000) and potassium phosphate. The effects of the PEG molecular weights and TLL on lipase and recombinant human interferon alpha-2b partition performance have been explored previously (Ooi et al., 2009; Lin et al., 2012). The TLL values of various PEG (6000, 8000 and 10000) used in this part of study (Table 3.1) were referred from the previous publications (Ooi et al., 2009; Lin et al., 2012). Here, the main aim of the study was to examine the effects of the PEG molecular weights and TLL on *B. cereus* CGTase partitioning.

Generally, it can be seen that the P_{FT} of the CGTase decreases as the TLL increases (Table 1). At higher TLL, both the top phase PEG and bottom phase salt concentrations in the ATPF were also higher. Therefore, it is suggested that the higher salt

concentration caused a reduction in the solubility of the CGTase protein due to the higher salting-out ability of potassium phosphate. A similar observation has been reported in the previous microbial lipase and recombinant human interferon alpha-2b partitioning studies (Ooi et al., 2009; Lin et al., 2012). The results also showed a correlation between the PEG molecular weight and the P_{FT} of CGTase. The CGTase P_{FT} values in PEG 8000/phosphates ATPS was higher compared to the PEG with molecular weights of 6000 and 10000. Low molecular weight PEG (PEG 4000 and 6000) may draw the contaminant proteins to the PEG top phase, thereby resulting in a low P_{FT} for the CGTase (Ng et al., 2011). On the other hand, the higher molecular weight PEGs (PEG 10000 and 20000) are generally more viscous, causing a decrease in the free volume of the top phase (PEG phase) due to the volume exclusion effect. This forces more CGTase to partition to the bottom phase (Ng et al., 2011; Ooi et al., 2009). From the results, the highest CGTase P_{FT} value is 7.26 achieved in PEG 8000/phosphates at TLL of 27.2 % (w/w). This value corresponds to the optimum condition created by the hydrophobicity of PEG and salting out effect of potassium phosphate. PEG 8000 system at 27.2 % (w/w) TLL was thus selected for further investigation.

Table 3.1: The effects of PEG molecular weight and concentration on CGTase partitioning performance. The data obtained was presented as the average of triplicate readings with standard deviation of $\pm 5\%$.

PEG molecular weight (g/mol)	TLL	P_{FT}
4000	28.79	5.10
	32.31	5.52
	37.70	2.65
	39.23	2.25
6000	29.6	5.72
	32.6	4.64
	35.2	2.20
	37.9	2.10
8000	27.2	7.26
	30.9	6.18
	33.5	5.60
	36.1	5.73
10000	27.5	4.82
	29.9	6.11
	31.9	4.56
	34.4	3.45
20000	27.8	3.23
	31.6	2.51
	34.6	2.16
	38.3	2.08

3.3.2 The effects of V_R on P_{FT}

Figure 3.2 showed the effects of V_R on CGTase P_{FT} . The CGTase P_{FT} values generally increased as the V_R increased. It is suggested that more CGTase had been attracted to the top phase since higher V_R resulted in a higher free volume of PEG at the top phase, which in turn increase the P_{FT} value of the CGTase. However, low P_{FT} (5.3) was found at a V_R of 4.0. A possible reason of this is an excessive free volume in the top phase

causing more contaminant protein to be partitioned to the PEG-rich top phase in the ATPF (Azevedo et al., 2008). The V_R of 3.0 corresponding to the highest CGTase purity (P_{FT} of 9.2) was therefore selected for the subsequent studies.

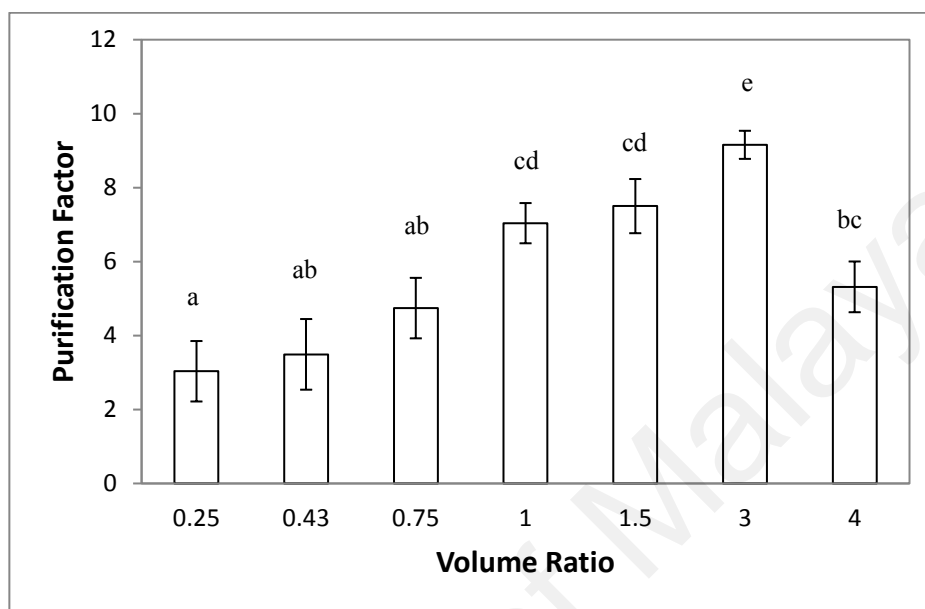


Figure 3.2: Effects of V_R on P_{FT} . The data obtained was presented as the average of triplicate readings. PFT with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

3.4.3 Effects of pH on P_{FT}

The P_{FT} of CGTase at different pH ranging from 5-9 was studied (Figure 3.3). The pH was adjusted by mixing different compositions of two potassium phosphates (KH_2PO_4 and K_2HPO_4) with a pH deviation range of ± 0.5 .

It has been reported that manipulation of the system pH will influence the target protein partitioning behaviour since it can affect the interaction between the net charge of target protein and ions present inside the system (Forciniti et al., 1991). The results in Figure 3.3 showed that highest P_{FT} (11.8) was obtained at pH 7. The iso-electric pH of CGTase is about 6.9, and it is therefore expected to exhibit low negative charge in a neutral environment. PEG tends to attract the negatively charged CGTase, thereby resulting in a

higher P_{FT} CGTase (Huddleston et al., 1991). The P_{FT} values of CGTase at basic pH values (pH 8 and 9) were low probably due to more contaminant proteins becoming negatively charged and moving up to the PEG phase. On the other hand, in an acidic environment (pH 5 or 6) the CGTase molecules become negatively charged and were repelled from the negatively charged PEG phase (Ng et al., 2011). Therefore, pH 7 was chosen for further investigation.

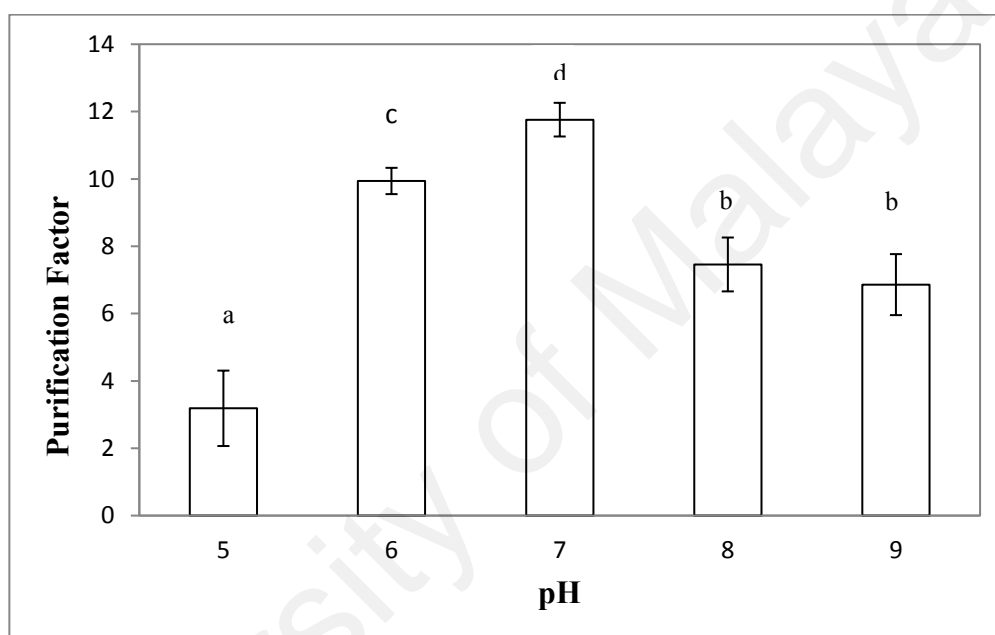


Figure 3.3: Effects of pH on P_{FT} . The data obtained was presented as the average of triplicate readings. P_{FT} with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

3.3.4 Effects of crude concentration on P_{FT}

Figure 3.4 showed the top phase P_{FT} crude concentration ranging from 10 to 40 % (w/w). The crude loaded into the ATPS can potentially change the V_R and composition of ATPS, thereby affecting the P_{FT} results (Walter et al., 1985). It was observed that the highest P_{FT} (15.9) was achieved at 20 % (w/w) concentration of crude loading. The result indicated that higher amount [>20 % (w/w)] of crude concentration reduced the V_R of ATPS. This ultimately decreased the free volume of PEG at the top phase of

ATPS, thereby reducing the amount of target enzyme partitioned to the top phase (Ooi et al., 2009). Hence, 20 % (w/w) crude concentration was designated for further studies.

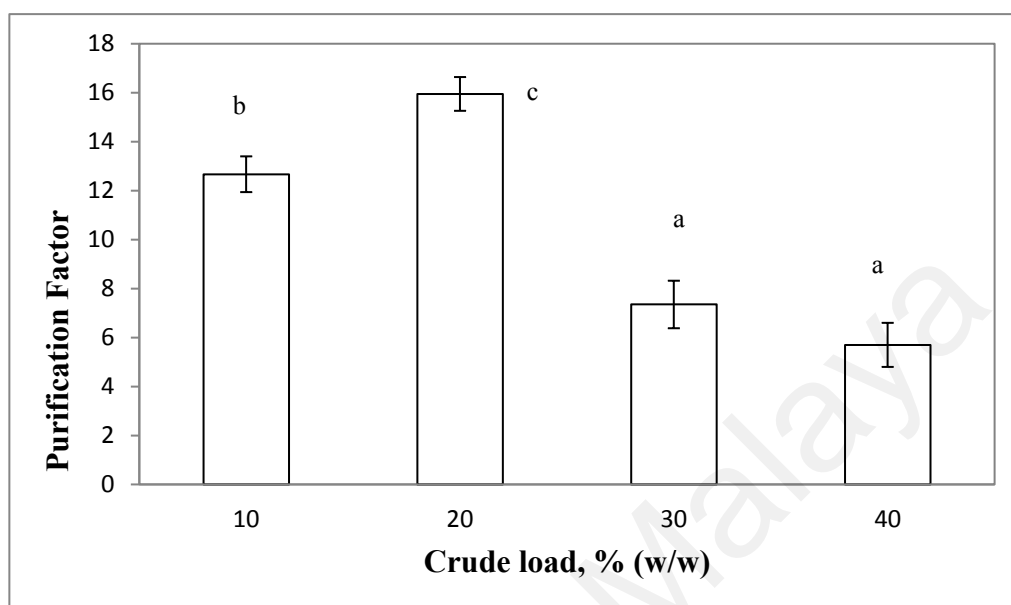


Figure 3.4: Effects of crude load on P_{FT} . The data obtained was presented as the average of triplicate readings. PFT with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

3.3.5 Effects of flotation time on P_{FT}

5 L/min of nitrogen gas flow rate was used to examine the effects of flotation time on P_{FT} . Figure 3.5 showed the effects of flotation time on the P_{FT} of CGTase. The P_{FT} increased with increasing flotation time, indicating that the CGTase compounds in bottom phase were adsorbed to the surfaces of the nitrogen bubble from the ascending gas stream before finally reaching the PEG top phase. No incremental increase in the P_{FT} value was observed after 80 min flotation time, and the highest P_{FT} value of 21.8 was achieved at 80 min flotation time.

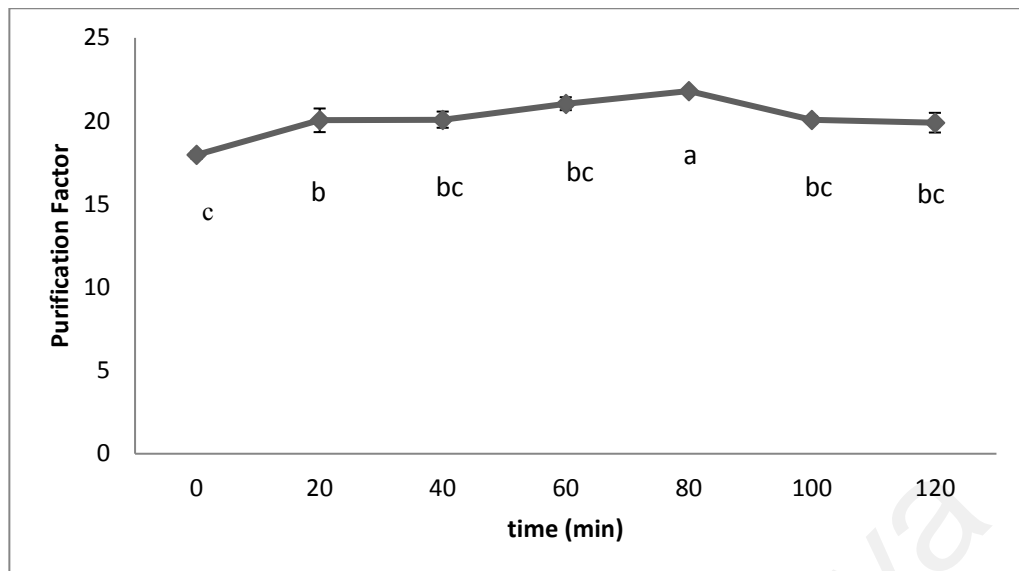


Figure 3.5: Effects of flotation time on P_{FT} of CGTase. The data obtained was presented as the average of triplicate readings. P_{FT} with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

ATPF is suitable for CGTase recovery since it comprises of hydrophobic amino acid residues that facilitate adsorption of the enzyme onto the nitrogen (N_2) bubble surfaces (Bart et al., 2001). In the experiment, nitrogen bubbles emerging from the pores at the bottom and passing through the aqueous phase caused the adsorption of hydrophilic molecules to the surface of the bubbles. Since the total density of the hydrophilic compound and the bubble is less than the density of the aqueous phase, the new compound (i.e. the bubble with hydrophilic compounds attached) moves up to the top phase where the hydrophilic molecules will be collected. It would be interesting to investigate the factor(s) affecting the amount of hydrophilic compounds moving up to the top phase.

Using Archimedes's Principle, if the new compounds are to move up to the top phase, it is required that

$$\text{density of aqueous phase} > \text{density of new compound.}$$

This implies

$$\frac{M_{p(bottom)}+M_o}{V} > \frac{M_{p(adsorption)}+M_b}{V_n} \quad (8)$$

where $M_{p(bottom)}$ is total mass of the hydrophilic molecules in the bottom phase, M_o represents the total mass of salt and feedstock (excluding the hydrophilic molecules) in the bottom phase, V represents the total volume of the bottom phase, $M_{p(adsorption)}$ represents the total mass of all hydrophilic molecules attached to a bubble, M_b is the mass of the bubble, and V_n is the total volume of the new compound (i.e. the volume of the new molecule).

The previous equation can be rewritten as

$$\frac{M_{p(total)}-N_{p(top)}M_p+M_o}{V} > \frac{N_pM_p+M_b}{V_n} \quad (9)$$

where $M_{p(total)}$ is the total mass of all hydrophilic molecules in the entire system, $N_{p(top)}$ is the number of hydrophilic molecules at the top phase, M_p is the mass of a hydrophilic molecules, and N_p is the number of hydrophilic molecules attached to one bubble. Rearranging equation (9), we have

$$N_{p(top)} < \frac{M_o+M_{p(total)}}{M_p} - \frac{V}{V_n} \left(N_p + \frac{M_b}{M_p} \right) \quad (10)$$

where all are constants except $N_{p(top)}$, V_n , N_p and M_b . To investigate the number of molecules that can be attached to a bubble, a schematic diagram of molecules lining up on the surface of a bubble is considered as shown in Figure 3.6.

In Figure 3.6, the big circle represents the bubble, and the small circles correspond to the hydrophilic molecules. r_p and r_b are radii for the hydrophilic molecule and bubble

respectively. It is obvious that the radius of both the bubble and the hydrophilic molecules play an important role in the number of hydrophilic molecules that can be adsorbed to the surface of the bubble. In particular, the number of hydrophilic molecules that can be adsorbed to the surface of the bubble can be found by dividing surface area of the bubble by the surface area of the square below each hydrophilic molecule (see Figure 3.6).

$$r_p = \lim_{r_b \gg r_p} \frac{4\pi r_b^2}{(2r_p)^2} = \frac{\pi r_b^2}{r_p^2}. \quad (11)$$

Also,

$$V_n = \frac{4}{3} N_p \pi r_p^3 + \frac{4}{3} \pi r_b^3 = \frac{4}{3} \pi r_b^2 (r_p + r_b). \quad (12)$$

Combining Eqs. (11) and (12) give

$$\frac{N_p}{V_n} = \frac{3}{4r_p^2(\pi r_p + r_b)}. \quad (13)$$

Putting Eq. (13) into Eq. (10) results in

$$N_{p(top)} < \frac{M_o + M_{p(total)}}{M_p} - \frac{3V}{4r_p^2(\pi r_p + r_b)} - \frac{3VM_b}{4\pi M_p r_b^2(\pi r_p + r_b)} \quad (14)$$

and all are constants except $N_{p(top)}$, r_b , and M_b .

It can be seen in equation (14) that the type of gas used to create the bubble directly affects the number of hydrophilic molecules that can be moved to the top phase. In particular, from equation (14) it can be seen that the larger the mass of the bubble, the fewer the number of hydrophilic molecules that can be moved to the top phase. This is in agreement with intuition as the heavier the object in a phase, say a liquid, the harder

it is for the object to float on the surface of the liquid. Thus, the choice of gas for the bubble thus plays a role in improving the efficiency of the CGTase extraction. Since nitrogen is lighter than both oxygen and carbon dioxide, it is the most ideal among the three for extraction of the CGTase in this work.

Also, from equation (14) there is an optimum r_b for the maximum number of hydrophilic molecules to move to the top phase. From this equation, one can also see that the number of hydrophilic molecules that can be collected decreases rapidly with the increase of the size and type of hydrophilic molecules involved. Future research on protein extraction in ATPF using gas bubbles may reasonably include experimentation on the type of gas molecules and size of hydrophilic molecules involved.

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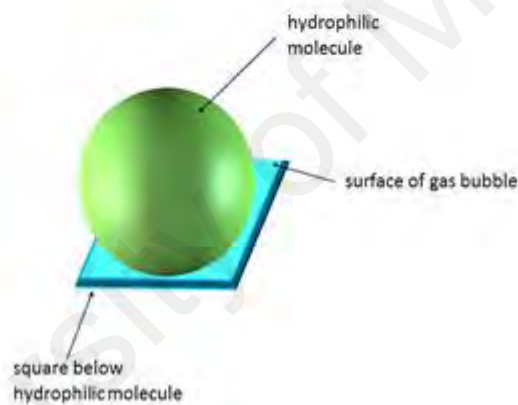
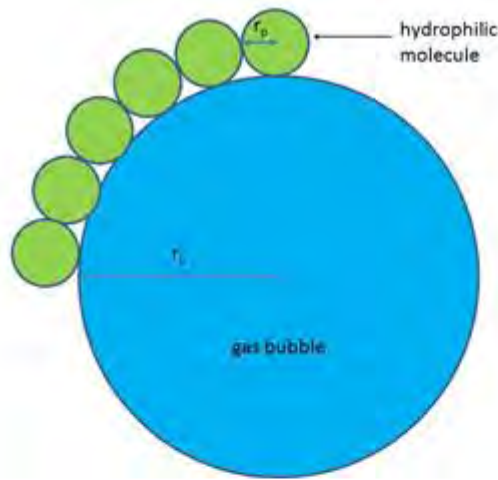


Figure 3.6: Diagram showing adsorption of hydrophilic molecule to the gas bubble, the square beneath a hydrophilic molecule and the area of occupancy of the molecule over the surface of the bubble.

3.3.6 Purity of CGTase recovered

The purity of CGTase from optimized ATPF was assessed using SDS-PAGE. Figure 3.7 illustrates showed the standard protein markers (lane 1), top phase sample from optimized ATPF (lane 2), and crude sample (lane 3). Multiple protein bands were found in the crude sample, indicating the presence of contaminant proteins. A band of

approximately 75 kDa was found in the top phase sample (lane 2) and the molecular weight of CGTase was about 75.4 kDa (Fujiwara et al., 1992). Several bands of between 100 and 130 kDa were also present, and are believed to be the contaminant proteins that had partitioned into the top phase. However, as evidenced from the SDS-PAGE image, the CGTase protein was recovered through the PEG/phosphate ATPF process where the majority of the contaminant proteins were removed from the crude sample.

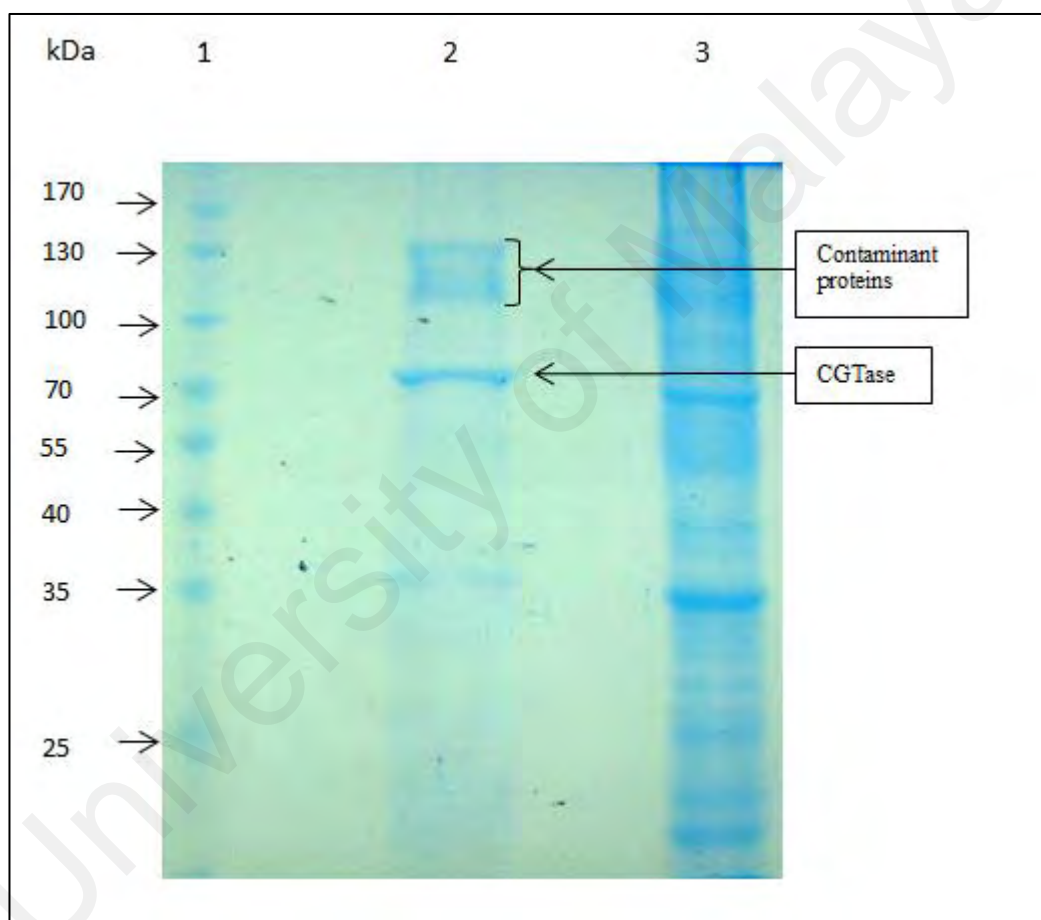


Figure 3.7: SDS-PAGE analysis. The purity of top phase partitioned CGTase was assessed by 12 % SDS-PAGE analysis. Molecular weight of standard protein marker ranged from 10 to 170kDa. SDS-PAGE-lane 1: protein molecular markers; lane 2: ATPF top phase sample; lane 3: crude sample.

3.4 Conclusions

Table 3.2 showed a summary of the P_{FT} and E (%) for ATPS and ATPF stages. In conclusion, the CGTase from *B. cereus* was successfully purified through the PEG 8000/phosphate ATPF process. The optimized condition for the purification was achieved at: 18.0 % (w/w) PEG 8000, 7.0 % (w/w) potassium phosphates, V_R of 3.0, 20 % (w/w) crude concentration at pH 7, and 80 min of nitrogen flotation at a flow rate of 5 L/min. The top phase P_{FT} of 21.8 and Y_T of 97.1 % revealed the potential of ATPF as a valuable primary purification step since it had successfully obtained higher P_{FT} and Y_T results as compare to previous CGTase purification study ($P_{FT} = 16.3$, $Y_T = 70\%$) (Ng et al., 2011).

Table 3.2: The effects of ATPS and ATPF upon CGTase E (%) and P_{FT} . The data showed the CGTase E (%) and P_{FT} results achieved for ATPS and ATPF stages.

ATP extractions studied (Optimum)	E (%)	P_{FT}
ATPS	69.76	15.95
ATPF	82.85	21.80

CHAPTER 4:

PRODUCTION OF GAMMA-CYCLODEXTRIN USING EXTRACTIVE BIOCONVERSION IN POLYMER/SALT AQUEOUS TWO-PHASE SYSTEM

4.1 Introduction

An ATPS is formed by mixing two incompatible aqueous solutions at a concentration exceeding a specific threshold (Albertsson, 1986). The ATPS provides a simple, low cost, and low pollution technique for purification, and it has proven to provide a biocompatible environment for the separation since both phases consist of a predominantly high water content (Andersson et al., 1990, Zijlstra et al., 1998). In this study, the production of γ -CD was performed using the enzymatic bioconversion method. Results of research on bioconversion have been used to facilitate the production of other bioproducts such as β -D-glucose-1-phosphate (Borghet et al., 2010), naringin (Tramice et al., 2008), bioethanol (Harun et al., 2014), L-DOPA (Acharya et al., 2008), and curcuminoids (Rodrigues et al., 2015). The application of ATPS extractive bioconversion provides a technique which integrates bioconversion and purification into a single step process (Martin, 2004). ATPS extractive bioconversion was designed to partition the biocatalyst and the desired product into the bottom and top phase respectively. In contrast with conventional methods (e.g., enzymatic bioconversion), ATPS extractive bioconversion integrated production and purification process into single step, the biocatalyst retained in one phase can be reused, prompting the possibility of a continuous extractive bioconversion process (Figure 1) (Charoenlap et al., 2004).

The CDs are cyclic oligosaccharides produced by CGTase enzyme *via* the transglycosylation process (Singh et al., 2002). CDs have a structural feature which

enables them to form inclusion complexes with a lot of guest compounds, promoting wide spread applications of CDs in different industries such as food, pharmaceuticals, cosmetics, agricultural and chemical products (Moriwaki et al., 2008).

There are three major types of CDs, namely, α , β and γ which are formed by 6, 7 and 8 glucopyranose units respectively (Li et al., 2007). Among these three types of CDs, the γ -CD has the premier solubility and the largest interior cavity. In addition, γ -CD is more expensive than α and β -CD due to its lower production compared to α and β -CD (Zijlstra et al., 1996, Ng et al., 2011). Unfortunately, there is no available study on ATPS extractive bioconversion which focusing on γ -CD as target product. Motivated by this, an ATPS extractive bioconversion was studied in this work to investigate approaches in improving the productivity and separation of γ -CD from *Bacillus cereus* CGTase (Ng et al., 2011). The approaches optimizing the γ -CD recovery involved investigating the effects of ATPS variables such as the pH (pH = 6.0, 6.5, 7.0, 7.5, 8.0), PEG molecular weight (PEG 3000, PEG 6000, PEG 8000), tie-line lengths (TLLs) [27.2-40.7 % (w/w)] and volume ratio (V_R) ($V_R = 0.3, 1.0, 2.0, 2.3, 4.0$ and 5.0).

4.2 Materials and methods

4.2.1 Materials

PEG with molecular weight 3000, 6000, 8000 was acquired from Fluka, Sigma-Aldrich (St. Louis, USA). Phenolphthalein and potassium phosphates were sourced from Merck (Darmstadt, Germany). Soluble starch was acquired from Becton, Dickinson and company (New Jersey, USA). The γ -CD was supplied by Sigma-Aldrich (St. Louis, USA).

4.2.2 *Bacillus cereus* cultivation

The *B. cereus* culture medium was prepared with 1 % (w/v) sago starch, 0.5 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.009 % (w/v) MgSO₄, 0.1 % (w/v) K₂HPO₄ and 1 % (w/v) Na₂CO₃ (autoclaved separately) (Ng et al., 2011). The inoculum was grown at 37 °C for 18 hours with 250 rpm continuous agitation. It was then transferred into a CGTase production medium containing 1 % (w/v) sago starch, 0.5 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.009 % (w/v) MgSO₄, 0.1 % (w/v) K₂HPO₄ and 1 % (w/v) Na₂CO₃, incubated at 37 °C for 30 hours with 250 rpm continuous agitation speed. The crude CGTase was harvested from supernatant by centrifugation at 4000 × g. The crude CGTase was then purified using ATPF method for extractive bioconversion.

4.2.3 CGTase activity analysis

CGTase cyclizing activity (β-CD production) was performed according to the method as described in section 3.3.3.

4.2.4 Partitioning experiments of CGTase and γ-CDs in ATPS

Partitioning experiments were carried out at room temperature (25 ± 1 °C) to determine the optimum ATPS for γ-CD production. Predetermined quantities of dissolved PEG, potassium phosphates, distilled water were added to reach a final total weight of 10 g ATPS containing 10 % (w/w) of CGTase and 10 % (w/w) of standard γ-CD (50 mg/mL). The established ATPSs were then shaken using vortex mixer followed by a 10 minute centrifugation (4000 × g). After phase separation, samples were collected for enzyme activity and γ-CD concentration analyses.

4.2.5 Production of γ -CD using ATPS extractive bioconversion

The ATPS extractive bioconversion of γ -CD production was carried out in a 250 mL Erlenmeyer flask. Predetermined quantities of dissolved PEG, potassium phosphate, and distilled water were added to reach a final total weight of 50 g ATPS containing 5 % (w/w) soluble starch and 20 % (w/w) of CGTase. A control (without ATPS phase-forming components) was conducted for comparison. The mixture solution was kept stirring at 250 rpm and temperature controlled at 55 °C for enzymatic (CGTase) conversion of soluble starch substrate into γ -CD. Samples of top and bottom phases were collected separately at regular time intervals and heated in boiling water for 5 min to thermally inactivate the CGTase. The quantification of γ -CD concentration was carried out using the reverse phase HPLC instrument.

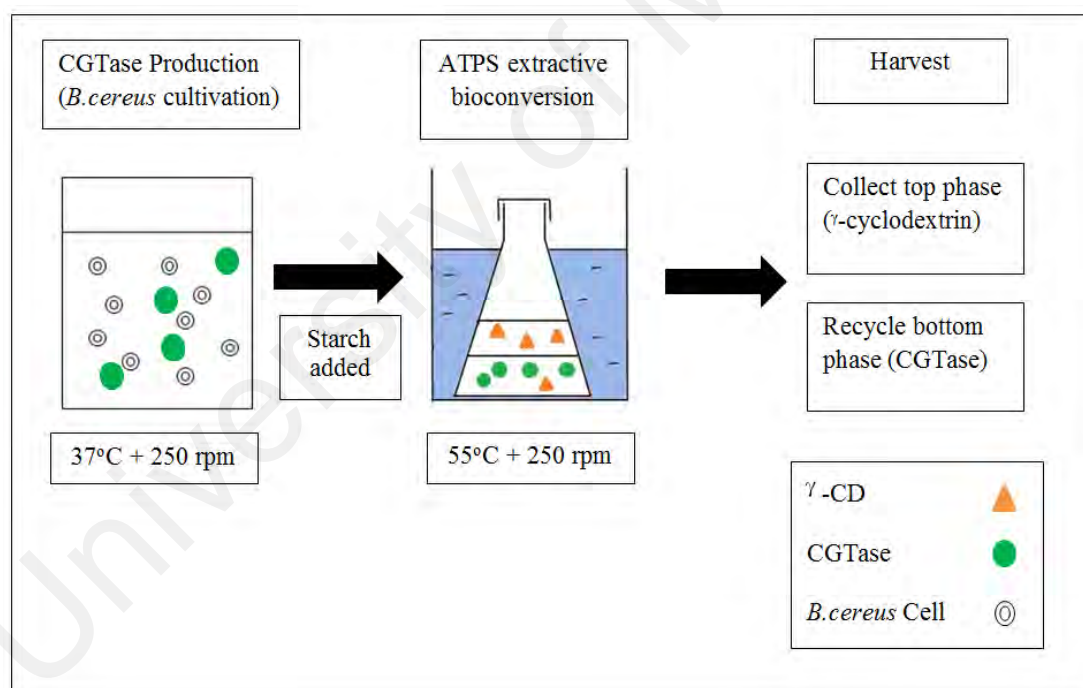


Figure 4.1: Schematic diagram of extractive ATPS soluble starch bioconversion.

4.2.6 Calculations

Relative CGTase activity is expressed as the fraction of the sample CGTase activity (U/mL) to the control.

V_R and K_{CGTase} were calculated using formula as described in Section 3.3.6.

Partition coefficient of γ -CD (K_{CD}) is expressed as

$$K_{CD} = \frac{C_T}{C_B} \quad (3)$$

where C_T is the γ -CD concentration (mg/ mL) in the top phase. The C_B represents the γ -CD concentration (mg/ mL) in the bottom phase.

Yield of γ -CD in top phase (Y_T) is calculated using the definition

$$Y_T = \frac{1}{1 + (1/V_R * K_{CD})} * 100\% \quad (4)$$

4.3 Results and discussion

4.3.1 Effects of phosphate salt system pH on relative CGTase activity

Stable and optimum CGTase activity are necessary for the purpose of high γ -CD productivity. Therefore, enzyme stability study was carried out in a 20 % (w/w) phosphate salt solution before investigating the partitioning mechanism. Figure 4.2 showed the effects of 20 % (w/w) potassium phosphate salt at different pH conditions on the CGTase stability after 8 hour of incubation. The highest relative CGTase activity 1.0 was achieved at pH 7. The result also showed that relative activity of CGTase was highly influenced by the pH of the salt system. This may be due to the changes that occurred in the basic structure of the CGTase when the pH value is higher or lower than pH 7. The acidic amino acids have carboxyl functional groups while basic amino acids have amine functional groups, hence pH can have pronounced effects on the state of ionization of these amino acids. Consequently, the ionic bonds that help to determine the shape of CGTase were affected. In turn, this altered CGTase active site, which cause it to lose the ability to bind well with the substrate, thereby influencing its bioactivity

(Ooi et al., 2009). Thus, potassium phosphate salt system at pH 7 (relative CGTase activity 1.0) was selected for ATPS extractive bioconversion.

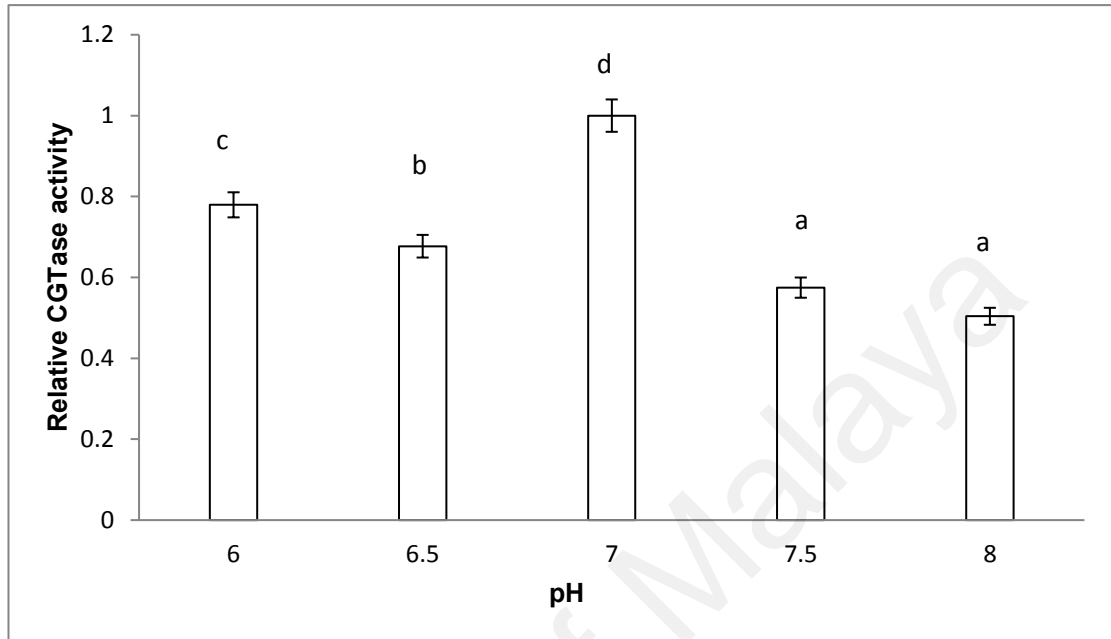


Figure 4.2: Effects of pH on CGTase activity. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

4.3.2 The distribution of biomolecules between two aqueous phases

The distribution of biomolecules between the two aqueous phases is defined as (Chow et al. (18))

$$\ln K_i = \frac{-\pi d_p^2 (1 - \cos \theta)^2 c (\Delta P_i)^{n+1}}{4kT} \quad (5)$$

where d_p is the diameter of the spherical particle, θ is the contact angle, c is a constant, ΔP_i is the difference in concentration of phase forming component i between the two aqueous phases, k represents the Boltzmann constant, and T represents the absolute temperature (K). K_i is the partition ratio associating the number of biomolecules between one bulk phase, N_i and the interface, N_j . Eq. (5) is applicable to a spherical

molecule with a cross-sectional area of $\pi d_p^2/4$, and for a molecule with a general cross-sectional area A , we have

$$\ln K_i = \frac{-A(1 - \cos \theta)^2 c (\Delta P_i)^{n+1}}{kT} \quad (6)$$

For a polymer-salt ATPS with a small but not negligible interface, $n \rightarrow 1$, giving us

$$\ln K_1 = \frac{-A(1 - \cos \theta)^2 c (\Delta P_1)^2}{kT} \quad (7)$$

and

$$\ln K_2 = \frac{-A(1 - \cos \theta)^2 c (\Delta P_2)^2}{kT} \quad (8)$$

where $K_1 = N_1/N_j$ is the ratio of the number of biomolecules in the top phase, N_1 , to the interface, N_j , $K_2 = N_j/N_2$ is the ratio of the number of biomolecules at the interface, N_j , to the bottom phase, N_2 . ΔP_1 and ΔP_2 are the concentration difference between the two aqueous phases for PEG and salt respectively. Adding (Eq. 7) to (Eq. 8) and substituting $\alpha = -(1 - \cos \theta)^2 c/kT$ yields

$$\ln K_1 K_2 = \alpha A (\Delta P_1)^2 + \alpha A (\Delta P_2)^2. \quad (9)$$

(Eq. 9) can be rewritten, using $K_1 = N_1/N_j$ and $K_2 = N_j/N_2$, as

$$\ln \left(\frac{N_1}{N_j} \times \frac{N_j}{N_2} \right) = \ln \left(\frac{N_1}{N_2} \right) = \alpha A (\Delta P_1)^2 + \alpha A (\Delta P_2)^2 \quad (10)$$

Since the partition coefficient, K and TLL are given by $K = N_1/N_2$ and tie line length (TLL) is defined as $TLL = \sqrt{\Delta P_1^2 + \Delta P_2^2}$ respectively, (Eq. 10) can be rewritten as

$$\ln K = \alpha A (TLL)^2. \quad (11)$$

From (Eq. 11), it can be seen that both the size of the molecule (and hence, to a small extent, molecular weight, since the two are slightly-correlated) and the TLL play a role in the number of molecules accumulated in the top and bottom phases. Since $\alpha = -(1 - \cos \theta)^2 c/kT$, we have $\alpha < 0$, and a plot of $\ln k$ versus $(TLL)^2$ is expected to give a negative slope. Thus, we have

$$\ln(K) - (TLL)^2. \quad (12)$$

Based on the theoretical prediction, the K_{CGTase} is expected to decrease gradually with TLL, which is in agreement with previous findings that a higher salt concentration may drive the CGTase protein towards a salt-rich bottom phase due to a high salting-out ability. This result is important as it reveals the effects of concentration difference in a phase forming component on the amount of K_{CGTase} available in the bottom phase, which can be reused for the continuous extractive bioconversion process.

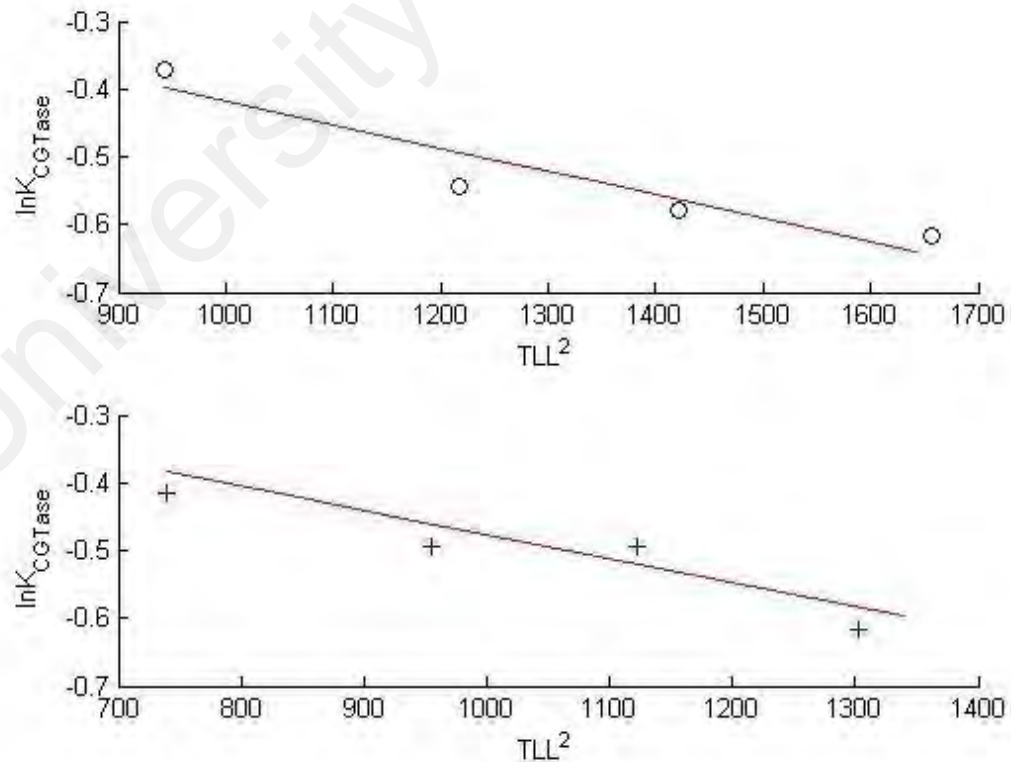


Figure 4.3: Plots of $\ln K$ against TLL^2 . The 'o' and '+' represent the data for 3000 g/mol and 8000 g/mol respectively. The lines correspond to theoretical plots.

4.3.3 Effects of PEG molecular weight and TLL on the CGTase partition and γ -CD yield

PEG with different molecular weights (3000, 6000, 8000) were tested. Previous study showed that the relative activity of CGTase was not much affected by the different PEG molecular weights (Ng et al., 2011). The TLL values of PEG/phosphates ATPSs in Table 4.1 were obtained from Ooi et al., (2009). The result showed the effects of TLL on the K_{CGTase} and the Y_T . It can be observed from the graphs in Figure 4.3 that K_{CGTase} decreases gradually with TLL. This is in agreement with Eq. (12), thereby confirming the theoretical prediction. The target of this study is to determine the TLL corresponding to the system, which gives the highest Y_T and lowest K_{CGTase} . A higher Y_T indicates a higher extraction efficiency of γ -CD while a lower K_{CGTase} suggests that more CGTase is retained in bottom phase, which can be reused for the continuous extractive bioconversion process. In other words, this study on TLL allowed the optimization of the K_{CGTase} generation. Through this optimization, it was found that the optimum ATPS was obtained in PEG 3000/phosphates at TLL of 37.7 % (w/w) with K_{CGTase} of 0.56 and Y_T of 61.02%.

Table 4.1: Effects of the PEG molecular weight and TLL on the K_{CGTase} and Y_T (%). The data obtained was presented as the average of triplicate readings with standard deviation of ± 5 %.

PEG molecular weight (g/mol)	TLL (% w/w)	K_{CGTase}	Y_T (%)
3000	30.7	0.69	48.68
	34.9	0.58	54.93
	37.7	0.56	61.02
	40.7	0.54	59.22
6000	29.6	0.84	60.00
	32.6	0.96	54.41
	35.2	0.88	59.90
	37.9	0.63	59.96
8000	27.2	0.66	55.97
	30.9	0.61	56.94
	33.5	0.61	55.34
	36.1	0.54	58.05

4.3.4 Effects of V_R on γ -CD partitioning

The results of K_{CGTase} and Y_T at different V_R ($V_R = 0.3, 1.0, 2.0, 2.3, 4.0$ and 5.0) for TLL 37.7 % (w/w) were shown in Figure 4.4. It was observed that the highest yield of γ -CD ($Y_T = 81.88\%$) was achieved at $V_R = 4.0$, which may be due to the higher free volume in the top phase, causing more γ -CD to be partitioned towards the top phase. The lowest γ -CD yield was observed at $V_R = 0.3$, the reduction of top phase free volume in low V_R condition may have limited the partition of the γ -CD to the PEG-rich top phase (Marcos et al., 1999). The V_R of 4.0 will therefore be selected for subsequent studies.

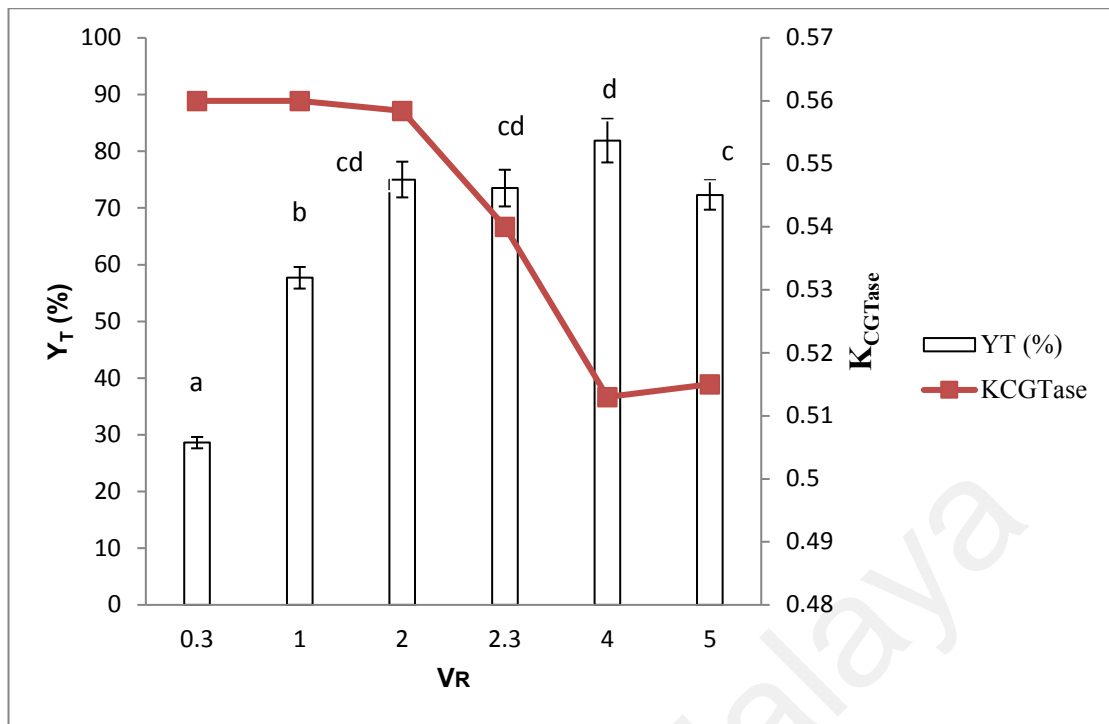


Figure 4.4: Effects of V_R on K_{CGTase} and Y_T (%). The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

4.3.5 Effects of ATPS extractive bioconversion on γ -CD production over time

Figure 4.5 showed γ -CD concentration in the top phase of ATPS against the extractive bioconversion process time. A 50 mL of PEG 3000/ potassium phosphate at TLL of 37.7 % (w/w) with V_R of 4 was applied in this study. 20 % (w/w) CGTase and 5 % (w/w) of soluble starch were added in the ATPS following Ng et al., 2011 (Ng et al., 2013). A γ -CD concentration of 2.02 mg/mL was achieved in the ATPS top phase, which was higher than the control (1.59 mg/mL). However, 1 hour (1.60 mg/mL) was suggested to be the harvesting time since the γ -CD concentration showed no significant increment afterwards. A comparison between CD concentration ratios (for different CDs) with the control sample and the 1 hour ATPS top phase sample is shown in Table 4.2. The γ -CD concentration ratio of the top phase sample (19.0%) was higher than the control sample (6.6 %), indicating that a higher γ -CD concentration ratio product is achievable through ATPS extractive bioconversion.

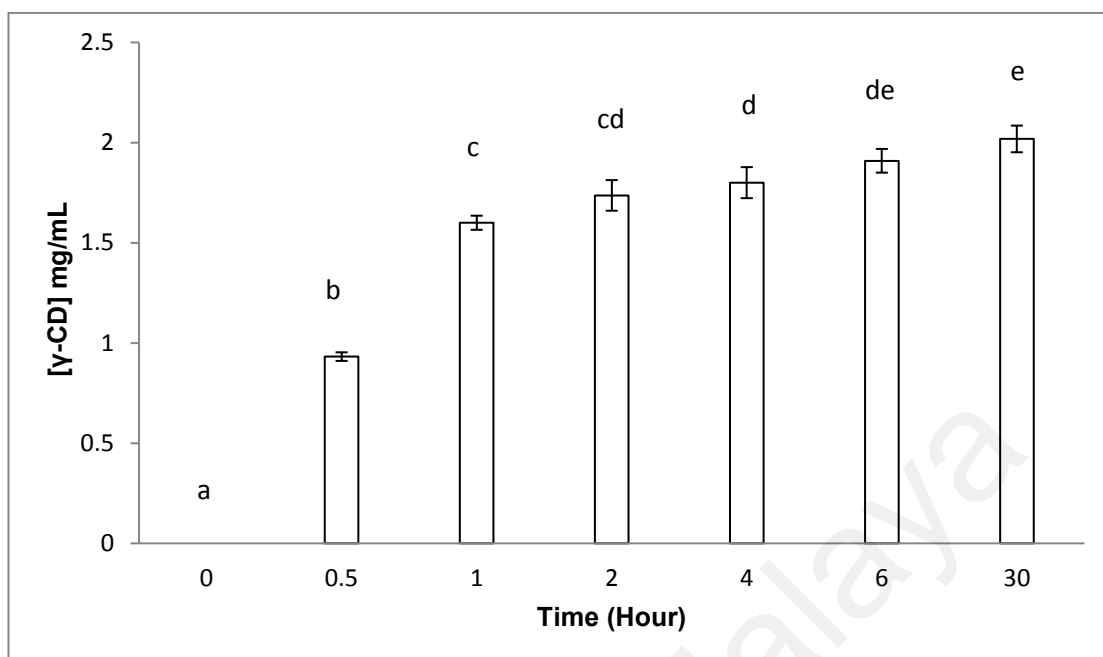


Figure 4.5: Concentration of γ -CD on top phase of extractive bioconversion ATPS over time. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

Table 4.2: A comparison of CDs concentration ratio (%) between ATPS top phase sample and control sample. The data obtained was presented as the average of triplicate readings with standard deviation of $\pm 5\%$.

CD type	Control (%)	1 hour ATPS Top phase (%)
α	67.1 (12.20 mg/ml)	55.4 (4.67 mg/ml)
β	26.3 (4.78 mg/ml)	25.6 (2.16 mg/ml)
γ	6.6 (1.20 mg/ml)	19.0 (1.60 mg/ml)
Total	100.0 (18.18 mg/ml)	100.0 (8.43 mg/ml)

4.3.6 Repetitive batch for γ -CD production

Repetitive batch of ATPS was carried out to study the recycling of CGTase enzyme in the bottom phase. Repetitive batch of γ -CD recovery was carried out in PEG 3000/potassium phosphate ATPS at 37.7 % (w/w) TLL with V_R of 4. A 20 % (w/w) of CGTase, and 5 % (w/w) of soluble starch were also added into the ATPS as described in Ng et al., 2011. Top phase extractions were done at a regular interval of 1 hour bioconversion process. Figure 4.6 showed the CGTase activity after each batch of top phase extraction, and it can be seen that the CGTase activity decreases with the following batch. The relative CGTase activity dropped to 0.76 in the third batch of extractive bioconversion. This result showed that there was a loss of CGTase *via* the removal of the top phase for the γ -CD recovery (Ng et al., 2013). CGTase enzyme was successfully recycled three times in this study.

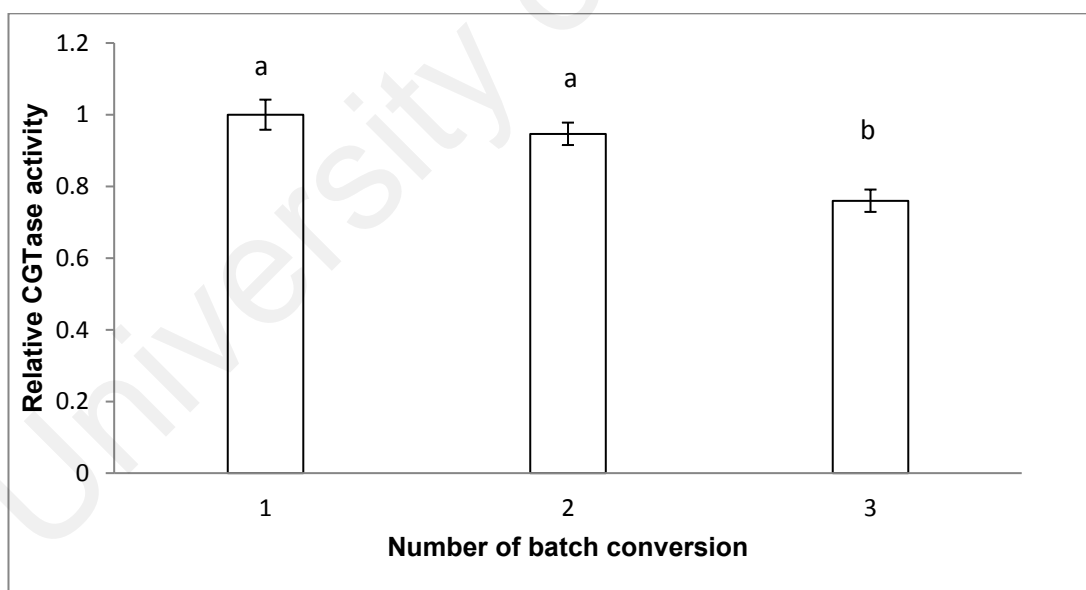


Figure 4.6: Relative CGTase activities in each batch of soluble starch bioconversion by recycling of the phase components and CGTase. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

4.4 Conclusions

Extractive bioconversion of γ -CD from soluble starch with CGTase enzyme was successfully applied in this study using ATPS. The optimized condition of ATPS extractive bioconversion was achieved by PEG 3000/ potassium phosphate at TLL of 37.7, composed of 30.0 % (w/w) PEG 3000 and 7.0 % (w/w) potassium phosphate with pH 7 and V_R of 4.0. γ -CD concentration of 1.60 mg/mL with a 19 % concentration ratio was recovered after 1 hour bioconversion process. The γ -CD production did not increase further beyond 1-hour process. The comparison between different studies on the ratio of γ -CD (%) was shown in Table 4.2. The ratio of γ -CD (%) was directly proportional to the CGTase strain and the method used in the production and purification. Surprisingly, the ratio of γ -CD (%) obtained in this study was quite high in spite of *B. cereus* producing low ratio of γ -CD in control sample (6.6 %). Thus it can be seen that ATPS extractive bioconversion is an efficient and economical technique, which combined γ -CD production and purification into a single-step process.

CHAPTER 5:

USE OF ALCOHOL/SALT AQUOUES TWO-PHASE SYSTEM IN EXTRACTIVE BIOCONVERSION OF GAMMA-CYCLODEXTRIN

5.1 Introduction

The use of ethanol/phosphate ATPS to recover phosphate has been reported by Greve and Kula (1991). Short chain alcohols or hydrophilic organic solvents are able to form stable and adjustable ATPS with inorganic salt solution; this might be due to salting-out effect and the low solubility of inorganic salt in short chain alcohol. The top phase is rich in alcohol and the bottom phase is rich in inorganic salt when the ATPS was formed.

ATPS extraction is widely applied in proteins purification because of its mild conditions and high capacity (Louwrier, 1999). However, most of the ATPSs are based on either a PEG/salt system or a polymer/polymer system. These ATPSs possess several disadvantages, such as slower segregation of the two phases, difficulty in recycling, high cost of polymers and difficult to isolate extracted target protein from the polymer phase (Tianwei et al., 2002). The target protein extracted *via* alcohol/salt ATPS can be easily recovered by evaporation of alcohol, and it offers other advantages like low toxicity to environment and inexpensive cost of extractant (Zhi and Deng, 2006).

The main objective of the study was to establish a simple and effective method of producing and extracting γ -CD from crude *B.cereus* CGTase and soluble starch directly through alcohol/salt ATPS extractive bioconversion.

5.2 Materials and methods

5.2.1 Materials

Ethanol, 1-propanol, 2-propanol and di-potassium hydrogen phosphate (K_2HPO_4) were obtained from Merck (Darmstadt, Germany). Phenolphthalein and potassium phosphate were sourced from Merck (Darmstadt, Germany). Soluble starch was acquired from Becton, Dickinson and company (New Jersey, USA). The γ -CD was supplied by Sigma-Aldrich (St. Louis, USA). All the chemicals were of analytical grade.

5.2.2 *Bacillus cereus* production

The production of *B. cereus* cells and CGTase were performed according to the method as described in section 4.3.2.

5.2.3 CGTase activity analysis

The CGTase cyclizing activity (β -CD production) was determined according to the method as described in section 3.3.3.

5.2.4 Partitioning experiments of CGTase and γ -CDs in ATPS

ATPSs were prepared by adding 1 g crude feedstock, weighing appropriate amount of alcohol, potassium phosphate, and distilled water were added to reach a final total weight of 10 g ATPS containing 10 % (w/w) of CGTase and 10 % (w/w) of standard γ -CD (50 mg/mL). The components were thoroughly mixed by centrifugation at 4000 rpm for 10 minutes. After phase separation, samples were collected for enzyme activity and γ -CD concentrations analyses.

5.2.5 Production of γ -CD using ATPS extractive bioconversion

The ATPS extractive bioconversion of γ -CD production was carried out in a 250 mL Erlenmeyer flask. Predetermined quantities of dissolved alcohol, potassium phosphate, distilled water were added to reach a final total weight of 50 g ATPS containing 5 % (w/w) soluble starch and 20 % (w/w) of CGTase. A control (without ATPS phase-forming components) was conducted for comparison. The mixture solution was kept stirring at 250 rpm and temperature controlled at 55 °C for enzymatic (CGTase) conversion of soluble starch substrate into γ -CD. Samples of top and bottom phases were collected separately at regular time intervals and heated in boiling water for 5 min to thermally inactivate the CGTase. The quantification of γ -CD concentration was carried out using the reverse phase HPLC instrument.

5.2.6 Calculations

Relative CGTase activity is expressed as the fraction of the sample's CGTase activity (U/mL) to the control.

V_R and K_{CGTase} were calculated by using formula as described in Section 3.3.6.

K_{CD} and Y_T were calculated by using formula as described in Section 4.3.6.

The data obtained was presented as the average of triplicate readings with an approximate inaccuracy of $\pm 5\%$.

5.3 Results and discussion

5.3.1 Selection of alcohol/salt ATPS

The results of CGTase activity in different alcohol was shown in Figure 5.1. The effects of alcohol (30 %, w/w) on relative CGTase activity was investigated after 8 hour of incubation with distilled water used as a control. The highest relative CGTase activity was found in ethanol solution, hence it was selected for further study.

The effects of 20 % (w/w) potassium phosphate salts at different pH conditions on the CGTase stability after 8 hour of incubation was shown in Figure 5.2. The different mixing ratios of potassium diphosphate and dipotassium phosphate salts were used in order to manipulate the different pH conditions. The result showed that relative activity of CGTase was highly influenced by the salt pH system chosen and the highest CGTase activity was found in pH 7.

Thus, according to these results, we concluded that ethanol/potassium phosphates in pH 7 ATPS is the optimum conditions for further study.

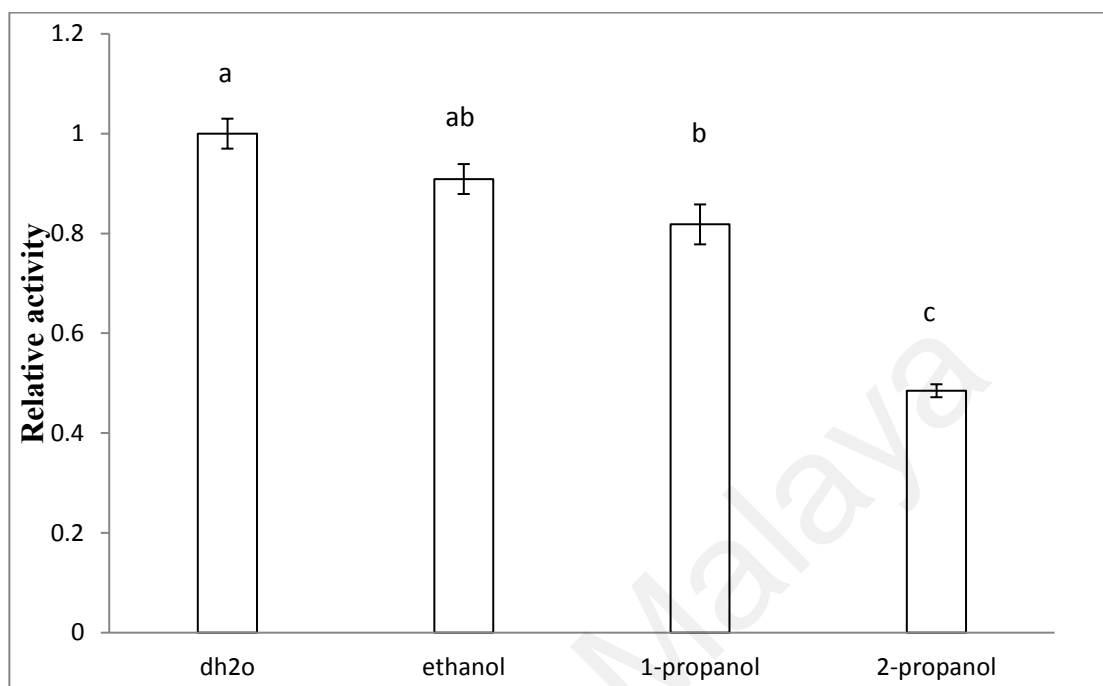


Figure 5.1: Effects of alcohol on relative CGTase activity. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

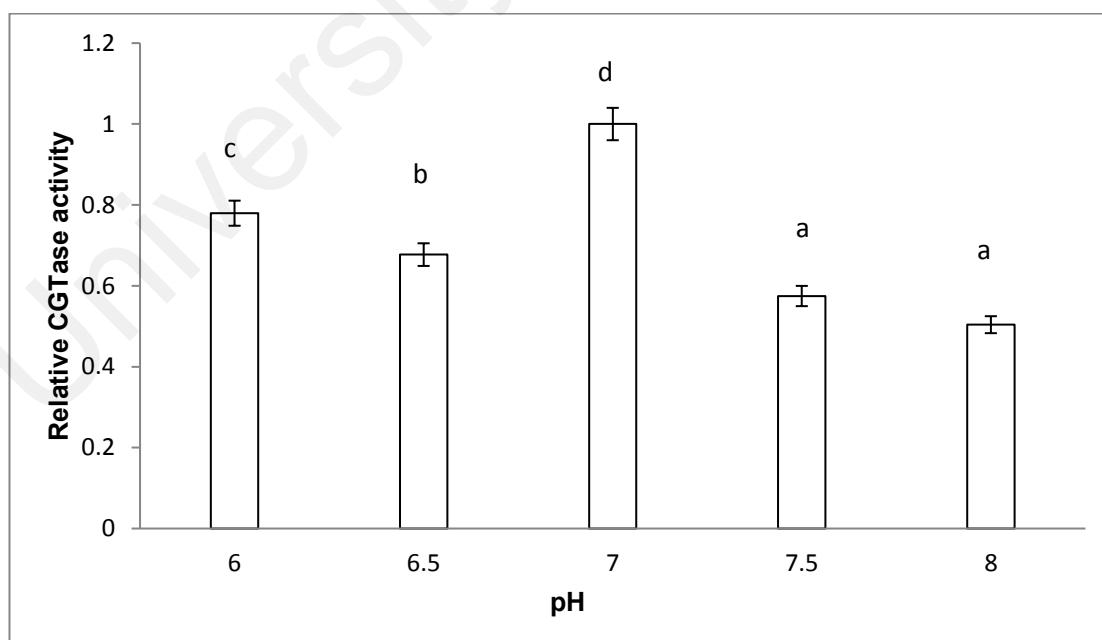


Figure 5.2: Effects of pH on relative CGTase activity. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

5.3.2 Optimization of ethanol/potassium phosphate ATPS

The effects of ethanol and potassium phosphate salt concentrations on Y_T (%) and K_{CGTase} were evaluated. The data obtained was presented as the average of triplicate readings. Highest Y_T and low K_{CGTase} were found in 24.0 % (w/w) ethanol with 20.0 % (w/w) potassium phosphate, it was therefore selected for further study.

The Y_T (%) and K_{CGTase} in ethanol/potassium phosphate ATPS was investigated in nine different combination in which various concentrations of ethanol and potassium phosphate were investigated systematically. The Y_T (%) of γ -CD generally increased when the concentration of ethanol in the system was increased, the γ -CD's surface contains hydrophobic region, which is highly associated with the ethanol hydrophobic tails, facilitating the interaction with γ -CD, and thus enhancing the partitioning of γ -CD to ethanol-rich top phase (Ooi et al., 2009). On the other hand, the K_{CGTase} generally decreased when the concentration of ethanol in the system increased, higher alcohol concentration in the top phase caused gradual dehydration in the bottom phase, hence favoring the retaining of CGTase in the bottom phase (Ooi et al., 2009). The introduction of alcohol contributes the leaving group OH^- during the bond heterolysis. Most of these OH^- anions are involved in the dehydration in the bottom phase as mentioned previously. The rest of these OH^- anions, just like the Cl^- , are radical anions that contribute to the overall electric field in the system (Chow et al., 2016).

The highest Y_T (%) was observed in 24 % (w/w) ethanol with 20 % (w/w) potassium phosphate system, with K_{CGTase} of 0.58, it was chosen based on its highest Y_T average value (sample size = 3).

ATPS phase-forming components		Y_T (%)	K_{CGTase}
Ethanol (%) (w/w)	Potassium phosphate(%, w/w)		
20	20	59.13	0.73
20	22	57.02	0.79
20	24	45.64	0.88
22	20	61.00	0.77
22	22	57.90	0.71
22	24	52.79	0.79
24	20	67.93	0.58
24	22	50.28	0.62
24	24	51.04	0.66

Table 5.1: Effects of ethanol and potassium phosphate concentrations on Y_T (%) and K_{CGTase} . The data obtained was presented as the average of triplicate readings with standard deviation of ± 5 %.

5.3.3 Effects of NaCl on Y_T of γ -CD

24 % (w/w) ethanol with 20 % (w/w) potassium phosphate system was used to study the effects of NaCl addition, ranging from 0 % (w/w) to 4 % (w/w), on Y_T of γ -CD. From Figure 5.3, maximum Y_T (87.45 %) was achieved at 3.0 % (w/w) NaCl. The partitioning behavior of γ -CD in ATPS can be related to the hydrophobicity differences between the phases caused by the addition of NaCl, where it modified the electrical potential difference between the two phases, which can in turn affect the partitioning of γ -CD (Zaslavsky, 1995). On top of that, NaCl generated electrostatic potential is suggested to have driven γ -CD to be partitioned to the top phase, thereby improving the top phase partitioning of γ -CD in ATPS (Zaslavsky, 1995). The interaction between the ethanol hydrophobic chain and the γ -CD's hydrophobic surface area would be enhanced by the

addition of NaCl. This is mostly attributed to the effects of salt addition on the water solvent structure and the hydrophobic interactions, and the target protein would be therefore attracted to the alcohol-rich top phase (Albertsson, 1986).

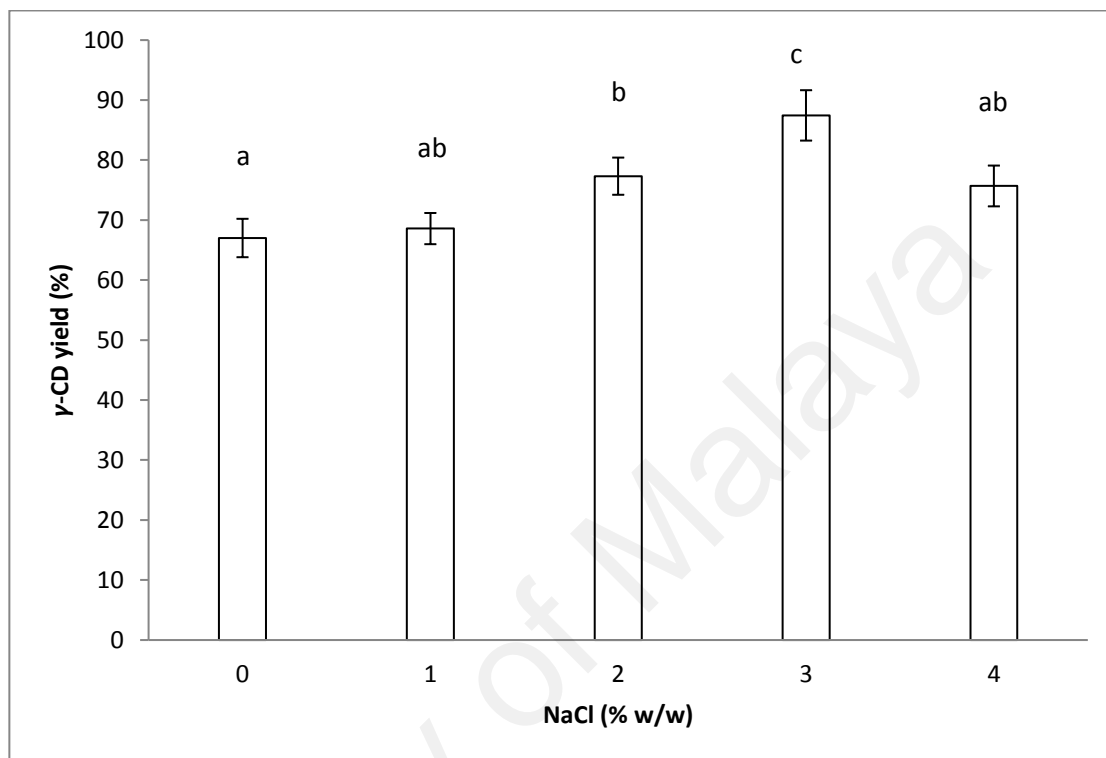


Figure 5.3: Effects of NaCl concentration on Y_T (%). The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

5.3.4 Effects of ATPS extractive bioconversion on γ -CD production over time

Figure 5.4 showed γ -CD concentration in the top phase of ATPS against the extractive bioconversion process time. A 50 mL of 24.0 % (w/w) ethanol, 20.0 % (w/w) potassium phosphate with 3.0 % (w/w) of NaCl ATPS was applied in this study. Generally, it was observed that there was a significant increase in γ -CD concentration during the process up to 1 hour. The increment became insignificant afterwards. After 24 hour of extractive bioconversion, γ -CD concentration of 0.24 mg/mL was obtained. However, 1 hour (0.23 mg/mL) was suggested to be the harvesting time since the γ -CD

concentration showed no further increment after that. The comparison between different studies on the ratio of γ -CD (%) was shown in Table 5.2. The ratio of γ -CD (%) was directly proportional to the CGTase strain and the method used in the production and purification. Surprisingly, the ratio of γ -CD (%) obtained in this study was quite high in spite of *B. cereus* produced low ratio of γ -CD in control sample (6.6 %).

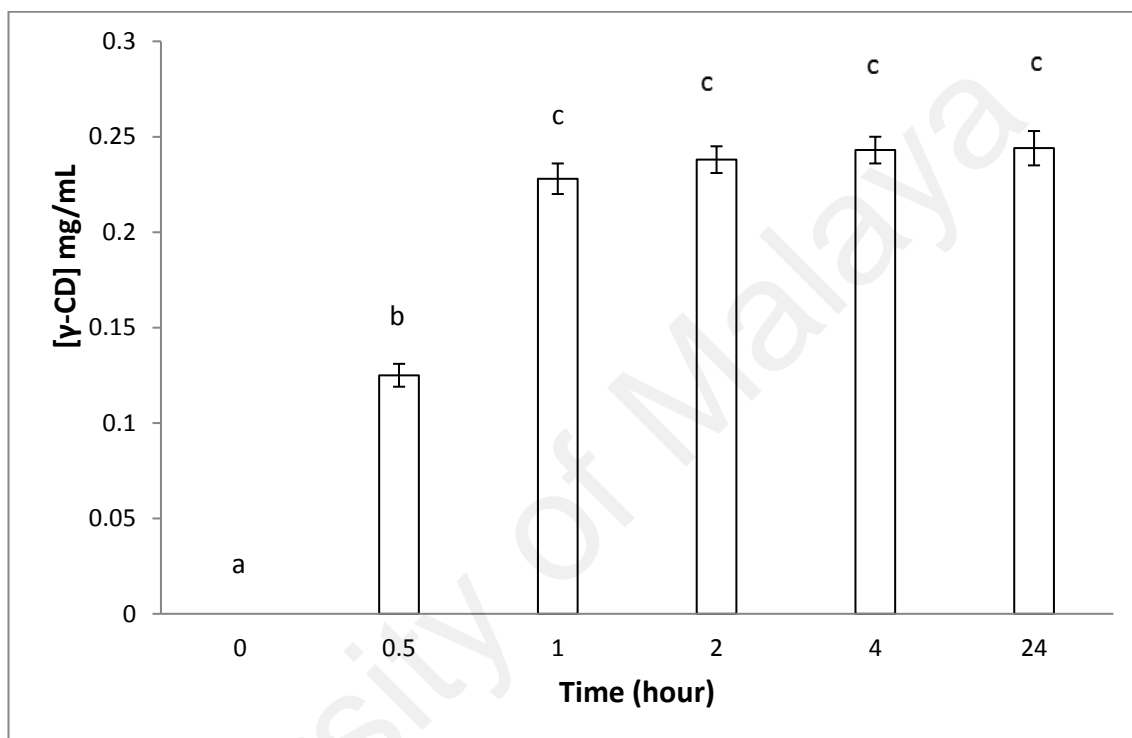


Figure 5.4: Concentration of γ -CD on top phase of extractive bioconversion ATPS over time. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

Table 5.2: A comparison of CDs concentration ratio (%) between ATPS top phase sample and control sample. The data obtained was presented as the average of triplicate readings with standard deviation of ± 5 %.

CD type	Control (%)	1 hour ATPS top phase (%)
α	67.1	62.5
β	26.3	24.4
γ	6.6	13.1
Total	100.0	100.0

5.3.5 Repetitive batch for γ -CD production

Repetitive batch of ATPS was carried out to investigate the recycling of CGTase enzyme in the bottom phase. Repetitive batch of γ -CD recovery was carried out in 24.0 % (w/w) ethanol, 20.0 % (w/w) potassium phosphate with 3.0 % (w/w) of NaCl ATPS. 20 % (w/w) of CGTase was added into the ATPS. Top phase extractions were done at a regular interval of 1 hour bioconversion process. Figure 5.5 showed the CGTase activity after each batch of top phase extraction, and it can be seen that the CGTase activity decreases with the following batch. The relative CGTase activity reduced to 0.66 in the third batch of extractive bioconversion. This result showed that there was a loss of CGTase *via* the removal of the top phase for the γ -CD recovery. CGTase enzyme was successfully recycled three times in this study.

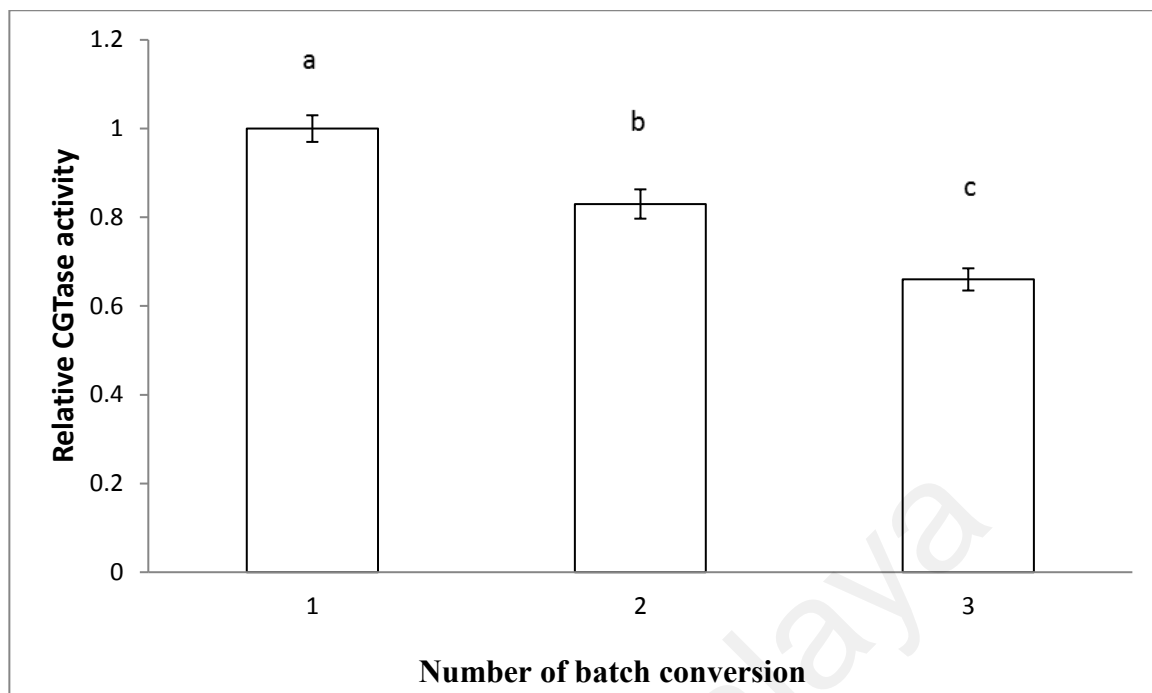


Figure 5.5: Relative CGTase activities in each batch of soluble starch bioconversion by recycling of the phase components and CGTase. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

5.4 Conclusions

The optimized conditions of ATPS extractive bioconversion was achieved at 24.0 % (w/w) ethanol, 20.0 % (w/w) potassium phosphate with 3.0 % (w/w) of NaCl ATPS. γ -CD concentration of 0.23 mg/mL was recovered after a 1 hour bioconversion process. Thus it can be seen that ATPS extractive bioconversion was successfully applied in this study using ethanol/salt ATPS which combined γ -CD production and purification into a single-step process.

CHAPTER 6:

EXTRACTIVE BIOCONVERSION OF GAMMA-CYCLODEXTRIN AND RECYCLING OF CYCLODEXTRIN GLYCOSYLTRANSFERASE IN AQUEOUS TWO-PHASE SYSTEM USING THERMO-SEPARATING POLYMER

6.1 Introduction

In this study, ATPS extractive bioconversion was carried out to partition the target product and biocatalyst into top and bottom phases, respectively. These molecules are selectively partitioned based on the surface properties of the molecules and particles such as size, charge, hydrophobicity (Walter et al., 1994). The ATPS extractive bioconversion combined production and recovery technique into a single step. In contrast to conventional processes (e.g., enzymatic bioconversion), the biocatalyst that retained in one phase is reusable and recyclable, encouraging a continuous extractive bioconversion process (Charoenlap et al., 2004).

Currently, polymer/polymer ATPS is one of the partitioning systems extensively studied and utilized. For example, PEG/dextran ATPS is the common polymer/polymer ATPS widely utilized for the purification and separation of diversified biomolecules such as proteins, nucleic acids and cell organelles (Charoenlap et al., 2004; Lu et al., 1996). Nevertheless, the application of PEG/dextran ATPS at industrial scale has been hampered by the high cost of the phase-forming chemicals (i.e. dextran) (Lu et al., 1996). Recovery of valuable biomolecules such as gamma-cyclodextrin (γ -CD) by ATPS could be more environmental and economical friendly using a recyclable ATPS, where ethylene oxide-propylene oxide (EOPO) is used instead of PEG, in addition to retaining the biological activity of the enzyme.

EOPO is a copolymer capable of separating into two phases when the temperature is higher than the lower critical solution temperature (LCST), which is around 50 °C (Johansson et al., 1997). After heating the system above a certain temperature, the EOPO split up into two phases, allowing the polymers to be recovered and reutilized in subsequent ATPS, simplifying the purification steps and reducing the cost of the environmental impact (Dembczynski et al., 2010; Johansson et al., 1999).

To date, some studies have shown that EOPO ATPS have been successfully applied on other biological products such as lipase (Show et al., 2012) and CGTase (Ng et al., 2012). However, no study regarding EOPO ATPS on extractive bioconversion of γ -CD has been found. Therefore, an ATPS extractive bioconversion was carried out to enhance the separation and productivity of γ -CD from *Bacillus cereus* CGTase. The effects of EOPO molecular weight, tie-line lengths (TLLs), volume ratio (V_R) and introduction of NaCl on the γ -CD recovery were investigated followed by its optimization.

6.2 Materials and Methods

6.2.1 Materials

γ -CD standard was bought from Acros Organic (New Jersey, USA). The poly(ethylene glycol-ran-propylene glycol) (2500, 12000 g/mol) and poly(ethylene glycol-ran-propylene glycol) mono butyl ether (970 g/mol) were bought from Sigma-Aldrich Co. (St. Louis, MO, USA). The potassium phosphate salts (K_2HPO_4 , KH_2PO_4) were bought from Merck (Darmstadt, Germany). Phenolphthalein was acquired from Merck (Darmstadt, Germany). Soluble starch was bought from Becton, Dickinson and Company (New Jersey, USA). All chemicals used in this study were of analytical grade.

6.2.2 *Bacillus cereus* production

The production of *B. cereus* cells and CGTase were performed according to the method as described in section 4.3.2.

6.2.3 Partitioning of CGTase and γ -CD in ATPS

Experiments were carried out at room temperature (24 ± 1 °C) with pre-determined amounts of dissolved EOPO, potassium phosphate and distilled water mixed in a 15 mL centrifugation tube. 10 % (w/w) of CGTase and 10 % (w/w) of standard γ -CD (50 mg/mL) were then added into the ATPS for a total weight of 10 g. The mixtures were shaken using vortex mixer and then subjected to centrifugation for 10 min at 4000 rpm. After the phase separation, bottom and top phases were collected for γ -CD concentrations and CGTase activity analyses.

6.2.4 CGTase activity analysis

The CGTase cyclizing activity (β -CD production) was determined according to the method as described in section 3.3.3.

6.2.5 Extractive bioconversion of γ -CD in ATPS

Extractive bioconversion of γ -CD was carried out in a 250 mL Erlenmeyer flask. The reaction temperature was 55 °C. Predetermined dissolved EOPO, potassium phosphates, distilled water, 5 % (w/w) soluble starch substrate and 20 % (w/w) of the CGTase were added into the ATPS for a final total weight of 50 g reaction mixture. A control flask (without ATPS phase-forming components) was also prepared for soluble starch enzymatic conversion. Top phase samples were collected at regular time interval and heated in boiling water for 5 min to thermally inactivate the CGTase. The quantification of the γ -CD concentration was carried out using HPLC.

6.2.6 Calculations

Relative CGTase activity is expressed as the fraction of the sample CGTase activity (U/mL) to the control.

V_R and K_{CGTase} were calculated by using formula as described in Section 3.3.6.

K_{CD} and Y_T were calculated by using formula as described in Section 4.3.6.

6.3 Results and discussion

6.3.1 Effects of EOPO molecular weight and TLL on Y_T of γ -CD and CGTase partitioning

Phase diagrams of ATPS at different EOPO molecular weights and phosphate salt were referred to Show et al., (2012). TLLs were constructed for the Y_T and K_{CGTase} tests. EOPO 970, EOPO 3900 and EOPO 12000 were selected for this study due to their compatibility with biomaterials and capability in developing two phases with the salt components (Persson et al., 2000; Dembczynski et al., 2010).

From Table 6.1, the highest Y_T of γ -CD at 63.1 % and CGTase with K_{CGTase} at 3.14 were achieved in EOPO 970/potassium phosphate ATPS at TLL of 54.6 % (w/w). As seen from the results in Table 1, the highest Y_T value was achieved in ATPS comprising of EOPO 970. The EOPO 970 with lower PO content (50 %) resulted in higher Y_T compared to EOPO with higher PO content (EOPO 3900, EOPO 12000). The lower PO content of EOPOs (EOPO 970) allowed for maximum solubility of γ -CD in the polymer phase, thereby avoiding the γ -CD precipitation in the interphase (Huang et al., 2002). The K_{CGTase} values were decreased as TLL increased, and this was attributed to the

increase in the polymer concentration causing the decrease in free volume of ATPS top phase, thus drawing more CGTase to the ATPS bottom phase (Forciniti et al., 1991). Hence, EOPO 970/potassium phosphate in TLL of 54.6 % (w/w) was selected for subsequent experiments since it exhibited the optimal condition for highest Y_T with low K_{CGTase} .

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Table 6.1: Effects of the EOPO molecular weight and TLL on K_{CGTase} and Y_T (%). The data obtained was presented as the average of triplicate readings with standard deviation of $\pm 5\%$.

EOPO molecular weight (g/mol)	TLL (% w/w)	K_{CGTase}	Y_T (%)
970	52.3	5.16	47.6
	54.6	3.14	63.1
	58.0	4.20	58.4
	61.8	4.06	53.0
3900	36.4	8.44	46.5
	41.2	8.69	46.3
	44.6	6.06	56.4
	48.5	4.19	55.9
12000	31.6	3.74	52.1
	34.4	3.65	54.7
	42.5	2.79	51.6
	50.2	2.04	47.5

6.3.2 Effects of V_R on the Y_T of γ -CD and partitioning of CGTase

The K_{CGTase} and Y_T at different V_R (0.3 to 4.0) for EOPO 970/potassium phosphate TLL 54.6 % (w/w) were shown in Figure 6.1. The highest Y_T of γ -CD at 68.7 % was reached at $V_R = 2.0$. Generally, the Y_T increased with V_R , this may attributed to the fact that a higher V_R (> 1) resulted in more free volume in the top phase, thereby increasing the solubility limit of Y_T , which in turn caused more γ -CD to be drawn to the top phase (Schmidt et al., 1994). The optimal $V_R = 2.0$ was selected for subsequent studies.

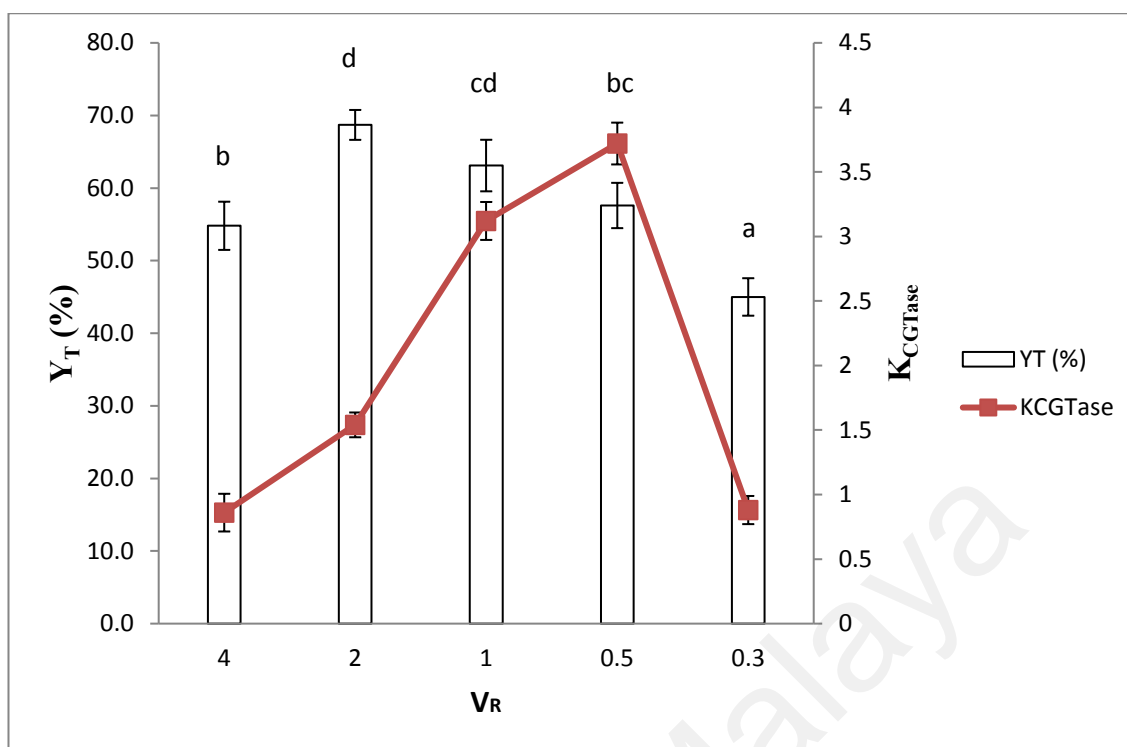


Figure 6.1: Effects of V_R on the Y_T of γ -CD and partitioning of CGTase. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

6.3.3 Effects of NaCl on Y_T of γ -CD

EOPO 970/potassium phosphate in TLL 54.6 % (w/w) with $V_R = 2.0$ was used to examine the effects of adding NaCl, ranging from 0 % (w/w) to 4 % (w/w) on the Y_T . The partitioning behavior of γ -CD in ATPS was due to the hydrophobicity difference between the phases caused by the addition of NaCl. In addition, the electrostatic potential created by NaCl had forced γ -CD to the top phase, thereby increasing the top phase partitioning of it (Zaslavsky et al., 1991). The interaction between the hydrophobic chain of the EOPO and hydrophobic surface of γ -CD was enhanced by the addition of the salts. This enhancement was attributed to the effects of the salt on the hydrophobic interactions and the water solvent structure. The target biomolecules would thus partition to the polymer rich top phase (Albertsson et al., 1986). Similar to PEG-salt system (Huddleston et al., 1991), when the NaCl

concentration is lower, the electrostatic field due to the Na⁺ and Cl⁻ ions will play a greater role in the γ -CD extraction. However, a high NaCl concentration causes a lot of γ -CD to partition to the top phase. This large number of γ -CD molecules bind with the EOPO polymers through hydrophobic interactions, thereby forming larger hydrophobes and repelling the water molecules to the bottom phase. With a reduction in the water molecules in the top phase, there is less water for dissolution of γ -CD molecules, thereby causing a reduction in the yield of the γ -CD in the top phase. Thus, although the NaCl serves to help increase the yield of γ -CD, too high a salt level will have the reverse effect of reducing the yield. From Table 6.2, it can be seen that optimal yield of γ -CD occurred at NaCl concentration of approximately 2 % (w/w).

Table 6.2: Effects of NaCl on Y_T (%). The data obtained was presented as the average triplicate readings with standard deviation of ± 5 %. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

NaCl concentration %(w/w)	Y_T (%)
0	68.3 ^a
1	71.7 ^{ab}
2	74.4 ^b
3	72.8 ^{ab}
4	72.1 ^{ab}

The effect V_E of the electric field (Lemeshko et al., 2013) on the molecules is given as

$$V_E = -\underline{E} \cdot \sum_n q_n \underline{r}_n \quad (5)$$

where \underline{E} is the electric field, q_n is the charge of electron n , \underline{r}_n is the position vector of electron n . By approximating the perimeter of a γ -CD molecule to a circle with the origin of \underline{r} at the center of the circle and taking the charge of an electron to be a constant k_e (i.e. the Coulomb constant), and the radius of the γ -CD molecule denoted as

R , Eq. (5) can be rewritten as

$$\begin{aligned} V_E &= -\underline{E} \cdot \sum_n q_n r_n \hat{r}_n = -\underline{E} \cdot \sum_n k_e R \hat{r}_n = -k_e R \sum_n \underline{E} \cdot \hat{r}_n \\ &= -k_e R \sum_0^{\frac{\pi}{2}} |\underline{E}| \cos \theta = -k_e R A |\underline{E}| \end{aligned} \quad (6)$$

where θ is the angle between \underline{E} and \hat{r}_n and $A = \sum_0^{\frac{\pi}{2}} \cos \theta = \text{constant}$. Thus, from Eq. (6), it can be seen that the higher the NaCl concentration, the larger the electric field, and hence the larger its effect on the molecule. Hence, it is expected that γ -CD, which has a larger R than α - and β -CD, to be more influenced by the presence of NaCl in terms of the electric field generated.

6.3.4 Extractive bioconversion on γ -CD production over time

Figure 6.2 showed γ -CD concentration in ATPS top phase against the extractive bioconversion process time. 50 mL of EOPO 970/potassium phosphate at TLL of 54.6 % (w/w) with $V_R = 2.0$ was employed in this study. 2 % (w/w) NaCl, 20 % (w/w) CGTase and 5 % (w/w) of soluble starch were added in the ATPS. About 1.10 mg/mL concentration of γ -CD was collected in the ATPS top phase sample after 30 hours extractive bioconversion process. Nevertheless, the harvest time was proposed at 2 hour (0.87 mg/mL) since the increase in γ -CD concentration was insignificant after this time. A comparison of the CD concentration ratios (for different CDs) between the control sample and ATPS top phase sample at 2 hour was shown in Table 6.3. The γ -CD concentration ratio of the top phase sample (17.5 %) was much higher than that of the control sample (6.6 %). This showed that higher γ -CD product ratio was achievable by using the ATPS extractive bioconversion process.

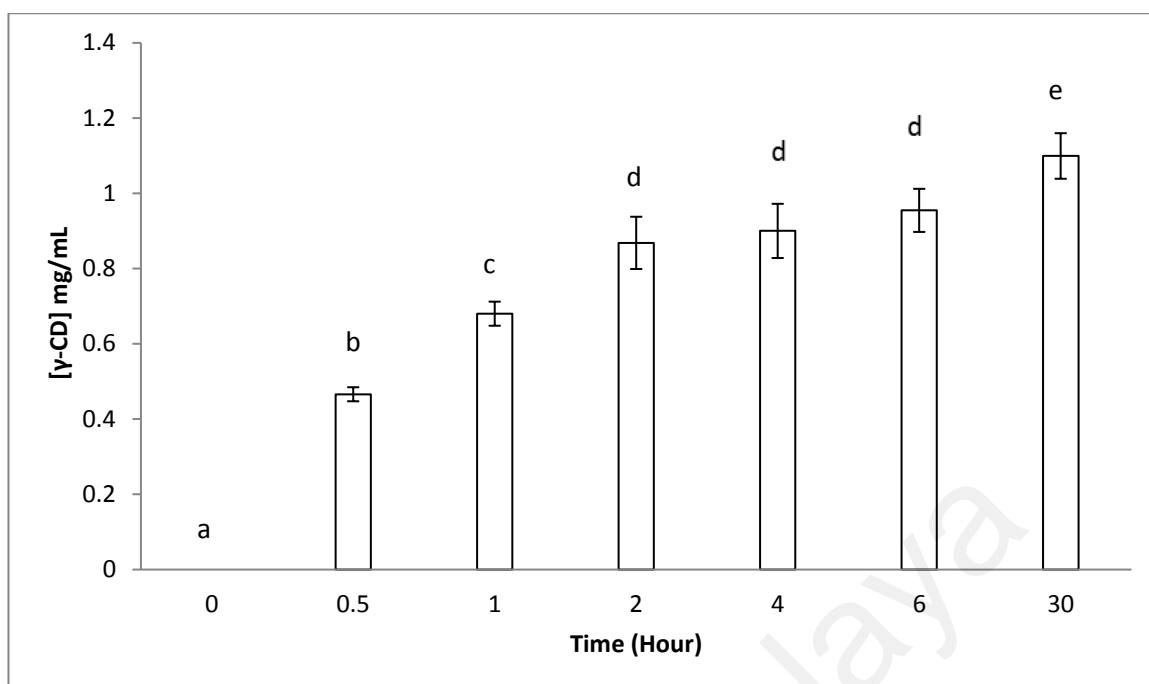


Figure 6.2: Effects of ATPS extractive bioconversion on γ -CD production over time. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

Table 6.3: A comparison of CDs concentration ratio (%) between ATPS top phase sample and control sample. The data obtained was presented as the average of triplicate readings with standard deviation of ± 5 %.

CD type	Control (%)	2 hour ATPS top phase (%)
α	67.1	59.0
β	26.3	23.5
γ	6.6	17.5
Total	100.0	100.0

6.3.5 Repetitive batch bioconversion

ATPS repetitive batch study was carried out to investigate the recycling of the CGTase enzyme in the bottom phase. The repetitive batch of the γ -CD recovery was carried out using EOPO 970/potassium phosphate ATPS at 54.6 % (w/w) TLL, $V_R = 2.0$, added with 2 % (w/w) NaCl, 20 % (w/w) of CGTase, and 5 % (w/w) of soluble starch. Top

phase extractions were done at a regular interval of 2 hours extractive bioconversion process. Figure 6.3 showed CGTase activity of the bottom phase after extraction of the top phase, and it can be seen that the CGTase activities continued to decline in subsequent batches. The relative CGTase activities went down to 0.45 in the fourth batch of extractive bioconversion. The loss of CGTase activities was observed with the removal of the top phase (which contained a significant amount of CGTase) (Tramice et al., 2008). Therefore, to ensure the effectiveness and sustained performance of bioconversion, the CGTase in the bottom phase should not be recycled more than once.

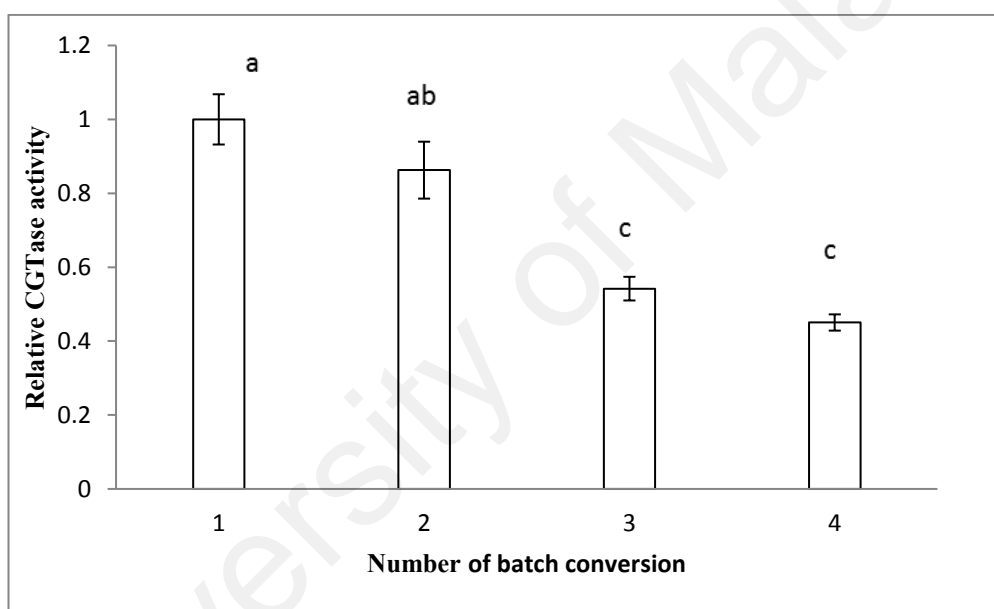


Figure 6.3: Relative CGTase activities in each batch of soluble starch bioconversion by recycling of the phase components and CGTase. The data obtained was presented as the average of triplicate readings. Value with different letter(s) are significantly different by the Tukey's HSD ($p < 0.05$). Uses harmonic mean sample size: 3.000.

6.4 Conclusions

In this study, EOPO polymer has been applied to the ATPS extractive bioconversion of γ -CD. The partitioning of γ -CD in EOPO-potassium phosphate ATPS was influenced by the EOPO molecular weight and concentration of the EOPO used. The optimal extractive bioconversion of γ -CD in top phase of the ATPS was obtained in EOPO

970/potassium phosphate ATPS of 54.6 % (w/w) TLL, V_R of 2.0 with 2 % (w/w) NaCl. The γ -CD concentration of 0.87 mg/mL with 17.5 % concentration ratio was recovered after 2 hours of extractive bioconversion process. Thus, the objective of this study, which was to produce and recover γ -CD directly using ATPS, has been achieved. In particular, it has been shown that the EOPO 970/potassium phosphate ATPS extractive bioconversion is a better alternative technique for producing the γ -CD compared to the conventional method as it merges production and purification into a single-step process. For future work, the thermo-separating properties of EOPO/potassium phosphate ATPS could be exploited upon to further study the recovery of γ -CD in the water phase as well as the recyclability of EOPO.

CHAPTER 7: CONCLUSIONS

High commercial values of CGTase and γ -CD have necessitated the development of cost-efficient purification technique that meets the increasing demands and challenges from the fast-growing global enzyme market. In order to develop a purification and production technique with lower cost and higher efficiency, the potential of ATPS as the alternative tool for bacterial CGTase and γ -CD has been evaluated within the scope of this dissertation.

It can be concluded that:

1. The *Bacillus cereus* CGTase was successfully purified through ATPF process. The most optimum condition for the purification was achieved using 18.0 % (w/w) PEG 8000, 7.0 % (w/w) potassium phosphates, V_R of 3.0, 20 % (w/w) crude concentration at pH 7, and 80 min of nitrogen flotation at a flow rate of 5 L/min. The utility of ATPF as a primary purification step was evidenced from P_{FT} of 21.8 and Y_T of 97.1 % achieved;
2. Extractive bioconversion of γ -CD was successfully applied in this study making use of ATPS. The optimized condition was reached by PEG 3000/ potassium phosphate at TLL of 37.7, composed of 30.0 % (w/w) PEG 3000 and 7.0 % (w/w) potassium phosphate with pH 7 and V_R of 4.0. γ -CD concentration of 1.60 mg/mL with a 19 % concentration ratio was recovered at top phase after 1 hour bioconversion;
3. The optimized condition of ATPS extractive bioconversion was obtained at 24.0 % (w/w) ethanol, 20.0% (w/w) potassium phosphates with 3.0 % (w/w) of NaCl ATPS.

γ -CD concentration of 0.23 mg/mL was recovered after a 1 hour bioconversion process;

4. The optimum extractive bioconversion of γ -CD in top phase of the ATPS was obtained at EOPO 970/potassium phosphate ATPS of 54.6 % (w/w) TLL, V_R of 2.0 with 2 % (w/w) NaCl. γ -CD concentration of 0.87 mg/mL with 17.5 % concentration ratio was recovered after 2 hours of extractive bioconversion process;
5. Based on these different types of ATPSs, PEG 3000/potassium phosphate ATPS provided the most efficient extractive bioconversion results in terms of the γ -CD production.

In the present study, γ -CD has been successfully recovered from soluble starch by implementing different types of ATPSs. However, further improvement can be done on γ -CD recovery using ATPS before practical implementation of ATPS in large-scale downstream processing of CGTase and γ -CD could be made.

1. Back-extraction step can be added to PEG/phosphate ATPS for the recovery of γ -CD from PEG top phase. The higher purity product would be obtained by back-extraction step. Besides, the back-extraction step allows for the recycling of PEG thus saving the chemical expenditure;
2. For the extractive bioconversion in ATPS, the partitioning behavior of CGTase that favor the bottom phase of the ATPS can be improved by employing the whole-cell CGTase rather than the cell-free CGTase. It was suggested that cell-containing CGTase feedstock will reduce the partition coefficient of the enzyme and thus

enhanced the retention of CGTase at the bottom phase of the ATPS. In addition, it is anticipated that the strategy of extractive bioconversion with ATPS can be further incorporated with the extractive fermentation of CGTase. This process integration encompasses fermentation and bioconversion could very well help to separate the product CDs from the CGTase while continuously producing CGTase at another phase of ATPS;

3. For thermo-separating EOPO, the salt bottom phase could be recycled through the establishment of a dialysis procedure. In this manner, both the top and bottom phase of the ATPS can be recycled and reused for the preparation of new ATPS for purification of CGTase;
4. The larger volume of ATPS would allow higher amount of γ -CD to be produced. Hence, the ATPS could be further scaled-up with the objective to attain a higher production amount of γ -CD.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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- 1) Lin, Y. K., Show, P. L., Yap, Y. J., Tan, C. P., Ng, E. P, Ariff, A., Suffian, M., & Ling, T. C. (2015). Direct recovery of cyclodextrin glycosyltransferase from *Bacillus cereus* using aqueous two-phase flotation. *Journal of Bioscience and Bioengineering*, 120, 684-689.
- 2) Lin, Y. K., Show, P. L., Yap, Y. J., Ariff, A., Suffian, M., Lai, O. M., Tang, T. K, Juan, J. C., & Ling, T. C. (2015). Production of γ -cyclodextrin by *Bacillus cereus* cyclodextrin glycosyltransferase using extractive bioconversion in polymer-salt aqueous two-phase system. *Journal of Bioscience and Bioengineering*, 121, 692-696.

Conference

- 1) Poster presentation, 12th Asian Congress on Biotechnology (ACB 2015), 15-19 November 2015, Kuala Lumpur, Malaysia.
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Direct recovery of cyclodextringlycosyltransferase from *Bacillus cereus* using aqueous two-phase flotation

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Purification of cyclodextrin glycosyl transferase (CGTase) from *Bacillus cereus* using polyethylene glycol (PEG)-potassium phosphates aqueous two-phase flotation (ATPF) system was studied in this paper. The effects of varying PEG molecular weight, tie-line length (TLL) value, volume ratio (V_R), pH value, crude concentration and gas nitrogen flotation time were investigated. The optimal condition for purification of CGTase was attained at 18.0% (w/w) PEG 8000, 7.0% (w/w) potassium phosphates, V_R of 3.0, 20% (w/w) crude load at pH 7, and 80 min nitrogen flotation time at a flow rate of 5 l/min. With this optimal condition, purification factor (P_{FT}) of 21.8 and a yield (Y_r) of 97.1% were attained. CGTase was successfully purified in a single downstream processing step using the ATPF.

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[Key words: Cyclodextringlycosyltransferase; Aqueous two-phase flotation; *Bacillus cereus*; Purification; Polyethylene glycol-salt system]

Cyclodextrin glycosyl transferase (CGTase, 1,4- α -D-glucopyranosyl transferase, cyclizing, E.C. 2.4.1.19) is an extracellular enzyme belonging to the glycosyl hydrolases of α -amylase family. This enzyme is capable of changing starch into cyclodextrins (CDs) through cyclization reactions involving bond cleavage, circularization and a new bond formation which produces the CDs product (1). In addition, due to the inability of CGTase to bypass α -(1,6) linkages in gelatinized starches which decrease the viscosity while maintaining the high molecular features of the starches (2), the CGTase can be used in the liquefaction of starch.

CGTase purification methods such as ion exchange chromatography (3) and column chromatography (4) have been studied extensively in recent years. However, there are major problems with these methods. For instance, due to the large amount of CGTase lost after multiple steps of the purification process (3), ion exchange chromatography was reported to generate low recovery yields of CGTase. To overcome this problem, the aqueous two-phase flotation (ATPF) system which combines aqueous two-phase system (ATPS) with solvent sublation was introduced (5). This ATPF system employs water-soluble phase components which results in the formation of two immiscible phases. The surface-active compounds are adsorbed to the surfaces of the nitrogen bubbles which

in turn dissolve in the polymer layer, resulting in a higher purity of the final product (Fig. 1) (5). Advantages of the ATPF technique include ease of operation, economic feasibility and environmental friendliness (6). Other applications of ATPF to separation studies include purification of lincomycin (7), penicillin G (8), puerarin (9), and lipase (10). In comparison to ATPS, ATPF [given its higher partition coefficient (9)] appears to be a more promising alternative for separating puerarin from a *Puerariae* extract.

Previous report has shown that ATPSs have been successfully used for the purification of CGTase (11). However, the use of PEG-potassium phosphate ATPF for the purification of *Bacillus cereus* CGTase has yet to be explored. Motivated by this, this paper aims to study the feasibility of using PEG-potassium phosphate ATPF for the purification of CGTase. In this paper, the partition behavior of CGTase was determined and optimized in ATPF with the goal of achieving a high purification factor (P_{FT}) of CGTase.

MATERIALS AND METHODS

Materials PEG of average molecular weight 4000, 6000, 8000, 10,000 and 20,000 g/mol were sourced from Fluka Co. (St. Louis, MO, USA). CDs were sourced from Sigma (St. Louis, MO, USA). Protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Potassium di-hydrogen phosphate (KH_2PO_4) and Dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck (Darmstadt, Germany).

CGTase production The CGTase was produced through *B. cereus* culture (11). The *B. cereus* culture was grown at 37 °C with a continuous agitation at 250 rpm for 30 h with 10% (v/v) of inoculum in a 250 ml baffled shake flasks (11). The crude enzyme supernatant was harvested after 15 min of 4000 \times g centrifugation.

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Production of γ -cyclodextrin by *Bacillus cereus* cyclodextrin glycosyltransferase using extractive bioconversion in polymer-salt aqueous two-phase system

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Aqueous two-phase system (ATPS) extractive bioconversion provides a technique which integrates bioconversion and purification into a single step process. Extractive bioconversion of gamma-cyclodextrin (γ -CD) from soluble starch with cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) enzyme derived from *Bacillus cereus* was evaluated using polyethylene glycol (PEG)/potassium phosphate based on ATPS. The optimum condition was attained in the ATPS constituted of 30.0% (w/w) PEG 3000 g/mol and 7.0% (w/w) potassium phosphate. A γ -CD concentration of 1.60 mg/ml with a 19% concentration ratio was recovered after 1 h bioconversion process. The γ -CD was mainly partitioned to the top phase ($Y_T = 81.88\%$), with CGTase partitioning in the salt-rich bottom phase ($K_{CGTase} = 0.51$). Repetitive batch processes of extractive bioconversion were successfully recycled three times, indicating that this is an environmental friendly and a cost saving technique for γ -CD production and purification.

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Keywords: Aqueous two-phase system; *Bacillus cereus*; Cyclodextrin; Cyclodextrin glycosyltransferase; Extractive bioconversion

An aqueous two-phase system (ATPS) is formed by mixing two incompatible aqueous solutions at a concentration exceeding a specific threshold (1). The ATPS provides a simple, low cost, and low pollution technique for purification, and it has proven to provide a biocompatible environment for the separation since both phases consist of a predominantly high water content (2,3). In this study, the production of γ -CD was performed using the enzymatic bioconversion method. Results of research on bioconversion have been used to facilitate the production of other bioproducts such as β -D-glucose-1-phosphate (4), naringin (5), bioethanol (6), L-DOPA (7), and curcuminoids (8). The application of ATPS extractive bioconversion provides a technique which integrates bioconversion and purification into a single step process (9). ATPS extractive bioconversion was designed to partition the biocatalyst and the desired product into the bottom and top phase respectively. In contrast with conventional methods (e.g., enzymatic bioconversion), ATPS extractive bioconversion integrated production and

purification process into single step, the biocatalyst retained in one phase can be reused, prompting the possibility of a continuous extractive bioconversion process (Fig. 1) (10).

The Cyclodextrins (CDs) are cyclic oligosaccharides produced by cyclodextrin glycosyltransferase (CGTase) enzyme via the transglycosylation process (11). CDs have a structural feature which enables them to form inclusion complexes with a lot of guest compounds, promoting wide spread applications of CDs in different industries such as food, pharmaceuticals, cosmetics, agricultural and chemical products (12).

There are three major types of CDs, namely, α , β and γ which are formed by 6, 7 and 8 glucopyranose units, respectively (13). Among these three types of CDs, the γ -CD has the premier solubility and the largest interior cavity. In addition, γ -CD is more expensive than α and β -CD due to its lower production compare with α and β -CD (14,15). The extractive bioconversion of cyclodextrins (α -CD, β -CD and γ -CD) by *Bacillus cereus* cyclodextrin glycosyltransferase in poly(ethylene glycol) [PEG] 20,000 and dextran T500 aqueous two-phase system has been explored (16). Unfortunately, there is no available study on ATPS extractive bioconversion which focuses on optimized production of γ -CD as target product. Motivated by this, an ATPS extractive bioconversion is studied in this work to

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APPENDIX A

STANDARD CALIBRATION

A.1 Protein determination standard calibration

The procedure for protein determination is described in section 3.2.6.

BSA was used as a standard in the study to determine the concentration of total protein.

A typical standard calibration is shown in Figure A1.

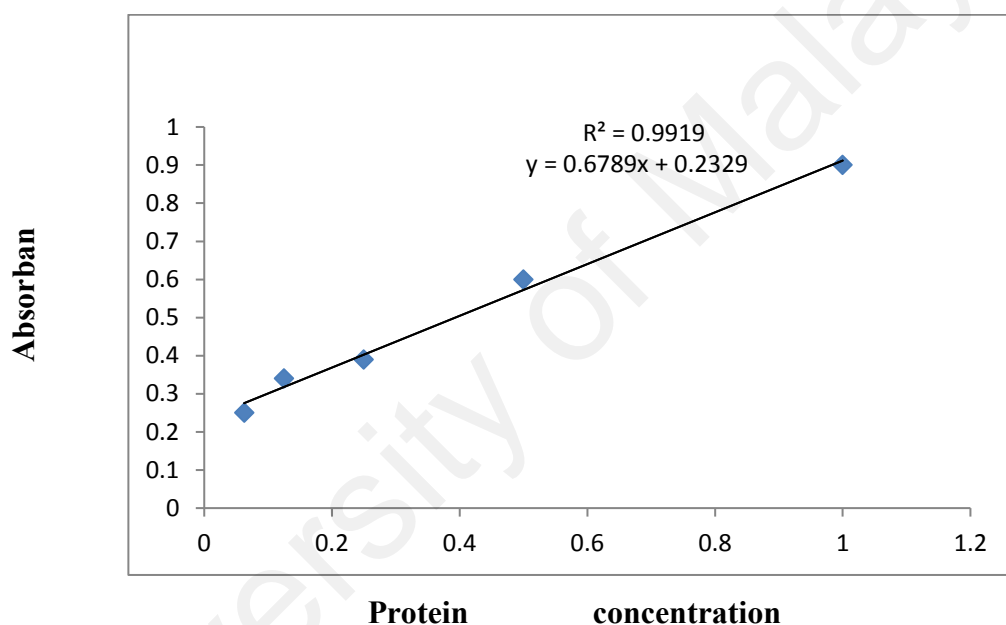


Figure A1: Standard calibration for protein determination

Note: The linear correlation and its R^2 are depicted in Figure A1.

APPENDIX B

B.1 Standard calibration of β -CD

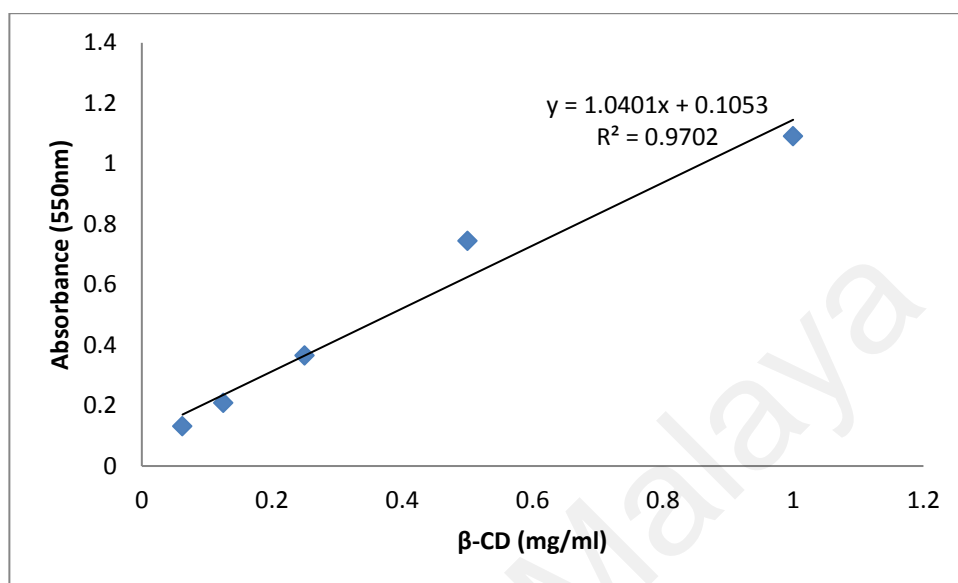


Figure B1: Standard calibration for β -CD determination

Note: The linear correlation and its R^2 are depicted in Figure B1.

APPENDIX C

C.1 Reverse phase HPLC chromatogram

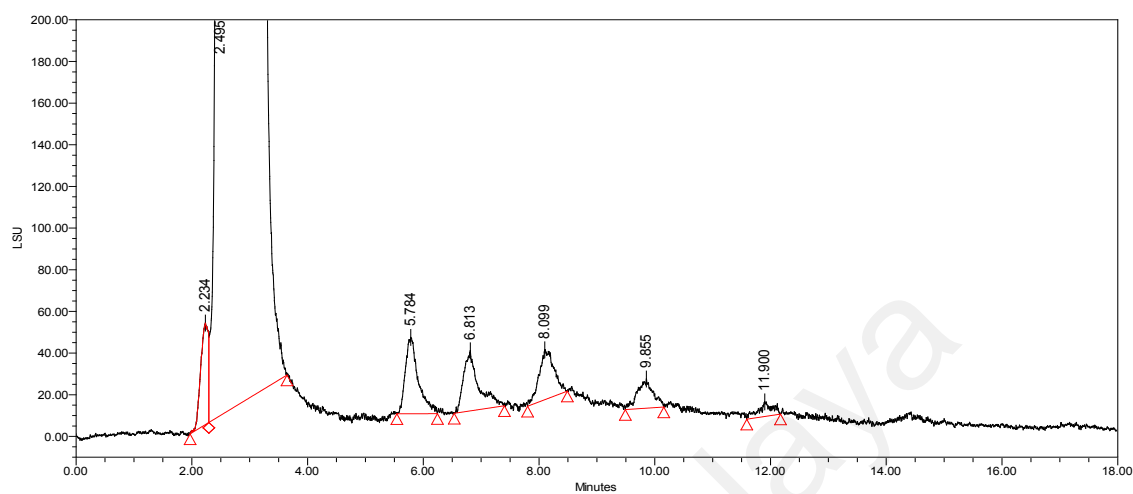


Figure C1: Reverse phase HPLC chromatogram of PEG3000/potassium phosphate ATPS top phase sample at initial stage.

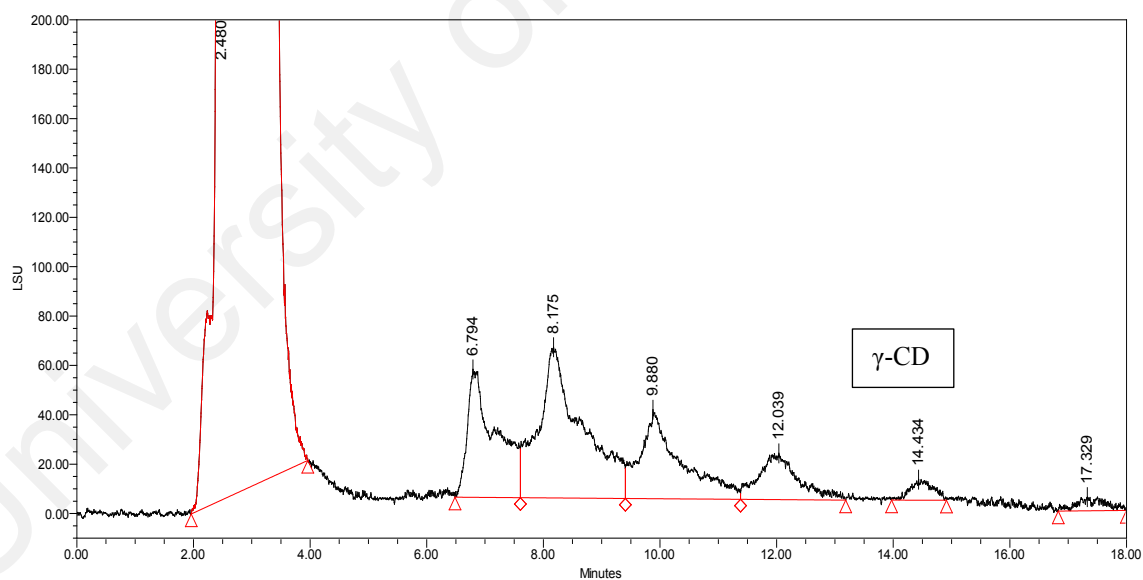


Figure C2: Reverse phase HPLC chromatogram of PEG3000/potassium phosphate ATPS top phase sample after 30 h.

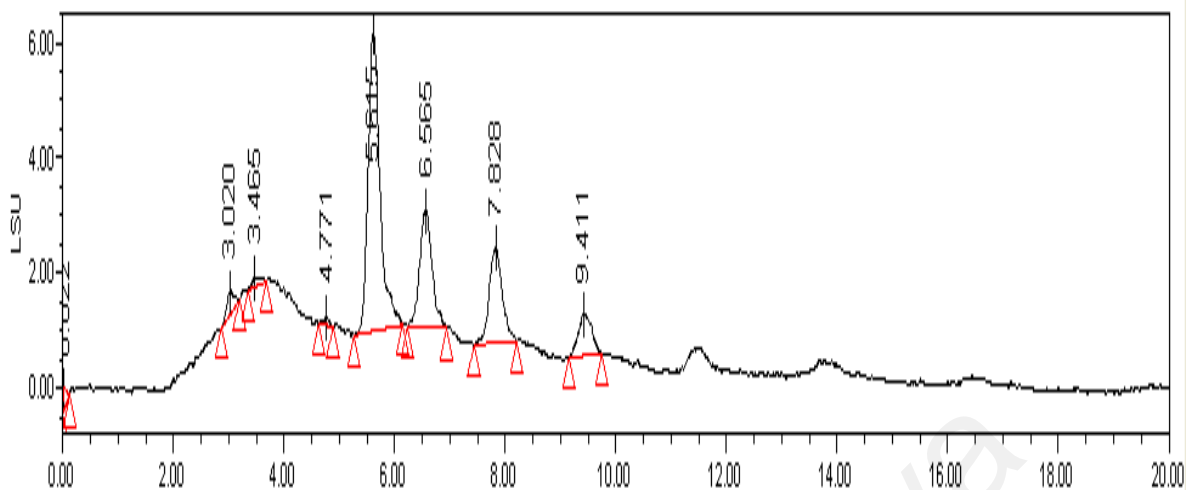


Figure C3: Reverse phase HPLC chromatogram of ethanol/potassium phosphates ATPS top phase sample at initial stage.

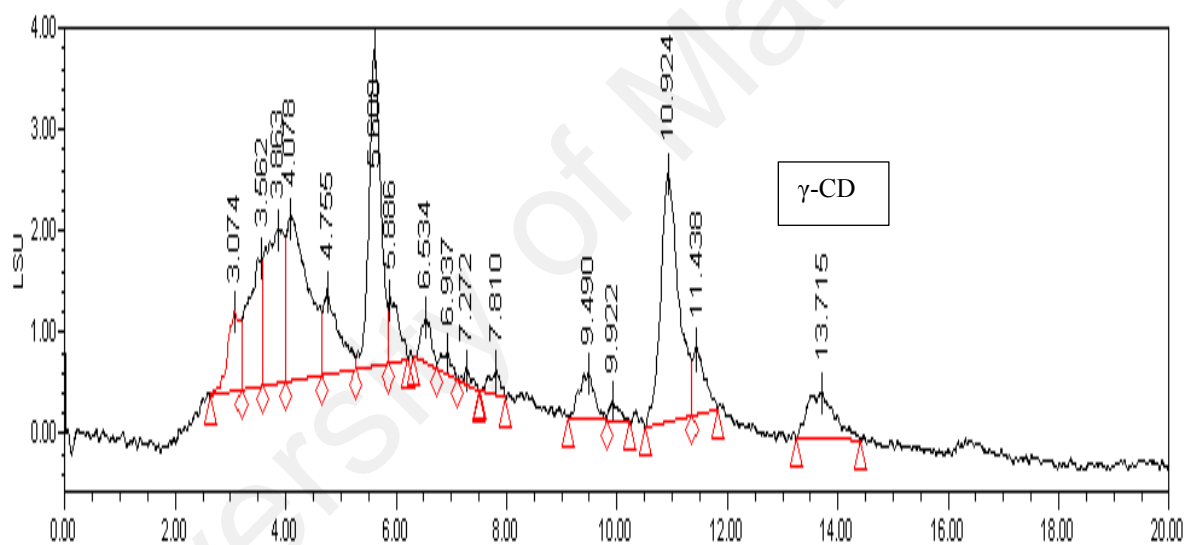


Figure C4: Reverse phase HPLC chromatogram of ethanol/potassium phosphates ATPS top phase sample after 24 h.

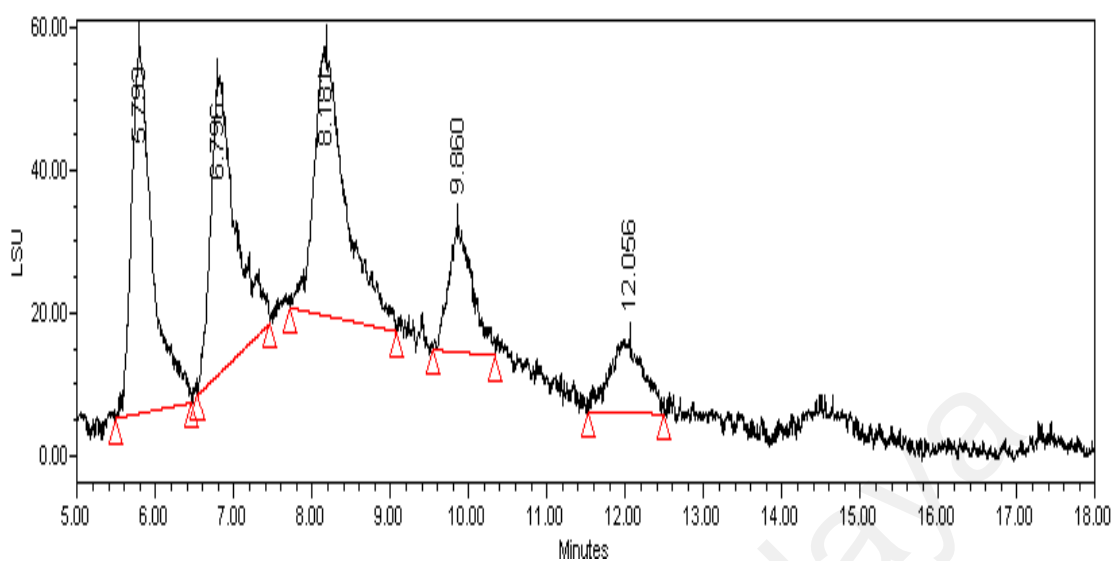


Figure C5: Reverse phase HPLC chromatogram of EOPO 970/potassium phosphate ATPS top phase sample at initial stage.

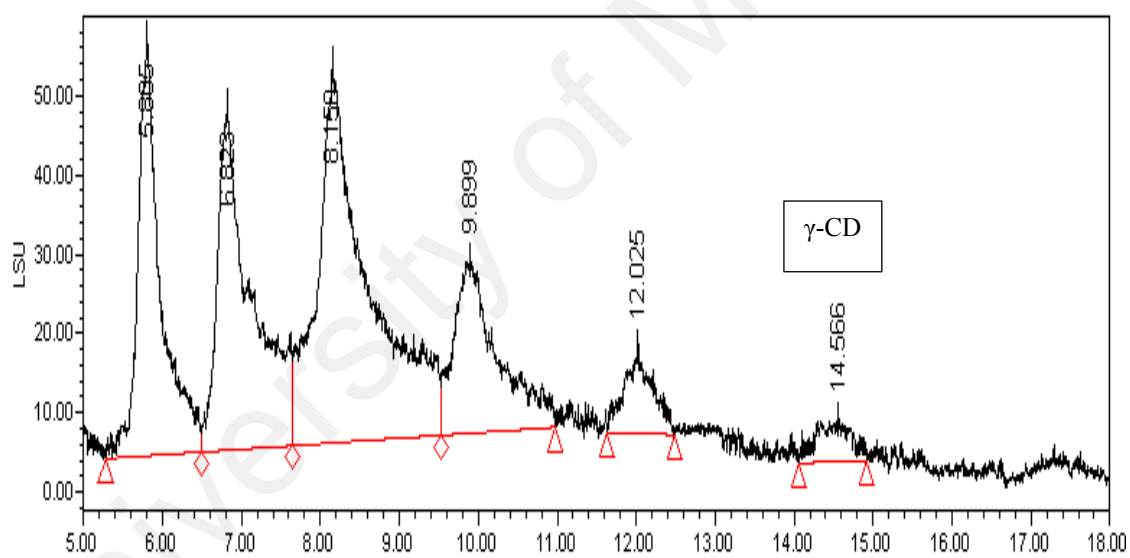


Figure C6: Reverse phase HPLC chromatogram of EOPO 970/potassium phosphate ATPS top phase sample after 30 h.