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Biotransformation of Ammonia and Carbon Dioxide to Carbamoyl Aspartate Using In Vitro Multienzyme Cascade

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of the author's knowledge, it contains no material previously published or written by another person, except where due reference is made in the text.

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Abstract

The removal of ammonia from wastewater is a major worldwide concern. Ammonia is toxic for organisms living in water and results in eutrophication in aqueous environments. Many novel technologies have been applied for nitrogen removal from wastewater such asbiological treatment. Yet it could not meet the effluent discharge standards efficiency due to multiple drawbacks such as high energy requirement, high material consumption and cost and inhibitory effects of chemicals in the Wastewater. We have recently shown hyperthermophilic carbamate kinase (CKase) mitigates environmental pollutants ammonia and carbon dioxide into value-added products in a green process. CKase transforms ammonia and carbon dioxide into carbamoyl phosphate (CP) via carbamate in an adenosine triphosphate (ATP)–dependent manner. CP is the key metabolic intermediate in the urea cycle, pyrimidine and arginine pathway; yet it decomposes easily in aqueous solution and, therefore, is not feasible for industrial applications. Hence, using an alternative enzyme to utilise CP is beneficial. Aspartate transcarbamoylase (ATCase) is an alternative enzyme to utilise CP by carbamoylation of aspartate to produce carbamoyl aspartate, a very important precursor for pyrimidine pathway and production of nucleotides.In this project a one-pot multienzymatic pathway was developed to convert ammonia from wastewater and carbon dioxide from air to carbamoyl aspartate (CA) using one recombinant lysate of *Esherichia coli*. The project also demonstrated the immobilisation of CKase and ATCase on a solid carrier for easy usage and recovery in wastewater treatment. The following steps have been conducted in this project:

First, the catalytic subunit of ATCase from three different hyperthermophilic microorganisms (the same as CKase) *Thermococcus sibiricus*, *Thermococcus barophilus* and *Pyrococcus furiosus* and one mesophilic microorganism, *Enterococcus faecalis*, have been cloned and expressed in *E. coli* BL21 (DE3). The enzyme kinetics properties were studied to confirm if they have the same stability (pH and temperature) as CKase from hyperthermophile.

Second, by confirming ATCase stability and activity at different pH and temperatures similar to hyperthermophilic CKase, ATCase and CKase pure enzyme were coupled to produce CA. Following that, the best combination of CKase and ATCase with respect to CA production yield was chosen for further study. In the next step, CKase crude lysate and pure ATCase were successfully coupled without adding any external ATP. CKase crude lysate contains sufficient endogenous acetate kinase that recycles cofactor ATP from adenosine monophosphate (AMP) (by engaging endogenous adenylate kinase) or adenosine diphosphate (ADP). Last, co-expressed crude lysate of recombinant CKase and ATCase

from a single bacterial culture was employed to convert ammonia, carbon dioxide and aspartate to CA without using external ATP (there is enough in the lysate to initiate acetate kinase activity). The usage of lysate substantially decreases limitations related to protein production and purification.

Third, aspartate production from fumarate and ammonia was developed (to remove more ammonia from wastewater) by using *E. coli* lysate as a source of aspartase. It was demonstrated that co-expressed crude lysate of recombinant CKase and ATCase contains all required enzymes, including acetate kinase and aspartase, to convert ammonia and carbon dioxide into CA.

Last, the CKase and ATCase on the Eupergit carrier were immobilised to recover the enzymes and applied crude lysate of recombinant CKase and ATCase on wastewater to convert ammonia to CA.

List of Abbreviations

AOB	Ammonia-oxidising bacteria
AP	acetyl phosphate
ATCase	aspartate transcarbamoylase
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
CA	carbamoyl aspartate
CANON	Completely Autotrophic Nitrogen Removal Over Nitrite
CKase	carbamate kinase
COD	chemical oxygen demand
CP	carbamoyl phosphate
CPCase	carbamoyl phosphate synthesis
DNA	deoxyribonucleic acid
DTT	Dithioreitol
DHO	Dihydroorotase
DHOD	dihydroorotate dehydrogenase
E. coli	*Escherichia coli*
HPLC	high-performance liquid chromatography
HPLC/MS	High-performance liquid chromatography/mass spectrophotometry
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
LB	Lysogeny Broth or Luria-Bertani Broth
MCS	multiple cloning sites
NEB	New England Biolabs
OPRT	orotate phophoribosyltransderase
OMPDC	OMP decarboxylase
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHARON	System for High-activity Ammonia Removal Over Nitrile
Tris	tris (hydroxymethyl) aminomethane
SRT	Solids retention time

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Chapter 1: Introduction

1.1. Background

The removal of nitrogen from wastewater has been a major challenge for municipal wastewater. Nitrogen in the form of ammonia is toxic for organisms living in the water. Excessive ammonia in the water induces eutrophication, resulting in harmful algal blooms, dead zones, killing fish, et cetera (Tchobanoglous & Burton, 1979). Many traditional and novel technologies have been conducted to remove ammonia from wastewater and run-off streams that release to rivers, seas, or other water bodies (such as Oxidation ditch, step, acid stripping and member filtration). However, there are no effective treatments that meet all the requirements for efficient ammonia removal from wastewater. Since, first, they do not efficienctly meet the discharge standard for sewage going into a river or the sea. Second, they are not economically efficient and use high amounts of energy, time and cost (Guibing et al., 2007; G. Zhu et al., 2008; Guibing Zhu, Peng, Wang, Wu, & Ma, 2007).

Consequently, water quality improvement remains a concern regarding human health, sea life and plants. In this study, the nature's catalyst, enzymes from ancient bacteria, was used to carry out the complex chemical and biological transformation of ammonia to value-added products. The biocatalytic transformation of ammonia has numerous advantages compared to other methods such as physical, chemical and biological methods for examples, struvite precipitation, membrane filtration and ion exchange (Lotfi et al., 2017; Luo et al., 2015; Mavinic, Koch, Huang, & Lo, 2007). First, it not only removes the ammonia from wastewater but also converts it into valuable products (such as carbamoyl aspartate, urea and arginine). Second, it has a niche of cost, energy and time efficiency (Guibing et al., 2007; G. Zhu et al., 2008; Guibing Zhu, Peng, Wang, Wu, & Ma, 2007). Third, it mitigates another environmental pollutant: carbon dioxide. Last, this method \comprises an attractive novel green technology.

In this project, it was shown that carbamate kinase (CKase) is an ideal candidate for sequestration of both ammonia and carbon dioxide (Hennessy et al., 2018) as CKase is able to convert ammonia and carbon dioxide to carbamoyl phosphate (CP), an important intermediate in the cell (Mani, Peruzzini, & Stoppioni, 2006; Matxalen Uriarte et al., 2001). However, although CP is a key intermediate metabolite, it decomposes easily in aqueous solution with a half-life of about 5 min at 37 ºC (Allen & Jones, 1964). Hence, it would be advantageous to determine an alternative enzymatic pathway such as aspartate transcarbamoylase (ATCase) to prevent CP decomposition. This may make CP more stable and able to be utilised in the central metabolism in microorganisms like mesophilic and thermophilic microorganisms. Therefore, a one-pot multienzymatic transformation of carbon

dioxide, ammonia and fumaric acid into carbamoyl aspartate (CA) using a single hyperthermophile or mesophile recombinant CKase/ATCase lysate from *E. coli* was investigated.

1.2. Wastewater Treatment

The amount of nitrogen must be controlled (Loganathan et al. 2014) to avoid eutrophication in water bodies and risks to human health (Correll 1998; Kwak & Lee 2015; Park et al. 2016; Campbell 1952). Moreover, ammonia, the main form of nitrogen in wastewater, needs to be removed because it can inhibit and kill a variety of microorganisms involved in wastewater treatment (Liu 2019). While various biological and chemical processes for removing nitrogen have been developed, they are still unsatisfactory with regard to effluent quality and cost (Yamashita & Yamamoto-Ikemoto 2014).

1.2.1. Nitrogen Removal by Biological Treatment

The removal of ammonia from wastewater is a major worldwide concern today. The ammonia concentration in wastewater varies from 30 to 560 mg/L, depending on the pH and temperature (Table 1.1) (Cervantes, 2009). Ammonia is toxic (from 0.1 mg/L to 2 mg/L depending on pH and temperature) for many organisms living in water and results in eutrophication in aqueous environments (Tchobanoglous & Burton, 1979). In addition, recovering the water from eutrophication is costly; $2.2 billion is spent annually on this in the USA (Dodds et al., 2009). The technologies for ammonia removal from wastewater have been categorised into three major groups: physical, chemical and biological treatments. So far, biological treatment has been the most effective, energy efficient, economical and environmentally friendly treatment to remove ammonia from wastewater. Yet, it currently does not efficiency meet effluent discharge standards (≤10 mg total ammonia/L) (G. Zhu et al., 2008; Guibing Zhu et al., 2007).

Table 1.1: Average Measurement of the Influent and Effluent of the Industrial Wastewater Treatment Plant (Elawwad, 2017)

Parameter	Influent ind. WW	Influent domestic WW	Mixed WW	Effluent
Average flow, m^3/d	3,680	480	4,160	4,080
COD_{tot}, mg L^{-1}	1,250 ± 120	580	1,173	174 ± 42
$COD_{filtered}$, mg L^{-1}	1,140	–	–	94
BOD, mg L^{-1}	676 ± 170	310	634	31.1 ± 10.4
Total ammonia, mg N L^{-1}	611 ± 140	–		515 ± 57
TKN, mg L^{-1}	925 ± 180	42	823	560 ± 79
Nitrate + nitrite, mg N L^{-1}	–	–	–	23.2 ± 12
Total phosphorus, mg P L^{-1}	6	8	7.1	1.6 ± 0.5
pH	8.7 ± 0.3	7.6 ± 0.4	8.7	7.1 ± 0.3
Alkalinity, mg L^{-1} as CCO_3	600	250	560	3 ± 2

Examples of biological technologies and processes to remove nitrogen, phosphate or organic materials from wastewater include:

- Oxidation Ditch—this removes biodegradable organics by using long solids retention times. This process is a modified activated sludge biological treatment process.
- Anoxic/Oxic—this removes nitrogen and organics by using microorganisms.
- Step feeding anaerobic/anoxic/aerobic (A2/O) and using a modified Bardenpho process and sequencing batch reactor—this removes both nitrogen and phosphorus.
- Bio-Denitro: this removes carbon, nitrogen and phosphorus.

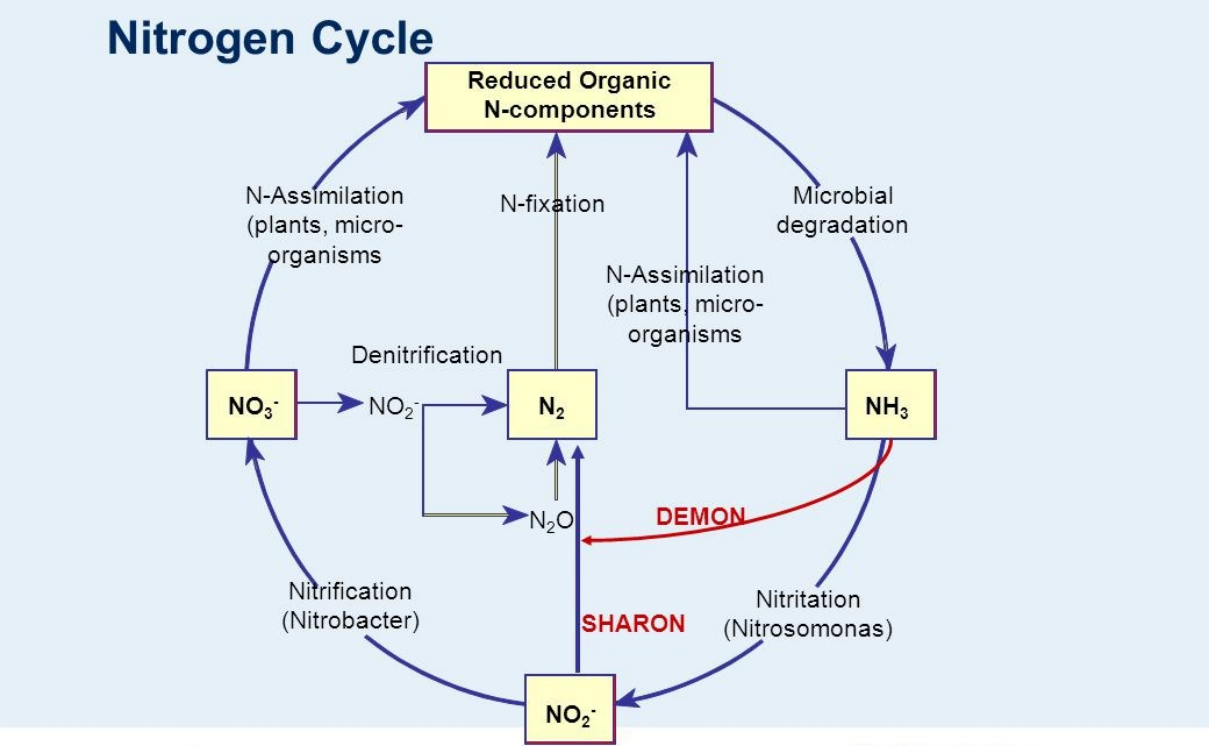

Figure 1.1: Microbial nitrogen cycle.These techniques have been established to remove nitrogen/ammonia from wastewater by understanding and applying the microbial nitrogen cycle and metabolism of inorganic nitrogen compounds (Figure 1.1) DEMON= The Deammonification System SHARON= single reactor system for High Ammonia Removal Over Nitrile (Pai et al., 2004; Tchobanoglous & Burton, 1979; Wentzel, Ekama, & Marais, 1992; S. Williams & Beresford, 1998).

Moreover, many new biological nitrogen removal methods have been established for nitrification and denitrification. However, many of these have limitations, for example:

- Simultaneous Nitrification and Denitrification (SND),an aerobic granular sludge system the combines nitrification and denitrification in the same reactor (Helmer & Kunst, 1998; Keller, Subramaniam, Gösswein, & Greenfield, 1997) and Oxygen-Limited Autotrophic Nitrification and Denitrification (OLAND) a nitrogen removal process combined with partial nitrification and anaerobic ammonium oxidation (Kuai & Verstraete, 1998; Muller, Stouthamer, & Van Verseveld, 1995). These processes can completely remove the nitrogen from wastewater. Still, their application is restricted by a low oxygen requirement (which reduces the nitrification rate) and difficulties cultivating the aerobic denitrifying bacteria.

- Single reactor system for High-activity Ammonia Removal Over Nitrile (SHARON) process is a partial nitrification process for the treatment of sewage from wastewater

flow stream. SHARON is a cost-effective treatment due to its low requirements for retention time, carbon source and oxygen concentration. Yet its application is limited due to the requirement of a high operation temperature (Van Dongen, Jetten, & Van Loosdrecht, 2001).

- Completely Autotrophic Nitrogen Removal Over Nitrite (CANON) is another example of a biological treatment for nitrogen removal. This process is performed by partial nitrification and anoxic ammonia oxidation and can be done using aerobic and anaerobic ammonia-oxidising bacteria (AOB). The advantages of this single-step process compared to SHARON and CANON are carbon saving and lower 'aeration' costs. However, providing anaerobic microorganisms is challenging (Dijkman & Strous, 1999; Nielsen et al., 2005; Pynaert, Sprengers, Laenen, & Verstraete, 2002; Pynaert, Wyffels, et al., 2002).

- The anaerobic ammonium oxidation (Anammox) process is another example of biological treatment. In this process, anaerobic AOB oxidises ammonia to nitrogen using nitrile as the electron acceptor. It also uses carbon dioxide as the carbon source. Therefore, there is no need to add a carbon source that causes problems due to the high amount of biomass yield. The shortcomings of this method are that there is nitrile remaining in the wastewater, and again, there is difficulty in cultivating the anaerobic bacteria (Fux, Boehler, Huber, Brunner, & Siegrist, 2002; Strous, Kuenen, & Jetten, 1999; Van de Graaf, de Bruijn, Robertson, Jetten, & Kuenen, 1996).

1.2.2. The Removal of Ammonia from Sidestream of Municipal Wastewater Treatment Plants

In wastewater treatment, sidestream produces from separation of liquids and solids. The separated liquid is returned to the treatment plant as sidestream. The various minor or major sidestream by-products are produced that need to be treated before being released into the environment (Eskicioglu, Galvagno, & Cimon, 2018). The sidestream always has a concentrated waste stream that makes it more valuable for nutrient recovery. However, the high concentration reduces the effluent quality of treated wastewater, resulting in increased energy costs to recycle or remove by-products. Therefore, the sidestream treatment is one of the bottlenecks in wastewater treatment (Eskicioglu et al., 2018). As an illustration, the side stream from sludge dewatering centrate (the liquid removed from thickened sludge) has a high concentration of ammonium and phosphate ions and a high chemical oxygen demand

(COD) as a result of the anaerobic digestion process (Table 1.2) (Fujimoto, Mizuochi, & Togami, 1991; Katehis, Diyamandoglu, & Fillos, 1998; Mavinic et al., 2007; Ueno & Fujii, 2001; Uysal, Yilmazel, & Demirer, 2010; Z.-Z. Zhang et al., 2016).

Table 1.2: Characteristics of High-Strength Centrate Wastewater Sidestreams from Full-Scale Wastewater Treatment Plants, Adapted from Eskicioglu et al. (2018)

Liquid sidestream	Flow (% of influent)	pH	Alkalinity (mg/L)	COD (mg/L)	TSS (mg/L)	Total nitrogen (mg/L)	NH_3-N/NH_4-N (mg/L)	Total phosphorus (mg/L)	ortho phosphate (mg PO_4^{3-}/L)	Reference
BNR[a] without sludge stabilisation	0.48	6.19	-b	1788	525	-	39	-	289.0	Kelowna WWTP, Kelowna (2017)[c]
BNR without sludge stabilisation	-	-	-	1100	879	-	40	-	202	Westside Regional WWTP, Kelowna (2017–2018)[c]
BNR with mesophilic AD	0.5	7.4	1900	930	790	670	535	220	220	Regina WWTP, Saskatchewan (2018)[e]
EBPR with mesophilic AD	-	7.3–7.5	-	-	-	-	756	-	207	Fujimoto et al. (1991)
Trickling filter with mesophilic AD	-	8.1	2087	-	250	-	989	-	88f	Mavinic et al. (2007)
Trickling filter with mesophilic AD	-	7.3	4854	-	1318	-	1039	-	-	WWTP in British Columbia, Canada (2017)[g]
Trickling filter with thermophilic AD	-	8.0	3295	-	500	-	1050	-	140f	Mavinic et al. (2007)
Trickling filter with ATAD & alum dosing	0.6	6.0	-	-	500	-	700	-	40	Salmon Arm WWTP[h]
Mesophilic AD	-	-	4284	648	-	1263	1038	-	177	King County, Washington, USA, Figdore et al. (2018)
Mesophilic AD & thermal	-	8.1	6278	-	283	-	1781	-	19.8	Blue Plains AWTP,

Liquid sidestream	Flow (% of influent)	pH	Alkalinity (mg/L)	COD (mg/L)	TSS (mg/L)	Total nitrogen (mg/L)	NH_3-N/NH_4-N (mg/L)	Total phosphorus (mg/L)	ortho phosphate (mg PO_4^{3-}/L)	Reference
hydrolysis										Washington, USA, Zhang et al. (2016)
Activated sludge with mesophilic AD[i]	-	7.9	-	-	-	-		707[f]	157[f]	Uysal et al. (2010)
AD	-	-	-	210	1.13	-	950	-	415	Castres, France, Uggetti et al. (2014)
Activated sludge with mesophilic AD	-	7.7	2943	431	1260	1039	886	79	-	Wards Island, New York, Katehis et al. (1998)

[a] EBPR = enhanced biological phosphorus removal; BNR = biological nutrient removal; AD = anaerobic digester; ATAD = autothermal thermophilic aerobic digester. [b] Indicates data not reported. [c] Data provided by City of Kelowna and Regional District of Central Okanagan, Canada. [d] Sludge taken off-site and composted with no return stream. [e] Data provided by EPCOR, Regina WWTP, Saskatchewan, Canada. [f] Calculated from authors' published data. [g] Data provided by a WWTP in British Columbia, Canada. [h] Data provided by City of Salmon Arm, Canada. [i] Characterisation based on the clear liquid component of lab-centrifuged sludge samples.

Many potential processes to remove nitrogen, phosphate and COD from the sidestream have been discussed, and each has its strengths and limitations. Since ammonia is the main form of nitrogen in wastewater, several processes have been developed to remove it from the sidestream. The following section considers key examples from either physical and chemical treatment or biological treatment of these ammonia removal processes.

1.2.3. Physical and Chemical Treatment

In this section, four techniques of physical and chemical treatments are described in wastewater treatment as follow: ammonia removal, struvite precipitation, membrane filtration and ion exchange. These

Struvite Precipitation

Struvite is composed of magnesium ammonium phosphate, which is a white crystal ($MgNH_4PO_46H_2O$). At a pH greater than 7.5, in solutions containing equal amounts of magnesium, ammonium and phosphate, struvite can precipitate. Recently magnesium has been added to digester supernatant to cause struvite precipitation. However, since the amount of nitrogen is always higher than phosphate, the ammonia removal efficiency is very low (Mavinic et al., 2007).

Ammonia Removal

Ammonium hydroxide is the reaction product of ammonia gas and water (Figure 1.3). The equilibrium direction depends on temperature and pH of the solution.

$$NH_3 + H_2O \rightleftharpoons NH_4^+OH^-$$

ammonia ammonium hydroxide

Scheme 1.1: Production of ammonium hydroxide from ammonia gas and water.

At high pH, the ammonia to ammonium ion ratio is 100:0; therefore, all ammonia is in the form of a gas and can be air stripped and released into the atmosphere. However, it can form smog and affect the local ecosystem (Krupa, 2003). Therefore, another process or method could be applied to trap the gaseous ammonia before entering the atmosphere, such as acid stripping, steam and hot air stripping, thermal stripping coupled with acid adsorption and ammonia recycling (see Table 1.3).

Table 1.3: Removal of Gaseous Ammonia Techniques Before Leaving to Atmosphere

Removal of gaseous ammonia techniques	
Acid stripping	In this technique, the gaseous ammonia will be trapped in acid solution to make an ammonium salt. The problem with this method is the supply of costly acid (Lei et al., 2010; Sagberg, Ryrfors, & Berg, 2006).
Steam and hot air stripping	The gaseous ammonia passes through an elevated temperature carrier air stream or liquid; therefore, ammonia gaseous converts to ammonium solution (Katehis et al., 1998). The issue with this technique is the high energy demand for heating and blowing, which is very costly (Guštin & Marinšek-Logar, 2011). The issue also applied to thermal stripping coupled with acid adsorption.
Thermal stripping coupled with acid adsorption	This technique combines acid stripping and thermal stripping. The heat supply can come from anaerobic digestion biogas, which covers the energy cost, but the acid supply remains costly (Tao & Ukwuani, 2015; Ukwuani & Tao, 2016).

Membrane Filtration

Membrane filtration occurs in different ways, as shown in Table 1.4.

Table 1.4: Different Membrane Filtration Techniques for Ammonia Removal from Sidestream Wastewater

Forward osmosis	In the forward osmosis method, osmotic pressure is applied to sidestream concentrate the feed solution containing nitrogen. The drawbacks of this technique are the need for frequent cleaning of the membrane and the need for additional pre-treatment or post-treatment to stabilise the concentrated solution (Holloway, Childress, Dennett, & Cath, 2007; Lotfi et al., 2017).
Membrane distillation	In membrane distillation, a microporous hydrophobic membrane is used. The heat volatilises the ammonia to pass through the membrane and to be collected from the other side using different methods. The problem with this method is the need to clean the membrane and 'membrane wetting' (El-Bourawi, Ding, Ma, & Khayet, 2006; Qtaishat & Banat, 2013; Zarebska et al., 2015).
Microporous hollow fibre membrane contactor	In the microporous hollow fibre membrane contactor, a membrane contactor adopts to separate ammonia from the sidestream. The pH should be kept above 9 to keep ammonia in the gaseous phase. The ammonia passes through a hydrophobic membrane to react with sulfuric acid to form an ammonium salt. The drawback of this technique is the cost of the acid and energy (Ashrafizadeh & Khorasani, 2010; Darestani, Haigh, Couperthwaite, Millar, & Nghiem, 2017; Ulbricht, Schneider, Stasiak, & Sengupta, 2013).
Electrodialysis using ion-exchange membrane	In electrodialysis using an ion-exchange membrane, an electrical current is applied to take ions from a central part through an ion-exchange membrane of opposite charge in the direction of a second separate outer chamber. The drawback of

	this method is the decrease in electrical conductivity of the membrane due to fouling and the high energy costs (Mondor, Ippersiel, Lamarche, & Masse, 2009; Mondor, Masse, Ippersiel, Lamarche, & Massé, 2008; Xie, Shon, Gray, & Elimelech, 2016).

1.2.4. Biological Treatment

Biological treatment uses microorganisms to remove ammonia from wastewater. It can occur in many ways, as shown in Table 1.5.

Table 1.5: Different Biological Treatments for Ammonia Removal from Sidestream Wastewater

Biological Treatment	
Bio-electrochemical system	In a bio-electrochemical system, organic materials are oxidised by anaerobic heterotrophic microorganisms, and the donated electron is collected by an anode (Kuntke et al., 2018). The electrons flow to an electron acceptor such as metal ions, nitrate or oxygen. An ion-exchange membrane separates the cathode and anode. In this system, ammonia migrates to the catholyte chamber, where ammonium is converted to ammonia gas due to the high pH, favouring the equilibrium towards ammonia gas rather than ammonium. Some bio-electrochemical system studies have demonstrated promising nutrient recovery and ammonia removal from wastewater with good energy efficiency (Kelly & He, 2014; Venkata Mohan, Velvizhi, Vamshi Krishna, & Lenin Babu, 2014; Virdis, Rabaey, Yuan, & Keller, 2008; Wu & Modin, 2013; Y. Zhang & Angelidaki, 2015).
Nitrification/ denitrification	Many nitrogen-free by-products are produced in anaerobic digestion, such as CH_4, CO_2, H_2 and H_2S. Yet, ammonium is the most abundant nitrogen. Conventional nitrification converts ammonia to nitrate (NO_3^-). Nitrate is then reduced to nitrogen gas under anoxic conditions. Therefore, the process converts ammonia to nitrogen gas. However, the high energy requirement of the system is a drawback of this method (Lackner et al., 2014).

Biological Treatment	
Bioaugmentation	In bioaugmentation, the excess nitrification sludge from the sidestream wastewater is returned to the mainstream process. The sidestream and mainstream combine, and, therefore, the mainstream temperature rises since the sidestream concentrate temperature is high due to the treatment. Elevating the temperature is a bonus in winter and cold climates, as nitrification is a highly temperature-dependent process (Kos, 1998).This is a good technique for ammonium-rich wastewater sources to enable an increase in the nitrifying capacity of wastewater treatment plants (Salem, Berends, Heijnen, & Van Loosdrecht, 2003).
Nitritation	The initial step in nitrification is nitritation, which transforms ammonia into nitrite by consuming oxygen. Nitritation occurs in dewatering the centrate stream, high in ammonia and during a continuous flow of SHARON (Hellinga, Schellen, Mulder, van Loosdrecht, & Heijnen, 1998). The most important factors in this system are temperature and pH: both need to remain high (temperature >30 ºC and pH >8), resulting in high energy, material consumption and cost (van Loosdrecht & Heijnen, 1999; Y. Zhou, Oehmen, Lim, Vadivelu, & Ng, 2011).
Partial nitritation and anaerobic ammonium oxidation (Anammox)	In the Anammox method, the bacterium that can oxidise ammonia under anaerobic conditions is applied to the sidestream. When this system is combined with SHARON, the ammonium is converted to nitrogen gas without the addition of COD (Lackner et al., 2014). The advantages of this system compared to traditional nitrification/denitrification methods are less sludge production, lower demand, lower oxygen, less energy consumption and not requiring an added carbon source. The drawbacks of this system are the inhibitory effect of phosphate, nitrate and high pH (>7.6) on Anammox and the emission of N_2O during the partial nitritation stage (Lotti, van der Star, Kleerebezem, Lubello, & van Loosdrecht, 2012; Ma et al., 2016; Puyol et al., 2014). Further studies could be conducted to find a microorganism with broad-range pH stability.

Biological Treatment	
Nitritation-Anammox process configuration	In this method, a single reactor tank nitritation, the Anammox process, has been used instead of a conventional two-stage reactor process. Two examples of this system are CANON and DEMON (DeamMONification). The bacteria can be added to the system in granular or biofilm forms.
Nitrous oxide emission from the Anammox process	In the nitrous oxide emission from the Anammox process during the nitritation stage, the nitrous oxide emission occurs due to the presence of oxygen. The CO_2 can be consumed in this process rather than providing a conventional carbon source (Joss et al., 2009; Peng et al., 2017). Yet, nitrous oxide emission is a problem that needs to be solved (Pijuan et al., 2014).
Algae production	The microalgae apply to anaerobic digestion dewatering liquor. The microalgae can use the concentrate as a substrate to produce biofuel and biogas (Chen, Zhao, & Qi, 2015; Uggetti, Sialve, Latrille, & Steyer, 2014). The ammonia removal rate with this technique is very high. However, the drawbacks of this method are the difficulty of microalgae acclimatisation to different climates; microbial diversity and abundance, which restrict the identification of the best candidate and the slight variation in different seasons, which creates instability in microalgae performance (Batan, Graff, & Bradley, 2016; Yellapu et al., 2018).

Overall, all microorganisms used in the abovementioned systems were not stable at broad range pH and temperature. Therefore they were unsuitable for use in industrial applications for wastewater treatment (Y. Liu et al., 2019). Finding microorganisms with good pH and temperature stability would be an advantage for industrial wastewater treatment applications.

Hence, this project aim is to investigate the hyperthermophilic bacteria that their enzymes have already been shown to have pH and temperature stability from our previous work (Hennessy et al., 2018). They can also survive in aerobic or anaerobic environments without affecting their performance (Karl O. Stetter, 2006).

1.3. Archaea and Hyperthermophiles

In the previous section, different Biological Treatments for Ammonia Removal from Sidestream using different microorganisms were discussed. Yet, the drawbacks of those involved in biological treatments are often being not stable in broad range pH temperature and therefore, cannot grow. Therefore, their usage in the wastewater treatment process is limited. Hence, the use of stable microorganisms will benefit the wastewater treatment process. In the previous project, it was already investigated the hyperthermophile proteins that have good pH and thermostability, making them good candidates for use in wastewater treatment.

Hyperthermophiles are a group of ancient bacteria called Archaea. The Archaea domain is an ancient group of bacteria that arose almost 2.7 billion years ago as the first inhabitants on Earth (Brocks, Logan, Buick, & Summons, 1999). They are single-cell microorganisms and include a large and diverse group of microorganisms distributed widely in nature and habitats. Although Archaea morphology is similar to bacteria, their metabolic pathways and some of their genes are closely related to Eukaryotes (Figure 1.2) (Koonin, Mushegian, Galperin, & Walker, 1997).

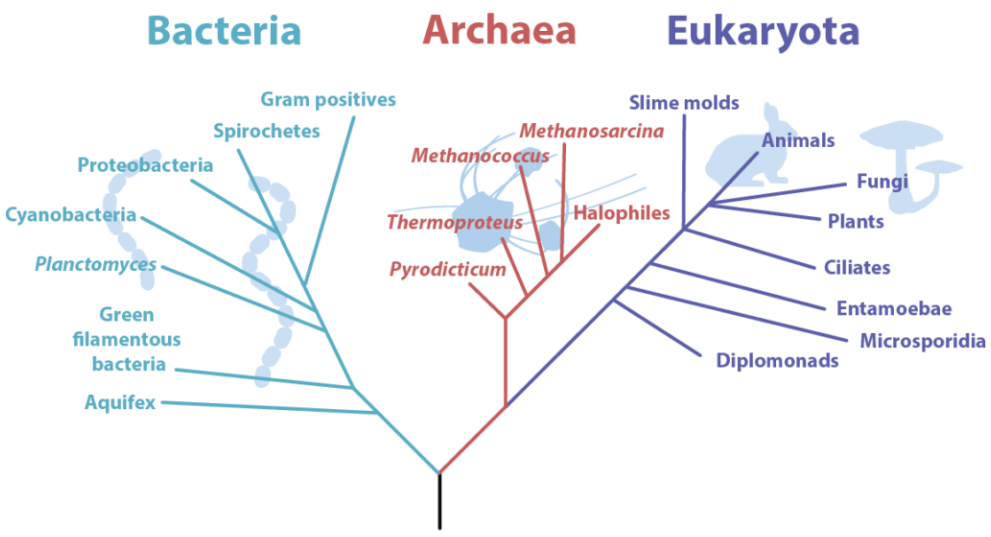

Figure 1.2: Phylogenetic tree of life. Source: Bruslind (2017).

Archaea can use a wider range of energy sources than Eukaryotes. Archaea can use compounds such as ammonia, metal ions and hydrogen gas, or use sunlight directly as an energy source to fix carbon (Minic & Thongbam, 2011; Straub et al., 2018). They can live in a broad range of habitats, such as soil, oceans, marshlands and human microbiota. They can live in harsh environments with extreme temperatures, pressures, pH values and

different solvents, such as hot springs and wells, salt lakes and deep within oceans (Arnone et al., 1997; Auerbach et al., 1997; Auerbach et al., 1998). Being able to survive and thrive in harsh environments made Archaea a great candidate for biological studies and applications such as gene cloning (Barns, Delwiche, Palmer, & Pace, 1996).

There are different types of Archaea, including methanogens (which produce methane as a waste product), halophiles (live in salty environments), psychrophiles (live at very cold temperatures) and thermophiles and hyperthermophiles (live at high temperatures)(Straub et al., 2018). Hyperthermophilic enzymes can form part of other enzyme categories, such as high salt tolerance (haloenzymes), high alkaline tolerance (alkalozymes) or other extreme conditions (pressure and acidity) (Lowe, Jain, & Zeikus, 1993).

The focus of this project is on hyperthermophilic bacteria since their properties are more suitable for the aim of this project. Hyperthermophiles have a high tolerance to extreme pressure and acidity. (Lowe et al., 1993; Reysenbach & Deming, 1991; Vieille & Zeikus, 2001). They also are stable in a broad range of temperature and pH which amplify their productivity in industry, as function at higher temperatures where more compounds and reagents are available (Lasa & Berenguer, 1993; Leuschner & Antranikian, 1995). Hyperthermophilic archaeon can grow at high temperatures between 80 ºC and 110 ºC (Vieille & Zeikus, 2001). They are isolated from different habitats such as hot marine environments, continental solfataras, oil wells and hot springs. They can also be located in shallow and deep-sea hot sediments and hydrothermal vents up to 4000 m below sea level with hydrostatic pressures ranging from 200 to 360 atm (Reysenbach & Deming, 1991). Hyperthermophiles could decompose the organic matter at early ages when life began on Earth and they can be facultative or obligate chemolithotrophs (e.g., sulfur oxidisers, sulfur reducers, methanogens) (Fischer, Zillig, Stetter, & Schreiber, 1983; Lowe et al., 1993).

Hyperthermophile enzymes are ideal candidates to be studied by biologists, chemists and physicists whose interests are in knowing about enzyme evolution and molecular mechanisms for protein thermostability since their molecular mechanisms are unique due to living in extremely harsh conditions (K. O. Stetter, 1996) (Huber, Huber, & Stetter, 2000). Hyperthermophile enzymes have many advantages: 1) easy purification using heat treatment after having been expressed in a mesophilic host (Vieille & Zeikus, 2001), 2) higher resistance to chemical denaturants (Vieille & Zeikus, 2001), and 3) enzymatic reactivity at higher temperatures that permit higher substrate concentrations, lower viscosity, less microbial contamination and higher reaction rates (K. O. Stetter, 1996, 1999).

Furthermore, thermodynamic and kinetic stability studies can determine an enzyme's thermostability. (Vieille & Zeikus, 2001).

1.3.1. Hyperthermophilic Enzymes with Commercial Applications

An ever-growing number of hyperthermophilic enzymes are being discovered, but only a few have been commercialised. Therefore, recent protein engineering tools aim to make these enzymes useful in a variety of applications (Vieille & Zeikus, 2001), such as:

1. Molecular biology—the hyperthermophilic enzymes can be used in cloning and expression as they have two major properties, processivity and fidelity. They are active in the 45 to 80 °C temperature range, which is very important in PCR technology. Examples include deoxyribonucleic acid (DNA) polymerase and DNA ligase (Larrick, 1997; Nuovo, 1994; Siebert, 1998).
2. Starch processing—usually operates at high temperatures and involves liquefaction and saccharification. The enzymes used in these processes are mostly from hyperthermophiles such as α–amylases, β–amylases, glucoamylases, α–glucosidases, pullulanases and amylopullulanases, cyclomaltodextrin glucanotransferases, xylase and isomerases (Bentley & Williams, 1996; Crabb & Mitchinson, 1997).
3. Other industrial and biotechnological applications like cellulose degradation and ethanol production (Coughlan, 1990), paper pulp bleaching (Godfrey & Reichelt, 1982), chemical synthesis and immunoassays in pharmaceutical and food industries (D. Cowan, Daniel, & Morgan, 1985).

The use of hyperthermophilic microorganisms and enzymes has attracted much attention in industry and biotechnology. Their enhanced stability makes them more suitable for harsh conditions or processes in industry. Furthermore, their stability is usually accomplice with a higher resistance to chemical materials commonly found in wastewater treatment. Therefore, the aim of this project is to use hyperthermophile enzyme to benefit from their stability in wastewater treatment.

1.3.2. Cell-Free System

Many factors and complex reactions in the whole cell make the studies difficult. Therefore, in this project, the aim is to use a cell-free system for protein synthesis, enzyme kinetic and protein biochemistry study to avoid complications in the study. A cell-free system is a tool that does not use the whole cell and uses the necessary ingredient and interaction that happens within cells in the controlled environment rather than a full cell system (Tinafar,

Jaenes, & Pardee, 2019). In the last decades, several different processes have evolved to replace the traditional cell-based system with purified enzymes in cell-free systems, also called in vitro systems. Today, a cell-free system is a key platform for synthetic biology applications. In a cell-free system, the isolated enzymes are used in combination with cofactors and substrates to produce valuable, desired products in a controllable and open environment (Gmelch, Sperl, & Sieber, 2019). Cell-free system usage in synthetic biology has many advantages:

- full control of reaction conditions by removing the unnecessary enzymes and cellular interreferences
- no restrictions regarding the cell wall and membrane transport system (Hold, Billerbeck, & Panke, 2016)
- enables the making of products that are unavailable in the cell due to their toxicity (Korman, Opgenorth, & Bowie, 2017; Korman et al., 2014)
- uses multiple enzymes from different organisms in multi-step in vivo pathways (Morgado, Gerngross, Roberts, & Panke, 2018)
- direct process control for optimisation in the enzyme cascade due to the absence of cell regulators (Y.-H. P. Zhang, 2015)
- determines enzyme ratio in the cascade for maximum productivity, resulting in avoiding unnecessary expenses (Beer, Pick, & Sieber, 2017; Z. Liu et al., 2017)
- synthesises hybrid biochemicals using organic and biological syntheses (Swartz, 2012)

Two types of cell-free systems dominate the synthetic biology study: purified enzyme and crude cell lysate (Figure 1.3) (Karim & Jewett, 2018).

The purified cell-free system, the individual overexpressed and purified enzyme, is used as a biocatalyst in the reaction. The crude cell lysate, the crude cell extract after cell lysis and centrifuge, is used, which does not contain lipid membrane and DNA genome.

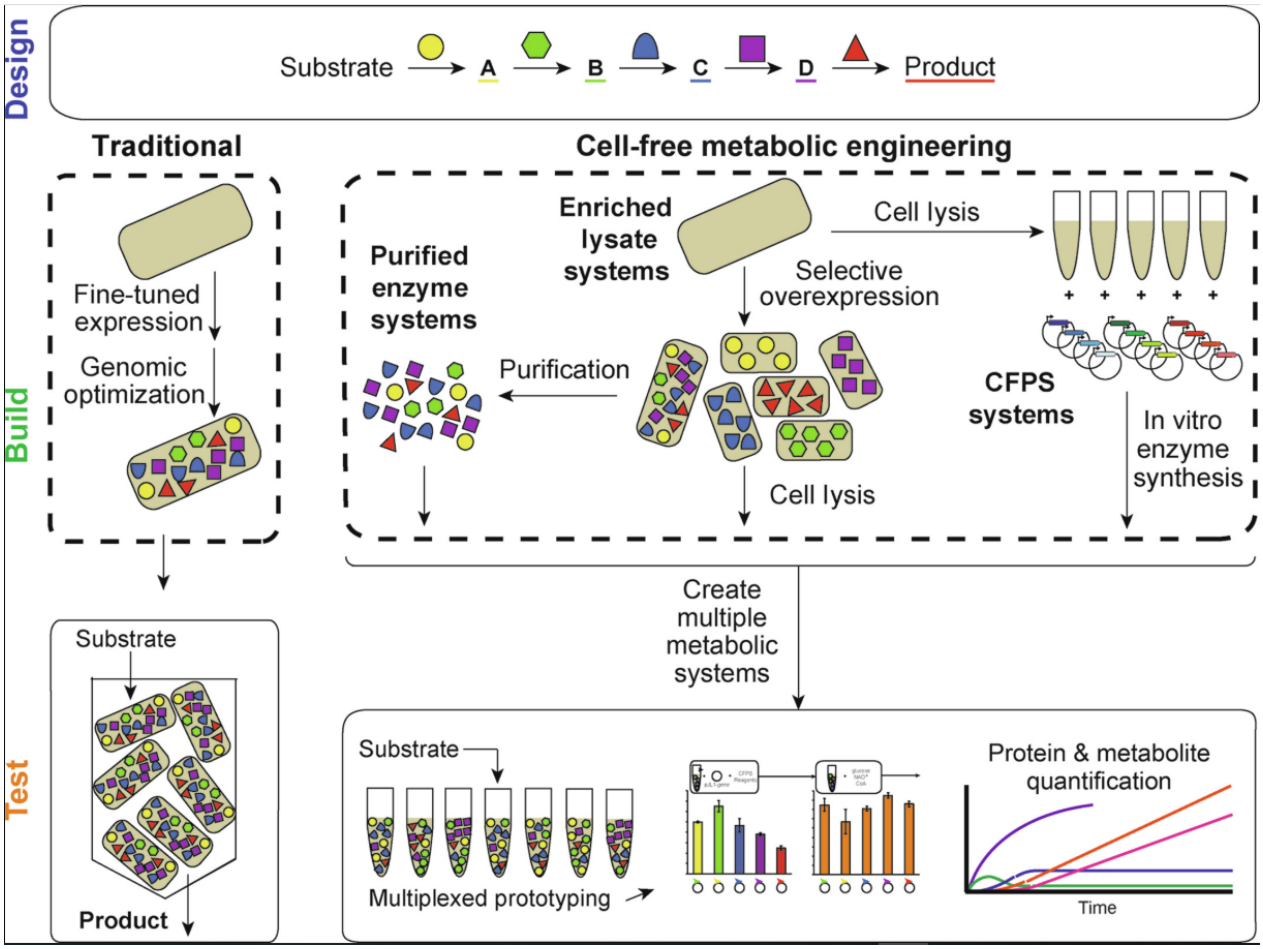

Figure 1.3: Traditional and cell-free biological methods for synthetic biology Source: (Karim & Jewett, 2018).

1.4. Enzymes and metabolic pathways

1.4.1. Enzymes and Biotechnology

The previous sections explained that hyperthermophiles are good alternatives to conventional microorganisms for the removal of ammonia from wastewater due to their pH and temperature stability as a result of living in harsh conditions.

Enzymes are the catalysts of metabolic reactions. Enzymes are the most important family of proteins that can catalyse biochemical reactions in cells (Eun, 1996). Most biochemical reactions occur at a very slow rate in the absence of enzymatic catalysis and would take years to occur under the mild conditions of the cells in organisms. Thus, in the presence of enzyme catalysis, the reaction rates accelerate and can occur in less than a second, which is over a million times faster than without the enzyme (G. M. Cooper & Hausman, 2004).

Enzymes were first reported over a century ago; however, the first enzyme in pure form was obtained in 1926 by James B. Summer (Sumner, 1926). His and the work of other two scientists was awarded the Nobel Prize in 1946. Therefore, the enzyme has been utilised in various industries, especially in the last three decades, with rapid strides in the field of biotechnology and, to a great extent, in the fields of genetic and protein engineering (Sanchez & Demain, 2011; Simoni, Hill, & Vaughan, 2002). There has been much exciting research work involving enzymes to develop new commercial, analytical and industrial processes. Enzymes are used in chemical products, health products, textiles, food and drink, detergents, hygiene products, biofuels and other industrial applications due to their elevated catalytic power, specific manner of operation, stereo and regioselectivity, cost-effectiveness, eco-friendly use and low-energy input (Li, Yang, Yang, Zhu, & Wang, 2012).

About half of the enzymes consumed in industry are extracted from fungi; one-third are from bacterial systems, and the remaining are from animals (8%) and plants (4%). As a source of industrial enzymes, microorganisms are usually prioritised over plants and animals due to their easy availability, fast growth rates and lower production cost. Moreover, enzymes from microorganisms are more amenable due to easy access to raw materials for their cultivation (Illanes, Cauerhff, Wilson & Castro 2012; Sanchez & Demain 2011; R. Singh, Kumar, Mittal & Mehta 2016). The presence of potentially harmful materials like phenolic compounds (in plants) and proteases and enzyme inhibitors in plant and animal tissues limit the use of plant and animal sources in enzyme production.

This thesis aims to study ATCase and CKase in which ATCase is a bi-substrate enzyme.

1.4.2. Enzyme Structure and Catalytic Activity

Enzymes are sequences of amino acids attached by peptide bonds (Eun, 1996). Many enzymes need other compounds to enable their catalytic activity, such as cofactors or coenzymes (G. M. H. R. E. Cooper, 2006). The basic enzymatic reaction can be illustrated as follows:

$$S + E \longrightarrow SE \Longrightarrow P + E$$

Where S represents the substrate, E the enzyme, SE the enzyme substrate complex and P the reaction product (G. M. H. R. E. Cooper, 2006).

The substrate binds to the enzyme's active site, where there is a cleft or groove on the surface of an enzyme, by non-covalent or covalent interaction. The binding accelerates the substrate conversion to the product by multiple mechanisms, such as providing a template in

the proper position and orientation to react, altering the conformation of the substrate to approach the transition state, and forming bonds between specific amino acid side chains in the active site and substrate to form a reaction intermediate. By doing the catalysis, the enzymes reduce the activation energy required for the reaction (Figure 1.3). Therefore, the reaction proceeds faster since each substrate molecule needs less energy to convert to a product (G. M. H. R. E. Cooper, 2006).

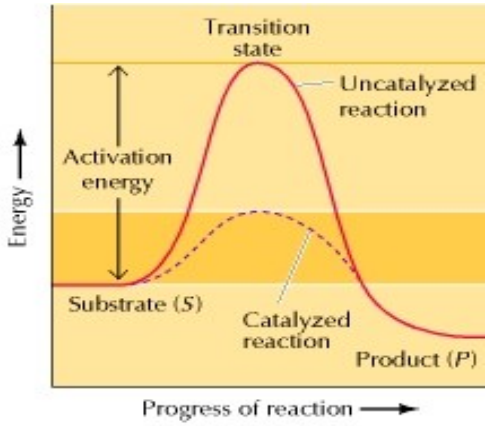

Figure 1.4: Energy diagrams for catalysed and uncatalysed reactions. Source: The cell: A Molecular Approach. Cooper GM (2004).

1.4.3. Thermostability Mechanisms of Proteins

Protein stability is essential for protein function at different physiological performance under various growth conditions. It is understood that there are forces that hold proteins together such as hydrogen bonds, ion pairs, and Van der Waals interaction (Vieille & Zeikus, 2001). In terms of thermostability, the forces are the combination of several factors performing in synergistic behavior.. although the main driving force in protein stability is the hydrophobic effect (Dill, 1990). Other factors that may be involved in protein thermostability include:

- amino acid composition and intrinsic propensity (Argos et al., 1979; Teplyakov et al., 1990)
- disulfide bridge (Kirino et al., 1994; Matsumura, Signor, & Matthews, 1989)
- hydrophobic interactions (Kirino et al., 1994)
- aromatic interactions (Burley & Petsko, 1985)
- hydrogen bonds (Shirley, Stanssens, Hahn, & Pace, 1992), ion pairs (Dill, 1990)
- prolines and reducing the entropy of unfolding (Sriprapundh, Vieille, & Zeikus, 2000)
- intersubunit interactions and oligomerisation (Rahman, Fujiwara, Nakamura, Takagi, & Imanaka, 1998; Vetriani et al., 1998)

- conformational strain release (Kawamura et al., 1996; Kimura, Kanaya, & Nakamura, 1992)
- helix dipole stabilisation (Nicholson, Becktel, & Matthews, 1988)
- packing and reduction in solvent-accessible hydrophobic surface (Karshikoff & Ladenstein, 1998; W. Zhu, Sandman, Lee, Reeve, & Summers, 1998)
- docking of N- and C-termini, and anchoring of loose ends (Ermler, Merckel, Thauer, & Shima, 1997; Lim et al., 1997)
- metal binding (Dong, Vieille, & Zeikus, 1997; Teplyakov et al., 1990)
- nonlocal versus local interactions (Eidsness, Richie, Burden, Kurtz, & Scott, 1997; Sanz-Aparicio et al., 1998)
- posttranslational modifications (Faguy, Koval, & Jarrell, 1992; Hettmann et al., 1998)
- extrinsic parameters (e.g., salts, high protein concentration, activators, substrates, coenzymes) or an extracellular environmental factor (e.g., pressure).

Yet, various microorganisms utilise different stabilising factors depending on their taxon, organism and even their protein (Trivedi, Gehlot, & Rao, 2006). Under extreme temperature, a thermostable protein maintains the structure and chemical properties of the polypeptide's chains (X.-X. Zhou, Wang, Pan, & Li, 2008)

1.4.4. Bi-Substrate Enzyme

More than 60 per cent of all enzymes are of the bi-substrate type and require two substrates for the reaction and yield two products (W. W. Cleland, 1977).

$$A + B \underset{}{\overset{E}{\rightleftharpoons}} P + Q$$

The majority of bi-substrate reactions have been categorised into two groups: transferase (one functional group transfers from one substrate to another) and redox reactions. Moreover, the reaction mechanisms in bi-substrate enzymes can be either sequential reactions (random or ordered) or ping-pong reactions (W. Cleland, 1970; W. W. Cleland, 1977, 1990). Therefore, measuring the bi-substrate reaction rates is more complex than uni-substrate reactions. For example, the initial velocity for uni-substrate reactions in the Michaelis-Menten model is

$$V = \frac{(Vmax * [S])}{(Km + [S])}$$

The reaction mechanism of bi-substrate enzymes (sequential or ping-pong) has more complex rate equations, explaining how and in what order the substrates bind. Therefore, to

measure the reaction, one substrate should be kept constant while varying the second substrate concentration, so the enzyme behaves like a uni-substrate enzyme (W. Cleland, 1970; W. W. Cleland, 1977).

For example:

Ordered bi-substrate reaction rate equation.

$$\left(\frac{1}{V}\right) = \frac{1}{Vmax} + \frac{Km^A}{(Vmax[A])} + \frac{Km^B}{(Vmax[B])} + \frac{(Km^A Km^B)}{(Vmax[A][B])}$$

Or

Ping-Pong reaction rate equation

$$\left(\frac{1}{V}\right) = \frac{Km^A}{(Vmax[A])} + \frac{Km^B}{(Vmax[B])} + \frac{1}{Vmax}$$

Where Km^A and Km^B are the [A] and [B] required for half maximal activity.

Many factors affect the bi-substrate reaction and make the kinetic study of bi-substrate enzymes very difficult. These include the reversible connection equilibrium, product inhibition, unstable transition state and enzyme promiscuity towards the substrate rather than their physiological substrates (W. W. Cleland, 1977; Leskovac, 2003).

Further, some bi-substrate enzymes can be regulated. The enzyme activity is regulated to function according to physiological demands, and it varies during the life of a cell. Allosteric enzymes are a group of enzymes with catalytic and regulatory sites and regulate their function by different kinds of regulation, such as feedback inhibition, where the product acts as a regulator (G. M. H. R. E. Cooper, 2006)). The regulator molecules (inhibitor or activator) bind to regulatory sites and change the conformation of enzymes, resulting in modification of the configuration of the active site and, therefore, the enzyme's catalytic activity.

Moreover, other parameters also affect the enzyme activity: temperature, pH and substrate concentration (G. M. H. R. E. Cooper, 2006). Enzymes catalyse many metabolic (catabolic and anabolic) pathways and cycles in the cell. The urea cycle and the pyrimidine and arginine pathways are examples of metabolic cycles and pathways in the cells that enzymes catalyse. In this project, enzymes from the pyrimidine pathway are used.

1.4.5. Metabolic Pathway

Different enzymes are responsible for the breakdown or build-up of substrates in different metabolic cycles or pathways. In 1940, Krebs depicted the enzymes performing a function in a series of reactions in a pathway known as metabolic pathways or cascades. (Krebs, 1940). He demonstrated that the product from the first enzyme is the substrate for the second enzyme in the cascade, the products of the second enzyme are the substrates for the fourth enzyme and so on. Later in 1940, Meyerhof and co-workers presented the glycolysis pathway, which converts glucose to pyruvate in 10 successive biochemical reactions (Kresge, Simoni, & Hill, 2005).

The study of the enzymatic cascade is very challenging. For example, enzyme activity in a cascade depends on the substrates, products, interactions with other metabolites from the cascade, enzyme ratio concentrations in the pathway and cell signals such as the one for enzyme regulation. Further, reversible reactions in the cascade are essential for cascade function but make the cascade study very difficult. Another problematic issue for studying a cascade is the rate-limiting enzyme, which can slow down the cascade catalysing rate (G. M. H. R. E. Cooper, 2006).

Apart from the problems mentioned, the complexity of the enzymes in the cascade makes the cascade study very complicated, for example, enzymes with more than one substrate or subunit. Many enzymes with multiple substrates are heterotrimeric and have different subunits that make them difficult to study (Guy & Phizicky, 2014).

The pyrimidine pathway is one of the important biosynthetic pathways in the cell that provides components for DNA and ribonucleic acid (RNA) in the cells. The first step of this pathway has been used in our project.

1.4.6. Pyrimidine Biosynthesis Pathway

One of the important metabolic pathways in the cell is the pyrimidine pathway, which produces pyrimidine nucleotides. Pyrimidine and purine nucleotides (Figures 1.5–1.6) have important roles in the cell. They are major energy carriers, precursors for nucleotide cofactors (e.g., Nicotinamide adenine dinucleotide and S -Adenosyl-L-methionine) and are required to synthesise and build nucleic acids DNA and RNA.

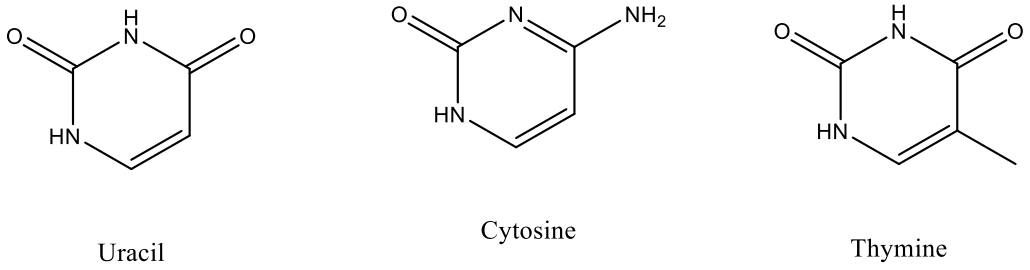

Figure 1.5: Nitrogenous base of pyrimidine nucleotide.

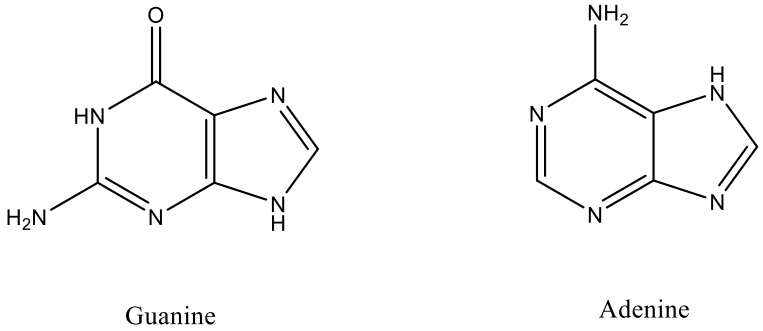

Figure 1.6: Nitrogenous base of purine nucleotide.

DNA and RNA are essential informational biopolymers for cellular function and growth and transfer genetic information from one generation to another (Moffatt & Ashihara, 2002). Therefore, pyrimidine and purine nucleotides are crucial for the cell, and their requirement is very significant for cells. The pathway for the synthesis of pyrimidine and purine nucleotides are similar in plants, animals and microorganisms (K. G. Wagner & Backer, 1992).

The pyrimidine biosynthesis pathway (Figure 1.7), through various enzymatic reactions, leads to the production of uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP) for RNA and deoxycytidine-5'-triphosphate (dCTP) and deoxythymidine triphosphate (dTTP) for DNA (O'Donovan & Neuhard, 1970; Theisen, Kelln, & Neuhard, 1987) Neuhard & Nygaard, 1987).

The pyrimidine biosynthesis pathway (also known as the orotate pathway) comprises nine steps. It is usually explained as the generation of uridine-5'-monophosphate (UMP) from CP in the first six reactions. The initial reaction prior to the pyrimidine pathway consists of CP production from glutamine, bicarbonate and ATP facilitated by CP synthetase (CPSase). CPSase can either use glutamine or ammonia as the amino donor. The CPSase affinity towards glutamine is higher than ammonia (Neuhard & Kelln 1996).

In the first step of the pyrimidine pathway, CP is converted to N-carbamoyl-L-aspartate (CA) and an inorganic phosphate facilitated by ATCase using L-aspartate. CA is converted to dihydroorotate in the second step, facilitated by dihydroorotase (DHOase). In this step, CA cyclises to produce L-dihydroorotate and one molecule of water. Then, in the third reaction (the only redox reaction in de novo), the pyrimidine ring, orotate, forms from dihydroorotate facilitated by dihydroorotate dehydrogenase (DODHase), which is a membrane-bound enzyme. The pyrimidine nucleotides form in the following two steps. First, the phosphoribosyl group of D-5-phosphoribosyl-1-pyrophosphate (PRPP) is added to orotidine 5'-monophosphate (OMP) facilitated by phosphoribosyltransferase (OPRTase). Secondly, OMP is decarboxylated to UMP, the first pyrimidine nucleotide, by OMP decarboxylase (ODCase). Subsequently, the highly specific UMP-kinase (UMPK) phosphorylates UMP to provide uridine diphosphate, which is then converted to UTP facilitated by the non-specific nucleoside diphosphate kinase (NDPK). Finally, the synthesis of CTP is catalysed by the transfer of an amino group from glutamine to UTP facilitated by CTP synthetase (CTPase) (Figure 1.6) (Moffatt & Ashihara, 2002)

In some organisms, the pyrimidine biosynthetic enzymes coding genes have been dispersed across the whole chromosome, while in other organisms, they are organised into clusters or operons. In *E. coli*, CPSase is encoded by the carAB operon, and ATCase is encoded by the pyrBI operon. The genes *pyrC, pyrD, pyrE, pyrF, pyrH, pyrG, ndk* and *pyrG* encode DHOase, DODHase, OPRTase, ODCase, UMPKase, NDPKase and CTPase, respectively (Moffatt & Ashihara, 2002). CKase and ATCase from the pyrimidine pathway are the enzymes catalysing the rate-limiting step in the pyrimidine pathway. The focus of this research is ATCase, the first enzyme of the pyrimidine pathway and the combination of CKase and ATCase together. The desired product of this research is carbamoyl aspartate which may be used directly as biofertiliser or converted to other products such as CTP, UTP and UDP.

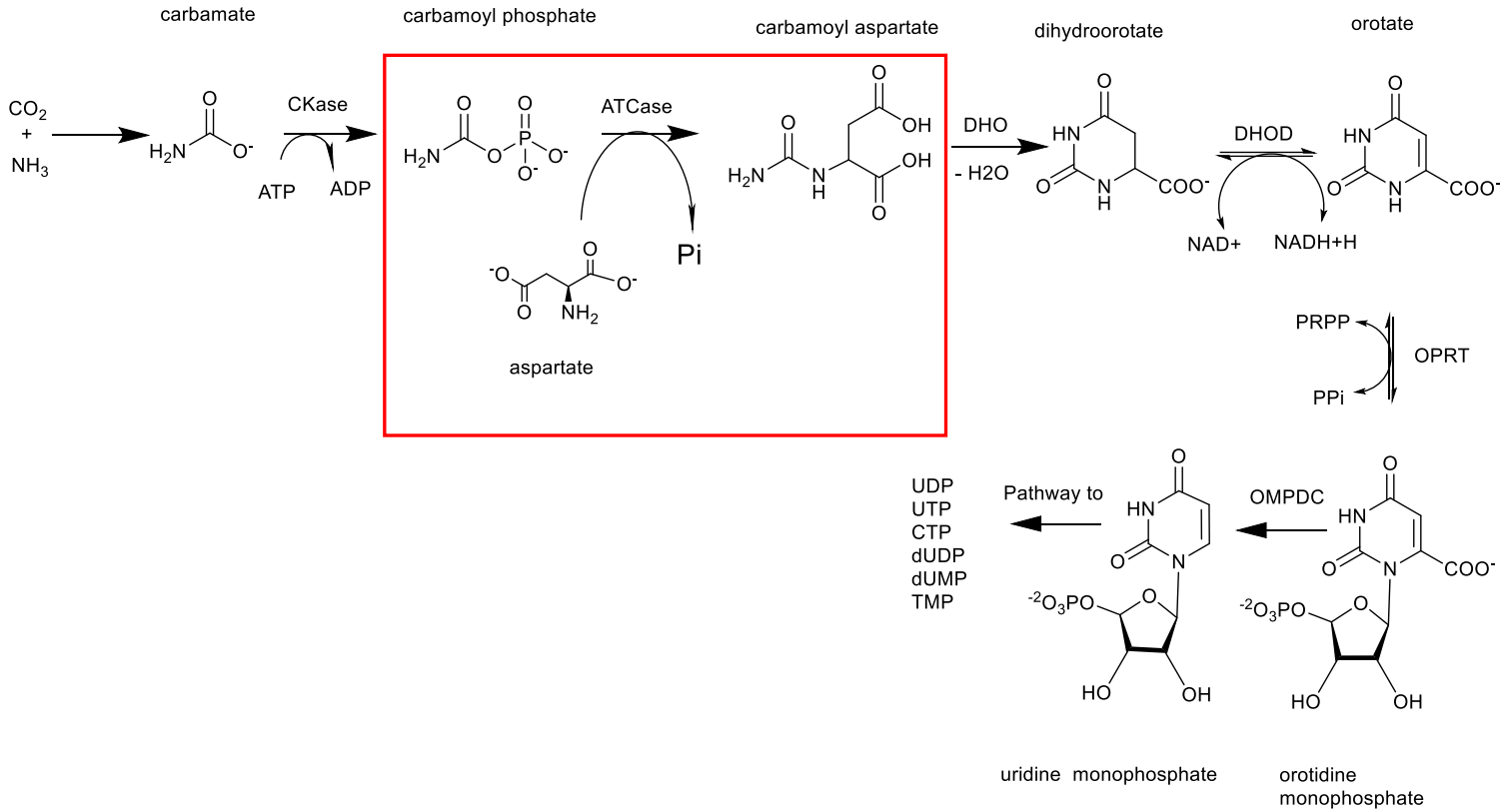

Figure 1.7: Pyrimidine nucleotide biosynthesis pathway. CP produced by CKase (equal to CPS in *E. coli*) using carbamate and ATP prior to pyrimidine pathway. Carbamate can be produced spontaneously by ammonia and carbon dioxide. Red circle: Aspartate transcarbamoylase (ATCase), the first step of the pyrimidine pathway, catalyse the condensation of CP with aspartate to form carbamoyl aspartate. DHO = dihydroorotase; DHOD = dihydroorotate dehydrogenase OPRT = orotate phophoribosyltransderase; OMPDC = OMP decarboxylase.

1.4.7. Carbamate Kinase

The initial reaction prior to the pyrimidine pathway consists of the production of CP by anabolic CKase. Anabolic CKase and CPSase can both synthesise CP. However, CKase uses only one mole of ATP instead of the two moles of ATP used by CPSase (Figure 1.7) (Matxalen Uriarte et al., 2001). CKase activity is reversible and uses carbamate and ATP as substrates in an anabolic pathway. Carbamate forms spontaneously from ammonia and carbon dioxide in aqueous solution (Mani et al., 2006). CKase was first identified from *Entercococcus faecalis* as catalysing ATP production in the arginine hydrolase fermentation pathway (Barcelona-Andres, Marina, & Rubio, 2002). CKase-like CPSase was subsequently identified from the hyperthermophile *Pyrococcus furiosus* (Virginie Durbecq, Legrain, Roovers, Piérard, & Glansdorff, 1997; M. Uriarte, Marina, Ramon-Maiques, Fita, & Rubio, 1999). It naturally catalyses anabolic CP formation (Alcantara, Cervera, & Rubio, 2000; Ramon-Maiques, Marina, Uriarte, Fita, & Rubio, 2000) in the absence of the arginine dihydrolase pathway and the presence of a relatively high concentration of ammonia (compared to ammonium; usually > 2.5 mM) and carbon dioxide (Ramon-Maiques et al., 2000; M. Uriarte et al., 1999). The crystal structure of CKase from *P. furiosus* has been described (Ramon-Maiques et al., 2000).

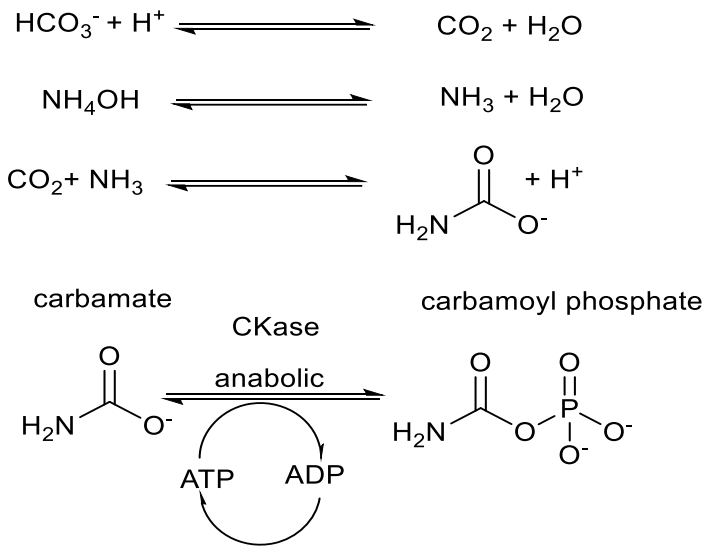

Figure 1.8: Carbamoyl phosphate production by carbamate kinase in aqueous solution in equilibria K_{eq}= 0.027, 25° C between CO_2/HCO_3^- and NH_4OH/NH_3.

1.4.8. Carbamoyl Phosphate

Carbamoyl phosphate (CP) is a precursor of the pyrimidine and arginine pathways and the urea cycle (Figure1.8) produced by CKase in our project. In arginine biosynthesis, ornithine

transcarbamoylase catalyses the conversion of CP and ornithine to citrulline, an arginine precursor. In the pyrimidine pathway, aspartate transcarbamoylase catalyses the condensation of CP with L-aspartate to form N-carbamoyl-L-aspartate (CA). CP is a highly thermolabile intermediate and decomposes at 100 °C in less than 2 seconds (Legrain et al., 1997). The CP decomposition at high temperature leads to a high and toxic amount of cyanate, which is a strong carbamoylating agent. The pH can affect the decomposition of CP at the mesophilic temperature range (25–37 °C) (Allen & Jones, 1964). In acidic environments (pH 1–5), the hydrolysis forms carbon dioxide, ammonia and orthophosphate, while in the base-catalysed reaction, cyanate and orthophosphate form (Figure 1.9) (Allen & Jones, 1964). Since CP is both extremely labile at high temperatures and potentially toxic, it requires metabolic protection *in vivo*; otherwise, such a decomposition can cause a very large energy cost for the cell. In extreme thermophilic bacterium, CP is protected from thermodegradation by being channelled towards synthesising citrulline or CA by ornithine transcarbamoylase or aspartate transcarbamoylase (Jan Massant et al., 2002; VandeCasteele, Chen, Roovers, Legrain, & Glansdorff, 1997; Q. Wang, Xia, Guallar, Krilov, & Kantrowitz, 2008).

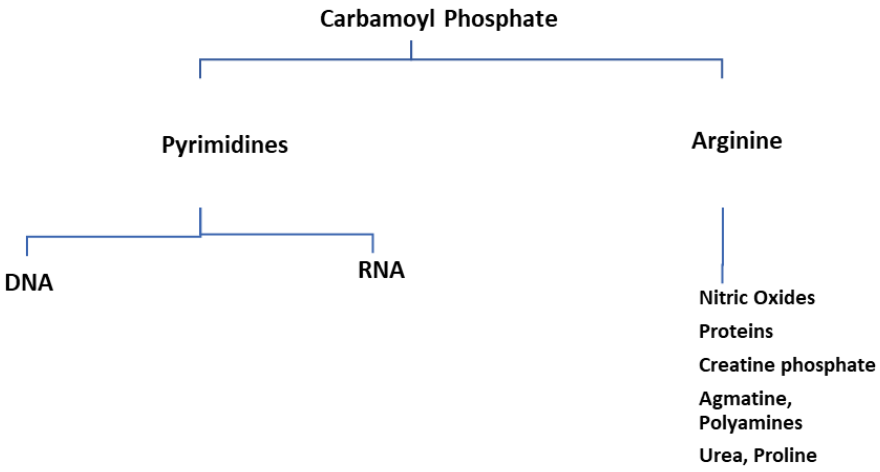

Figure 1.9: Metabolic conversions of CP.

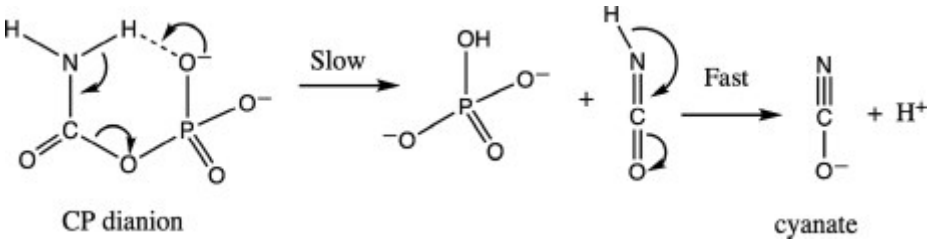

Figure 1.10: Proposed thermal decomposition of CP.

1.4.9. ATCase from *E. coli*

The first step in the pyrimidine reaction is the conversion of CP to CA and an inorganic phosphate facilitated by ATCase (E.C.2.1.3.2.) using L-aspartate. ATCase is the allosteric model enzyme that regulates pyrimidine nucleotide biosynthesis and has regulatory and catalytic subunits. ATCase uses 1) feedback control (inhibited by downstream metabolites CTP and UTP via binding of metabolites to ACTase active site and competing with the binding of the substrates) and 2) the cooperative binding of the substrate (L-aspartate) to regulate the pyrimidine pathway (such as activation of enzyme by ATP via allosteric regulation) (J. Gerhart, 2014). In the binding process, CP binds first and to the aspartate second and after completion of the reactions, CA is released before the phosphate (Wedler, 1974).

The enzyme catalytic and regulatory mechanism has been extensively studied in *E. coli*. The ATCase crystal structure has been studied in the presence and absence of substrates, products and analogues. Details of the conformational changes between its low-activity, low-affinity form (T state) and its high-activity, high-affinity form (R state) at the molecular level have been demonstrated (Ke, Honzatko, & Lipscomb, 1984; Perbal & Hervé, 1972) Further, the structural data have shown how the enzyme catalyses the reaction between CP and L-aspartate to yield CA and inorganic phosphate and how the allosteric regulator modulates this activity (J. C. Gerhart & Pardee, 1962, 1964; Pardee & Yates, 1956).

To study enzyme mechanisms, kinetic experiments must be performed to measure the key parameters, such as the Michaelis-Menten constant (Km) and catalytic rate constant (Kcat). The Michaelis-Menten constant Km describes the substrate binding affinity to the enzyme and the relationship between reaction rate and substrate concentration. In contrast, Kcat shows the rate of substrate conversion to the product by the enzyme (Cornish-Bowden, 2014).

Many kinetic studies have been performed on ATCase from *E. coli*. It has been a model of cooperativity and allosteric regulation. Yet, only a couple of studies have been done on hyperthermophile ATCase, such as that from *Pyrococcus abyssi* and *Aquifex aeolicus*. More details about ATCase structure, function and kinetics have been described in the introduction of Chapter 2.

Overall, CKase and ATCase from hyperthermophiles might be good candidates for ammonia removal from wastewater as they are stable in a broad range of pH and temperature, resistant to harsh condition and have good robustness. A few reasons why thermostability is relevant to wastewater treatment are as follow: first, as temperature varies in wastewater treatment, it is more beneficial to use more thermostable microorganism (Atomi, Sato, & Kanai, 2011). Second, hyperthermophile enzymes have high stability at high pH therefore, it makes them good candidate for the usage in alkaline wastewater stream (Hennessy et al., 2018). Third, hyperthermophiles enzymes have usually high resistance to chemical denaturants commonly used in wastewater treatment (de Miguel, Barros-Velázquez, & Villa, 2006). Therefore, the thermostable enzymes such as hyperthermophiles are good alternative to be used in wastewater treatment. However, to apply the enzyme directly to wastewater will not be practical if the enzymes are not immobilised to avoid enzyme loss in the solution.

1.5. Immobilisation

To feasibly remove ammonia from wastewater using an enzymatic pathway, CKase and ATCase should be immobilised. Immobilisation is a technique to improve enzyme performance as a biocatalyst (R. A. Sheldon, 2007). Immobilisation helps recover and reuse the enzymes, which significantly decreases the cost. Immobilisation also allows the enzyme to be easily separated from the reaction mixture, simplifying the enzyme application and providing an efficient and reliable process. (Tischer & Wedekind, 1999).

Moreover, immobilisation helps the enzymes be more stable in the soluble form. Immobilisation provides a protective matrix effect making the enzymes more resistant to environment changes (Guzik, Hupert-Kocurek, & Wojcieszyńska, 2014). Therefore, immobilisation induces enzyme stability) and activity under extreme conditions such as different pH values, temperatures and organic solvents (Cantone et al., 2013)

There are many methods for the immobilisation of enzymes. These include binding them onto prefabricated carrier material and in situ prepared carriers. The binding force can differ from weak to strong or by having a single or multiple binding points. The enzyme binding to

the carrier can be through reversible physical adsorption or an irreversible ionic linkage creating covalent bonds through ether, thioether, amide or carbamate bonds (Brena & Batista-Viera, 2006).

The choice of an appropriate immobilisation method is critical as it determines the enzyme activity and characteristics of the reaction (Chiou & Wu, 2004). Using different immobilisation methods depends on the desired application, but immobilisation is essentially performed in two classical ways: the physical and chemical methods. These are categorised into four different techniques: adsorption, entrapment, covalent bonding and cross-linking (Fernandez-Lafuente, Rosell, Rodriguez, & Guisan, 1995; Tischer & Wedekind, 1999).

One of the most important components to consider for immobilisation is the choice of enzyme carrier, which contributes to enzyme performance (Knežević-Jugović et al., 2017). The carrier varies from inorganic carrier to natural organic and synthetic organic carrier. In Chapter 5, immobilisation has been discussed in detail concerning methods, properties and the advantages and disadvantages of different carriers.

The aim of this project is to use synthetic organic carrier called Eupergit® C 250 L and covalent bonding method for irreversible immobilisation. The advantage of using Eupergit® C 250 L is the great physical and chemical properties, such as adaptation to a wide range of different enzymatic processes (Katchalski-Katzir & Kraemer, 2000). Moreover, it is resistant to microbial attacks and has a low swelling tendency in solvents and exhibits good performance in stirred batch reactors (Gemeiner, Rexová-Benková, Švec, & Norrlöw, 1994).

Furthermore, the covalent bonding method is the best method for this project as a powerful bond between the enzyme and the carrier is created (that allows the easy recovery and reuse of the enzymes and prevents the enzyme from leaching into the reaction), increased thermostability and unlimited covalent binding between the enzyme and substrate. (Ispas, Sokolov, & Andreescu, 2009; Ovsejevi, Manta, & Batista-Viera, 2013; R. Sheldon, 2007). Therefore, due to above mentioned reasons, the Eupergit® C 250 L and covalent bonding method are the most promising method used in this project for CKase and ATCase enzyme immobilisation for potential usage in wastewater treatment.

1.6. Thesis outline:

In summary, this project aims to investigate the conversion of ammonia to CA using a multienzymatic cascade and then immobilise CKase and ATCase, thereby enabling industrial application.

In this project the following questions are answered:

- Is it possible to clone, express and characterise the ATCases from three hyperthermophiles, *T. barophilus*, *T. sibiricus and P. furiosus,* and one mesophile from the mesophile in *E. faecalis*?
- Do hyperthermophiles enzymes have good pH and temperature stability?
- Is it possible to combine the pure enzyme CKase and the catalytic subunits of ATCase from the hyperthermophilic and the isoenzyme mesophilic to produce CA from ammonia and carbon dioxide (in the form of bicarbonate)?
- What is the best combination of CKase and ATCase with respect to different source organisms and the CA production rate?
- How could incorporate ATP recycling system from *E. coli* lysate to the one-pot combination of CKase and ATCase for the synthesis of CA?
- How could optimise the conditions of the CKase and ATCase combination to achieve the highest CA yield while ATP is being recycled?
- Is it possible to co-express the best combination of CKase and ATCase in a vector, transform it to *E. coli*, lyse the cell and use the lysate?
- Is it possible to apply enzymatic production of aspartate from fumaric acid and ammonia using aspartase?
- Is it possible to use *E. coli* lysate as a source of aspartase?
- Is it possible to combine aspartase with pure CKases and ATCases while ATP is recycling?
- Is aspartase active in recombinant co-expressed lysate of CKase and ATCase?

- Does CA production occur from ammonia, carbon dioxide and fumarate while ATP is recycling from one single co-expressed lysate?

- Is it possible to immobilise ATCase *T. sibiricus* and ATCase *E. faecalis* and CKase *T. sibiricus* on Eupergit® C 250 L as the carrier?

- Is it possible to immobilise the combination of CKase and ATCase (CKase *T. sibiricus* and ATCase *T. sibiricus*; CKase *T. sibiricus* and ATCase *E. faecalis*) on Eupergit® C 250 L?

- What is the characteristic of immobilised enzymes at different temperatures and pH values?

- Is it possible to use the immobilised enzymes in wastewater to produce CA from ammonia?

There are also a couple of gaps in this thesis that could be suggested as future works such as:

- application of immobilised enzymes in wastewater treatment plan

- recovery of CA from wastewater treatment

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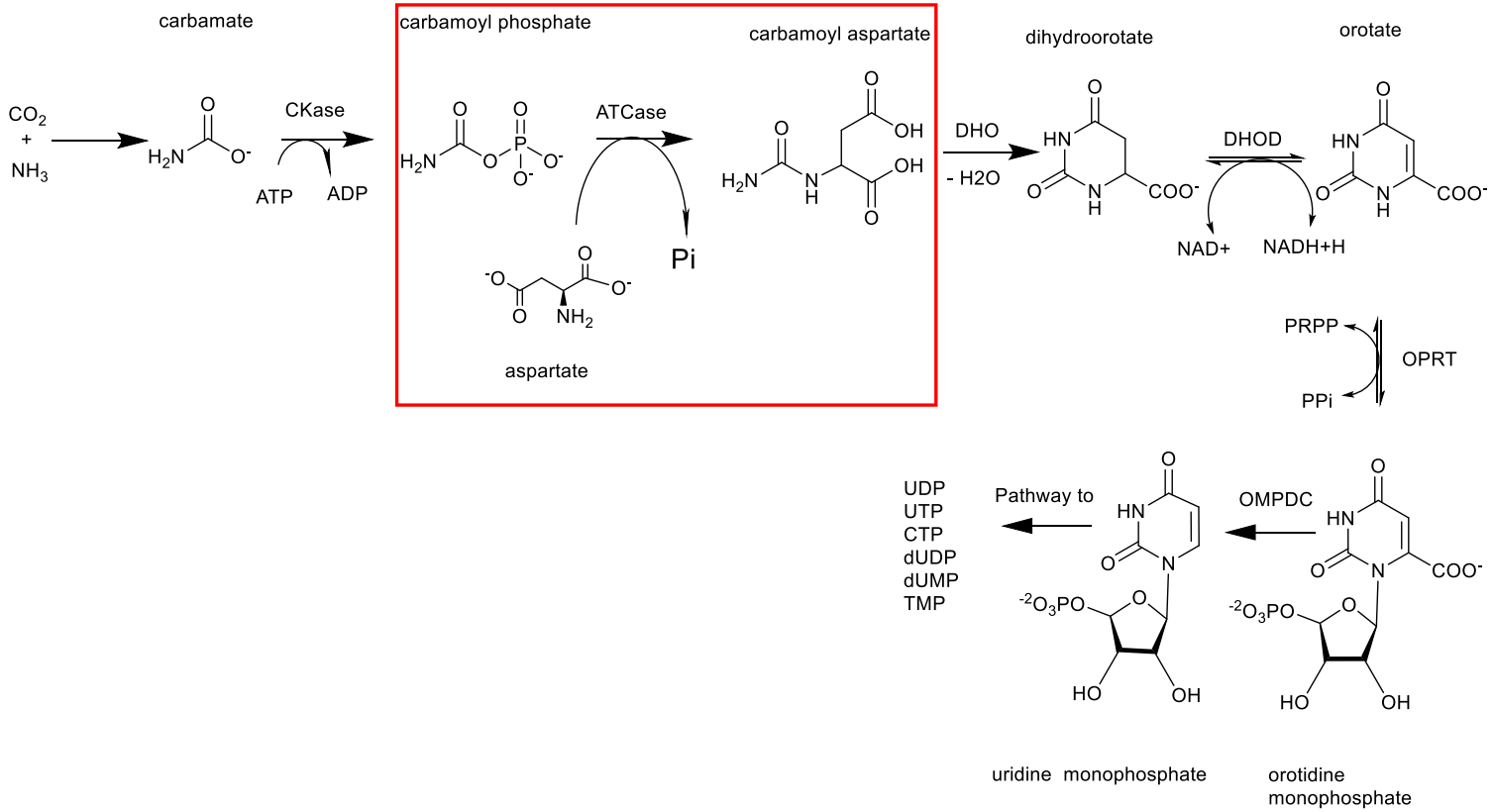
Chapter 2: Characterisation of Aspartate Transcarbamoylase from Hyperthermophiles and Mesophiles

2.1. Introduction

In the initial work of this project that was published (Hennessy et al., 2018), it showed that CKase is an ideal candidate for both ammonia and carbon dioxide sequestration. CKase can convert ammonia and carbon dioxide to CP via carbamate in an ATP-dependent manner across broad ranges of pH and temperature (Hennessy et al., 2018). Although CP is a key intermediate metabolite that can be transformed into valuable compounds (such as UDP, UTP, CTP, urea, arginine) through the urea cycle, pyrimidine and arginine biosynthetic pathways (Allen & Jones, 1964; Legrain, Demarez, Glansdorff, & Piérard, 1995; O'Donovan & Neuhard, 1970) (Scheme 2.1), it decomposes easily in aqueous solution with a half-life of ~5 min at 37 ºC (Allen & Jones, 1964), making it not feasible for industrial application. Hence, an alternative enzymatic pathway uses ATCase to prevent CP decomposition, making it more stable and able to be utilised in central metabolism in mesophilic and thermophilic microorganisms (Q. Wang et al., 2008).

The alternative enzyme to prevent CP decomposition is ATCase (E.C.2.1.3.2). ATCase is the first enzyme in the pyrimidine biosynthetic pathway and the key regulatory enzyme in purine, pyrimidine and arginine biosynthesis, which exists in all organisms. ATCase catalyses the transfer of the carbamoyl moiety from CP to the α-amino group of L-aspartate to yield CA and orthophosphate (Scheme 2.1) (W. N. Lipscomb, 1994; James R. Wild, Foltermann, & O'Donovan, 1980). CA is a valuable product as it could potentially use as nitrogen biofertilizer, convert to other valuable product such as UMP and nucleotides and be a promising target for for anti-malaria and anti-cancer (Wang et al., 2022)



Scheme 2.1: Pyrimidine pathway coupled with carbamate kinase enzyme. CP produced by CKase using carbamate and ATP. Carbamate can spontaneously produce ammonia and carbon dioxide. Red box: aspartate transcarbamoylase (ATCase) reaction. DHO = dihydroorotase DHOD = dihydroorotate dehydrogenase OPRT = orotate phophoribosyltransderase; OMPDC = OMP decarboxylase.

2.1.1. Background About ATCase Studies

There is a growing body of literature that recognised the importance of ATCase. ATCase from E. *coli* is one of the most widely used groups of ATCase that have been extensively studied for structure and kinetic properties. The following presents the history of ATCase from *E. coli*:

ATCase was first purified and crystallised from *E. coli* by Shepherdson and Pardee in 1960 with a molecular weight estimation of about 220 kDa based on sedimentation velocity measurements, while the catalytic reaction was discovered in 1955–1956 (Lowenstein & Cohen, 1956). More studies were conducted on catalytic and regulatory subunits after the purification of ATCase in 1967 (J. C. Gerhart & Holoubek, 1967; J. C. Gerhart & Schachman, 1965).

The study of ATCase structure demonstrated that ATCase has a quaternary structure C_6R_6 and is composed of six regulatory (R_6) and six catalytic subunits (C_6) (Weber, 1968). The molecular weight of regulatory and catalytic subunits is 17 and 34 kDa, respectively (Wiley & Lipscomb, 1968). ATCase has a pivotal role in metabolic pathways in prokaryotes and varies in different bacteria. Three different classes of ATCase (A, B and C) have been introduced according to their molecular mass and molecular organisation (Bethell & Jones, 1969; J. R. Wild & Wales, 1990). Class C is the simplest class and consists of a homotrimer of catalytic chain with 34 kDa molecular mass for each chain (Brabson & Switzer, 1975a, 1975b). Class A is a dodecamer of six subunits of 34 kDa and six subunits of 45 kDa polypeptide, in which 34 kDa polypeptide chains have catalytic and regulatory functions of enzymes and 45 kDa polypeptide functions are unknown (Adair & Jones, 1972; Bergh & Evans, 1993). Finally, class B is an allosteric enzyme and the most characterised class of ATCase, with two trimers of identical catalytic subunits (C_3) of 34 kDa polypeptides and three dimeric regulatory subunits (r_2) of 17 kDa polypeptides (Figure 2.1). The whole enzyme is a dodecameric quaternary structure of $2(C_3):3\ (r_2)$ (Figure 2.1). This group includes the ATCase from *E. coli*, other members of Enterobacteriaceae (Allewell, 1989; W. N. Lipscomb, 1994; Robin, Penverne, & Herve, 1989; Van Boxstael, Cunin, Khan, & Maes, 2003a; Van Boxstael, Maes, & Cunin, 2005) and Archaea (Xu & Glansdorff, 2002), including *P. abyssi* (Purcarea, Herve, Ladjimi, & Cunin, 1997), *Sulfolobus acidocaldarius* (V. Durbecq et al., 1999) and *Methanococcus jannashii* (Hack et al., 2000).

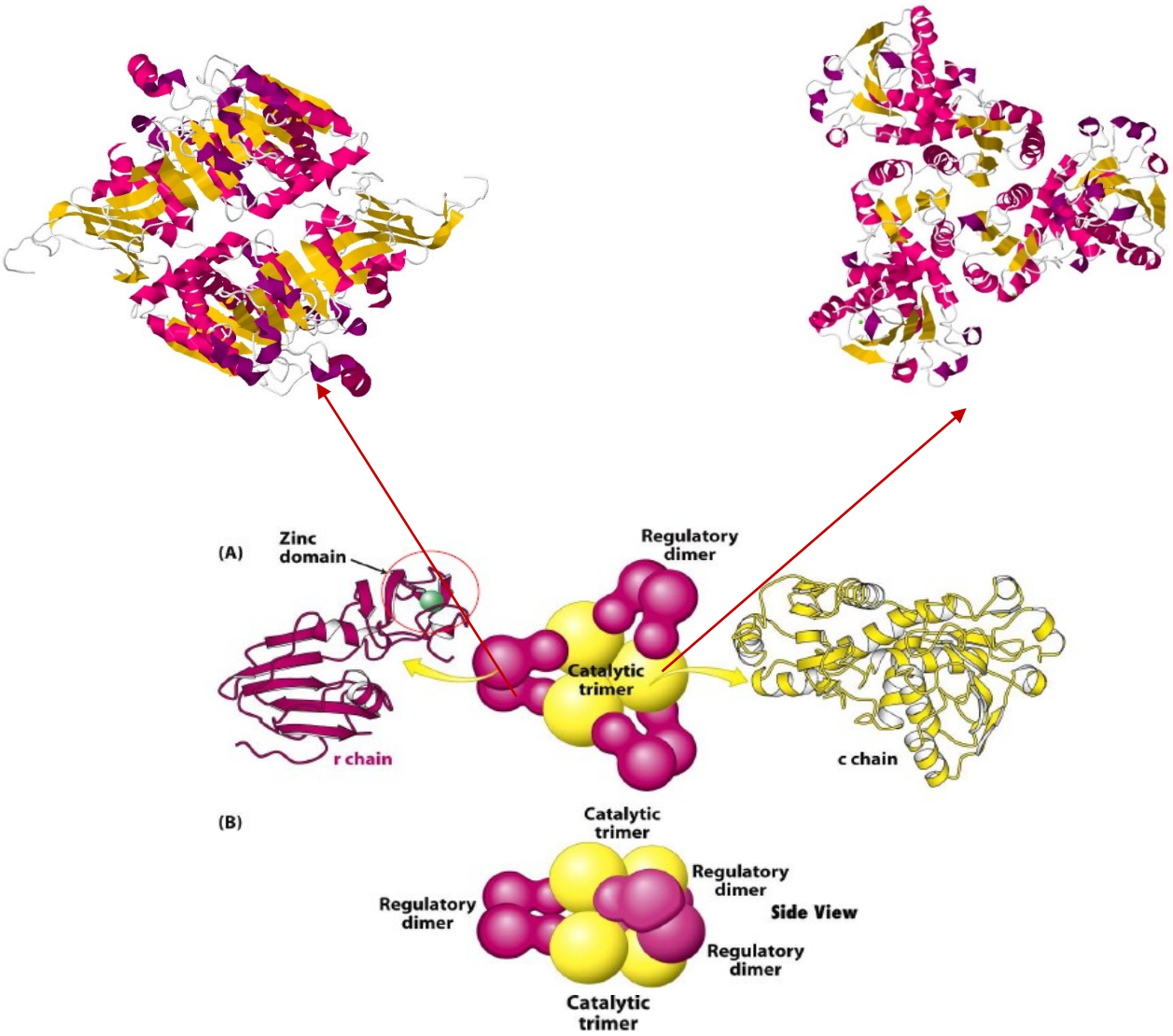


Figure 2.1: Schematic view of ATCase whole enzyme structure (bottom: A & B[1]). [1] Biochemistry, Sixth edition 2007. W.H. Freeman and company. Top: Crystal structure of the secondary structure of trimer catalytic subunits c3 (right) and dimer regulatory subunits r_2 (left) *E. coli* ATCase at 1.88 Å resolution from PDB 3CSU. (DOI: 10.2210/pdb3csu/pdb; 10.2210/pdb4wto/pdb).

As discussed, concerning the ATCase structure, ATCase has catalytic and regulatory subunits in which the two catalytic trimers are attached non-covalently by the three regulatory dimers. Each catalytic subunit consists of two domains: the aspartate and the CP, where each domain is associated with the binding of aspartate and CP, respectively. Also, the regulatory subunit has two domains: the zinc (Zn) and the allosteric (Allo), associated with the binding of zinc cofactor (essential for association of regulatory and catalytic subunits) and allosteric effector, respectively (e.g., ATP and CTP, which may increase or decrease the enzyme catalytic rate) (Figure 2.2) (Ke, Honzatko, & Lipscomb, 1984; Stevens, Gouaux, & Lipscomb, 1990). The aspartate, CP and Allo domains include α helix or β

sheets, while the Zn domain is categorised as rubredoxin-like, with a zinc or iron bound (Murzin, Brenner, Hubbard, & Chothia, 1995).

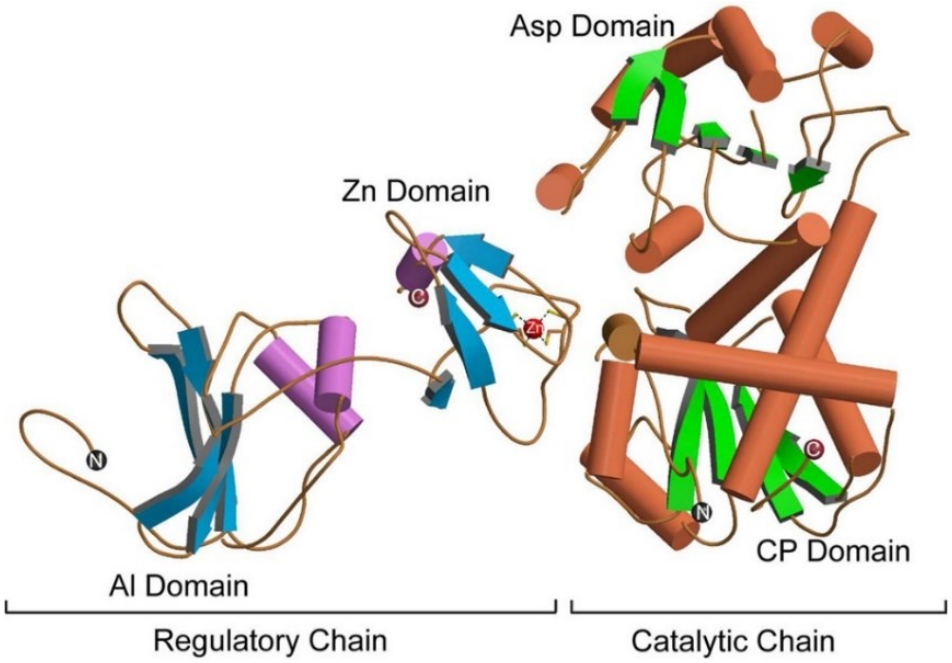


Figure 2.2: Schematic ATCase secondary structural from *E. coli* showing one catalytic and one regulatory subunit. Each catalytic chain has an aspartate (Asp) and a CP domain, and each regulatory chain has an allosteric (Al) and a zinc (Zn) domain (Lipscomb & Evan 2012).

ATCase structure study showed that it has six active and six allosteric sites. The active sites are positioned at the interface between neighbouring catalytic subunits, and six allosteric sites are positioned on each regulatory subunit. The crystal finding of ATCase indicated that the C_6R_6 represents the crystal structure of a threefold axis direction and one non-coincident twofold axis direction in the molecule (Galkin et al., 2009; Wiley et al., 1972; Wiley & Lipscomb, 1968).

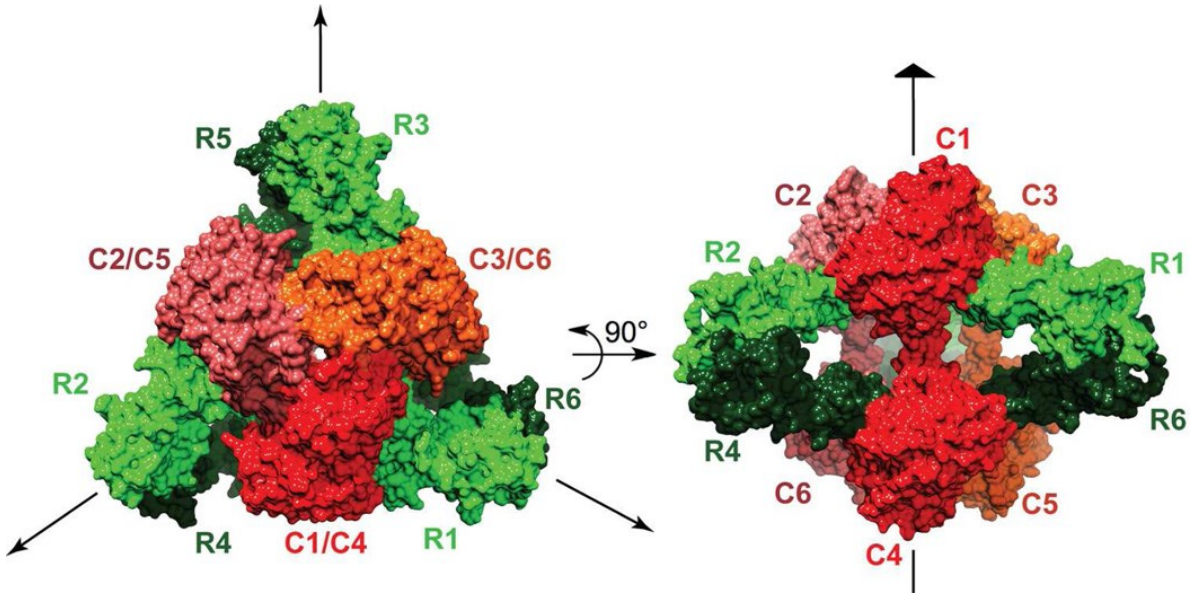


Figure 2.3: ATCase quaternary structure from *E. coli* in two different R (right) and T (left) states. Catalytic chains are composed of two catalytic trimers, C1–C2–C3 and C4–C5–C6, while three regulatory dimers are R1–R6, R2–R4 and R3–R5. Source: Lipscomb and Evan (2012).

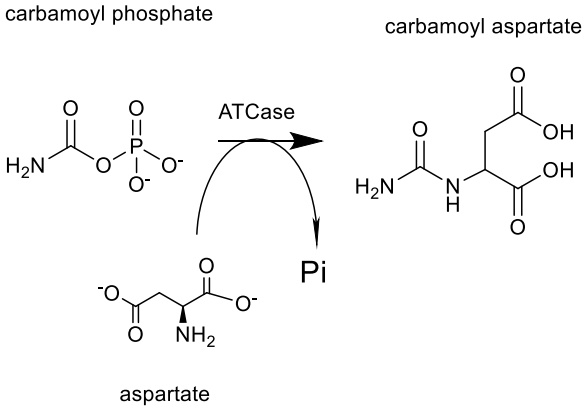
The investigation of the ATCase crystal structure revealed that the ATCase enzyme exists in two different structural states: low-activity, low-affinity form (T state) and its high-activity, high-affinity form (R state) at the molecular level. After aspartate binds to the enzyme in the presence of CP, the ATCase conformation changes from the T to the R state (Figure 2.3). The conformational change from the T to the R state comprises an elongation of 11 Å along the molecular threefold axis. One catalytic trimer rotates 12 degrees relative to the other catalytic trimer. Each of the three regulatory dimers moves about 15 degrees towards an approximate twofold axis, which occurs in the transformation. Therefore, elongation occurs along a threefold axis direction and the catalytic and the regulatory subunit domains. The two domains of the regulatory subunit open by 1.7 Å while the two domains of the catalytic subunit close by 6.8 Å between the T and R states (Ke et al., 1984; Ke, Lipscomb, Cho, & Honzatko, 1988; Krause, Volz, & Lipscomb, 1987).

2.1.2. Catalytic Mechanism of ATCase from *E. coli*

Having discussed the ATCase structure, the final section of this chapter introduction addresses the ATCase's catalytic mechanism. Before proceeding to the catalytic mechanism of ATCase, it is important to mention that the ATCase is structurally and catalytically very complex enzyme due to 1) the enzyme is an allosteric enzyme, 2) it is a multisubunit protein having 12 subunits including two separate catalytic and regulatory subunits, 3), it is a bi-

substrate enzyme that contains one cofactor (Zn) and nucleotide effectors in structure, or during catalytic activity, 4) it undergoes massive structural rearrangement during reaction, and 5) it has a complicated quaternary structure. Therefore, it is important to know the catalytic mechanism of ATCase.

The study of the ATCase catalytic mechanism revealed that in the binding process, CP binds first and aspartate second. After completion of the reactions, CA is released before the phosphate (Scheme 2.2) (Wedler, 1974). Also, previous studies observations have reported that there are several crucial residues for catalysis, including His 134, Gln 137, Ser 52, Thr 55, Lys 84, Arg 105, Thr 53, Arg 54, Arg 229, Ser 80, Glu 231 and Arg 167 from a neighbouring catalytic chain, which is important for a deep understanding of ATCase's catalytic mechanism (Figure 2.4 left) (J. Wang, Stieglitz, Cardia, & Kantrowitz, 2005). Moreover, the studies have shown that when CP binds, the enzyme undergoes structural changes. The 80s loop (a loop in the catalytic chain of ATCase composed of residues 73–88) restructure to place the Ser 80 and Lys 84 into the active site. Also, the 50s loop (a loop in the catalytic chain of ATCase comprising residues 50–56) and the 240s loop (a loop in the ATCase comprising residues 234–245) make some movement (Figure 2.4 right).



Scheme 2.2: Aspartate transcarbamoylase (ATCase) reaction. ATCase catalyses the transfer of the carbamoyl moiety from CP to the α-amino group of L-aspartate to yield CA and orthophosphate.

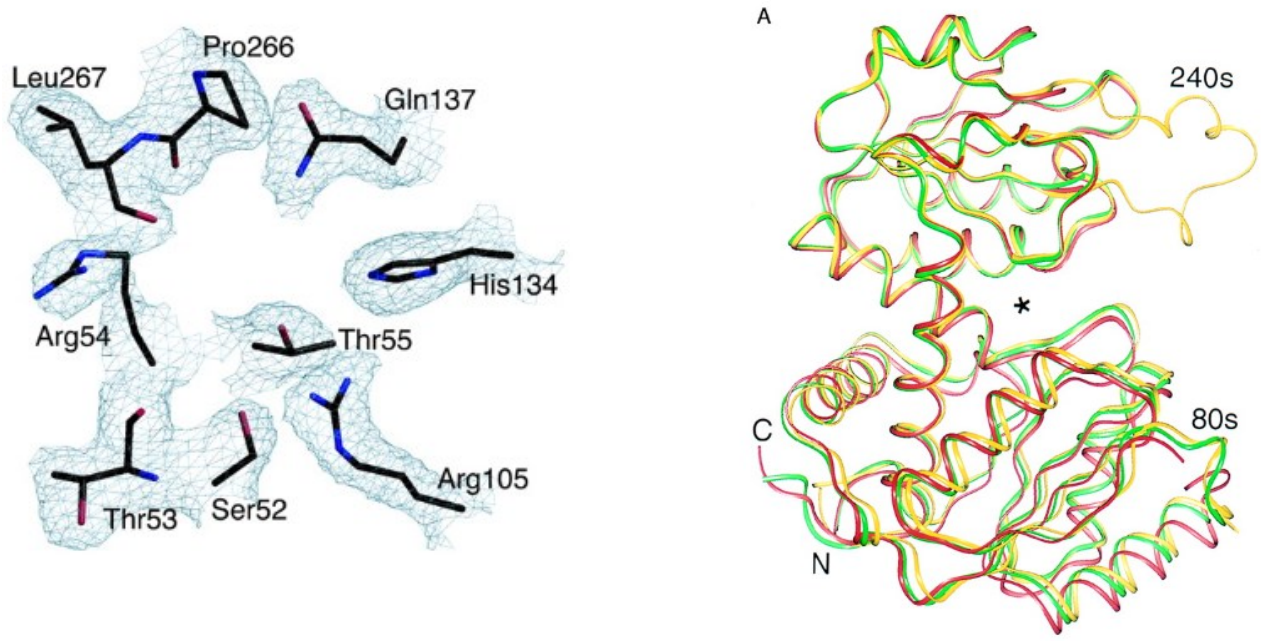


Figure 2.4: Left: aspartate Transcarbamoylase (ATCase) active site crucial residues (J. Wang et al., 2005). Right: ATCase structure from *E. coli* catalytic subunit showing one catalytic subunit and location of 80s, 240s loop (D. S. Shi, Allewell, & Tuchman, 2015).

Moreover, the study of the catalytic mechanism of ATCase demonstrated that after CP binds, conformation changes create a high-affinity binding site for aspartate, both structurally and electrostatically. This creates a positively charged binding site for aspartate in the active site (J. Wang et al., 2005). Therefore, the role of CP in preparing the active site for the binding of aspartate is critical (Stebbins, Xu, & Kantrowitz, 1989). After CP binding, aspartate binds to the ATCase CP complex (Figure 2.5). Aspartate binding changes the tertiary and quaternary structures. The active site electrostatic change induces the movement of the positively charged residue of the 240s and 80s loop to reposition closer to the substrate. The repositioning of the 240s loop results in the side chains of the active site necessary for catalysis to be in the high-activity, high-affinity position leading to quaternary conformational structure changes from T state to R state (Figure 2.6). These conformational changes facilitate the two domains of each catalytic chain approaching each other for catalysis. Therefore, the amino group o

N f aspartate attacks the amide carbon of carbamoyl and forms the tetrahedral intermediate. Following that, the intermediate converts to CA and inorganic phosphate, which leaves the active site, respectively (Gouaux, Krause, & Lipscomb, 1987).

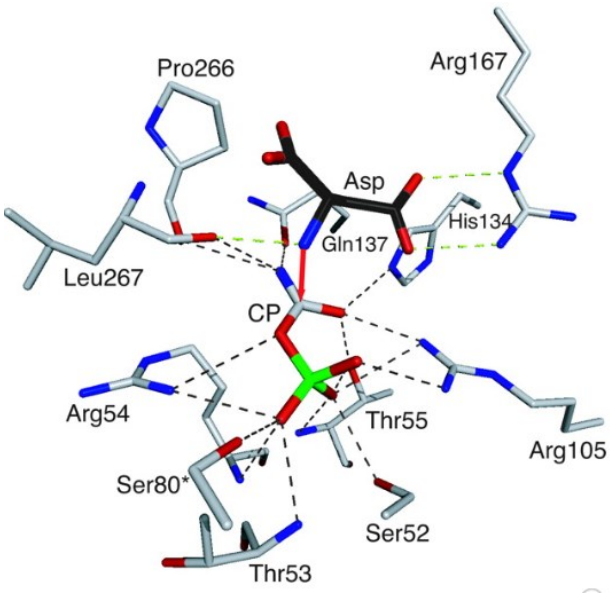


Figure 2.5: Autodocking of *E. coli* ATCase active site to CP and aspartate. The position of CP is from crustal structure and aspartate from autodocking. The red arrow shows the attack of nitrogen of aspartate to the carbonyl carbon of CP.

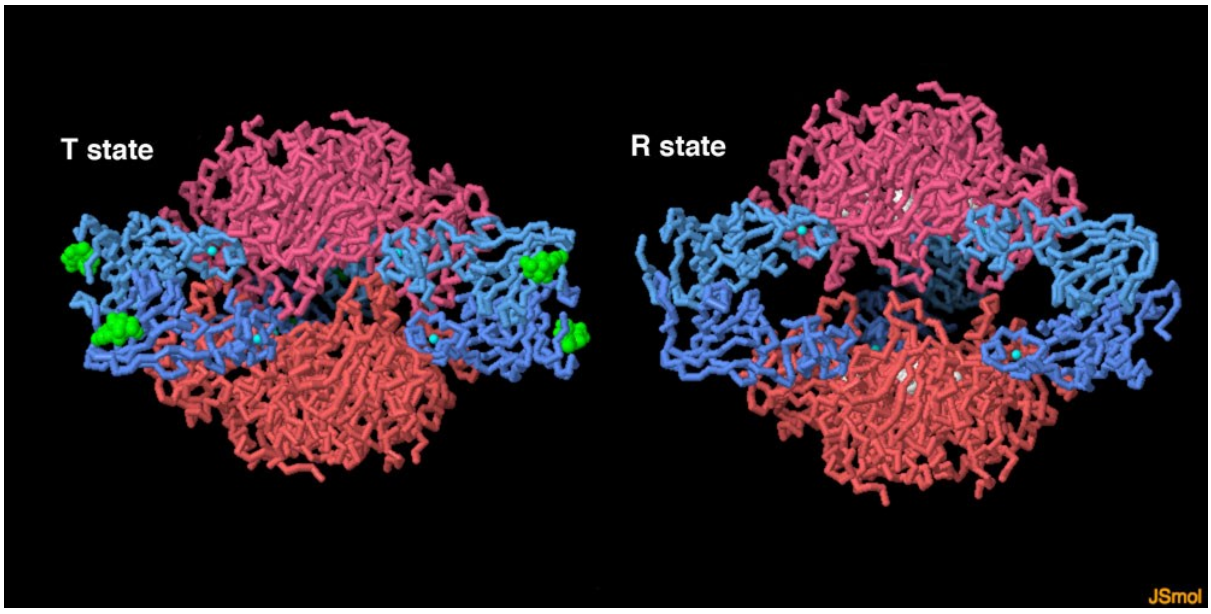


Figure 2.6: ATCase from *E. coli* in two different R and T states. The catalytic subunits are shown in red and regulatory subunits are in blue. The small atoms in bright turquoise are zinc ions. The R state has an inhibitor (white) bound to N-(Phosphonacetyl)-l-aspartate (Shi, Allewell & Tuchman, 2015).

2.1.3. Previous Studies on ATCase

Most studies on bacteria ATCase have only focused only on *E. coli*; therefore, our knowledge about ATCase from other bacteria is very limited. Hyperthermophilic ATCase is an example of bacteria where rare and limited studies have been conducted. So far, *P. abyssi* and *A. aeolicus* are the only two hyperthermophiles in which their ATCases have been briefly studied. Some kinetic studies on *P. abyssi* and *A. aeolicus* follow below.

The kinetic parameters such as k_m and V_{max} for aspartate and CP were determined for *P. abyssi* and compared with *E. coli*. The results demonstrated that k_m for *P. abyssi* was almost 10 times more than *E. coli* for Cp at 37 ˚C, and k_m for aspartate was similar in *P. abyssi* and *E. coli*. Moreover, the k_m and V_{max} value for aspartate in ATCase from *P. abyssi* and *E. coli* increased by elevating the temperature. However, the enzyme efficiency increased much more in *P. abyssi* than in *E. coli* at higher temperatures (Van Boxstael, Cunin, Khan, & Maes, 2003b).

Further, ATCase from *P. abyssi* showed higher thermostability than the homologue *E. coli* enzyme (Purcarea, Erauso, Prieur, & Hervé, 1994). The reason for this, investigated in the study, is the crystal structure of hyperthermophiles compared to *E. coli.* The crystal structure studies suggested several strategies for thermostability in hyperthermophile and thermophile organisms, such as:

- additional ion bonds
- low number of thermolabile residue, for example, asparagine, glutamine and tryptophan
- elevated number of proline residue
- increase hydrophobic residue to make a hydrophobic core (Matthews, 1993).

In other studies, the kinetic properties of catalytic subunit C_3 and whole enzyme C_6R_6 were investigated. The results revealed that the catalytic subunits, regulatory subunits and the whole enzyme from different microorganisms, including hyperthermophiles *P. abyssi* and *A. aeolicus*, could be expressed in *E. coli* by using either *pyrB* gene (responsible for catalytic subunit production), the *pyrI* gene (responsible for regulatory subunit production) or the *PyrBI* gene (V. Durbecq et al., 1999; Hack et al., 2000; Purcarea et al., 2003; Purcarea et al., 1994; Van Boxstael et al., 2005). Further, depending on the various conditions, the catalytic subunit C_3 and whole enzyme C_6R_6 have shown different behaviours. For example, the whole enzyme exhibits better thermostability at higher temperatures than the catalytic subunit in hyperthermophiles and *E. coli*. Further, it was demonstrated that the catalytic

trimer subunit manifesting hyperbolic kinetic and its activation energy was similar to the catalytic subunit from *E. coli* (Hack et al., 2000).

Another significant aspect of ATCase from hyperthermophile *P. abyssi* is its cooperative behaviour towards aspartate and exhibiting Michaelis-Menten saturation kinetics for CP. Both behaviours are similar to *E. coli*. In addition, *P. abyssi* exhibited that it is allosterically regulated by ATP as activator and CTP and UTP as inhibitors.

Conversely, although the elevating temperature has no significant effect on cooperativity or allosterically behaviour of ATCase from *P. abyssi*, it increases the V_{max} (Van Boxstael et al., 2005) and ATCase from *A. aeolicus,* the enzyme activity increased by increasing the temperature without a change in K_m for either aspartate or CP (Purcarea et al., 2003).

Having discussed some kinetic properties of hyperthermophiles *P. abyssi* and *A. aeolicus*, it is important to discuss the relationship of ATCase with CKase or CPSase (the enzyme responsible for the reaction prior to the pyrimidine pathway that converts ammonia and carbon dioxide to CP). Several studies have been conducted on the couple reaction between ATCase and CPSase or CKase.

Previously published studies indicated that CPSase or CKase can channel the CP from its active site to the ATCase active site without releasing CP to the environment. This channel occurs through physical association of the CPSase and CKase and the formation of enzymes complex to allow direct transfer of CP between active sites. One of the most important microorganism groups that take advantage of this mechanism and channelling is hyperthermophiles. This mechanism protects CP from decomposition at high temperatures as it is very labile (Purcarea et al., 2003; Purcarea, Herve, Cunin, & Evans, 2001; Q. Wang et al., 2008).

Moreover, some evidence suggests that hyperthermophilic enzymes are good catalysts due to their good stability in different pH and temperature ranges and good robustness (Lowenstein & Cohen, 1956) due to their unique properties living in a harsh environment. Our published paper characterised CKases from three hyperthermophiles (*T. barophilus*, *T. sibiricus, P. furiosus*) and two mesophiles (*E. faecalis* and *C. tetani*) organisms. It showed their ability to remove and use ammonia from wastewater and convert it to CP, a key intermediate in metabolic pathways. We also showed briefly in our paper that ATCase can couple with CKase to convert CP to CA. Since CP is very labile in aqueous solution, it must simultaneously be converted to another product to avoid decomposition in the solution.

Therefore, coupling CKase with ATCase is an ideal candidate for protecting CP from decomposition and converting it to a value-added product.

A considerable number of studies have been published on ATCase from *E. coli*. Therefore, ATCase from *E*. *coli* would not be my project focus as previous studies have established all its kinetic and structural aspects. However, for my project, another member of the Enterobacteriaceae (*E. coli* family class) has been chosen, namely, *E. faecalis*, which showed more than 40 per cent amino acid similarity with other hyperthermophiles ATCase.

2.1.4. Promiscuity

Traditionally enzymes are considered to catalyse specific reactions, by converting a single substrate to a single product with high efficiency. Hence the specificity of the enzyme is a key feature. However, many enzymes can catalyse structurally distinct substrates and catalysing reactions other than its physiological substrate which make them catalytically promiscuous(Koshland, 1958; Lichtenthaler, 1994) . Another type of promiscuity is functional promiscuity. Functional promiscuity is crucial for the evolution of new protein function, drug metabolism and the in-vitro engineering of biocatalysts (Bornscheuer & Kazlauskas, 2004; Hult & Berglund, 2007; Per & Seongsoon, 2005).

2.1.4.1. SPECIFICITY OF ENZYME

One of the important characterize of enzymes is its catalytic specificity. There are some enzymes demonstrating complete specificity which means that they only catalyse one particular reaction or substrate. And the rest of enzymes are specific to particular types of chemical bonds or functional groups. In general, there are four different types of specificity as follow (Hult & Berglund, 2007):

- Absolute specificity- The enzyme catalyses one particular reaction
- Group specificity- The enzyme operates on a particular functional group such as amino or phosphate.
- Linkage specificity- The enzyme takes action at a specific chemical bond.
- Stereo-chemical specificity- The enzyme acts on a special steric or optical isomer.

2.1.4.2. PROMISCUITY AND EVOLUTION

Some scientists consider the promiscuity of an enzyme the initial point for the divergence of enzyme evolution. New protein functions can be exploited from existing structural scaffolds during the evolution of enzyme promiscuity (James & Tawfik, 2003; O'Loughlin, Patrick, & Matsumura, 2006) . In the event of a single point mutation, the substrate- specific enzymes may show a promiscuous activity towards an unnatural substrate, thereby making the enzyme more efficient or even enabling the organism to survive. After gene duplication, the promiscuous enzyme can be subjected to more mutations to optimize or gain new function(Aharoni et al., 2005; Olga Khersonsky, Roodveldt, & Tawfik, 2006; O'Boyle, Holliday, Almonacid, & Mitchell, 2007; O'Brien & Herschlag, 1999a, 1999b)The new promiscuous activity of an intermediate evolution enzyme can evolve sufficient “native” function to give the organism a survival advantage. This may lead to the production and optimization of a new “specific” enzyme after gene duplication and mutation(Olga Khersonsky et al., 2006; O. Khersonsky & Tawfik, 2010).

Archaea are the oldest organisms on the earth and the source of promiscuity. New specialised functions of enzymes have been evolved from a small number of archaea enzymes. Archaea enzymes have many disordered proteins that can be seen in “hub proteins” in the metabolic regulation of many organisms. Therefore, promiscuity is one of the evolutionary tools for enzymes to assume specialised catalytic roles (Arora, Mukherjee, & Gupta, 2014).

2.2. Aim

In this chapter, we aimed to clone, express and characterise the ATCases from three hyperthermophiles, *T. barophilus*, *T. sibiricus and P. furiosus,* and compare them with their isoenzyme from the mesophile *E. faecalis*. Since our published paper has already studied the CKase from *E. faecalis*, this study focuses on ATCase from mesophile *E. faecalis,* a member of Enterobacteriaceae.

The r published research on CKase has determined that CKase from these three hyperthermophiles has good pH and temperature stability (Hennessy et al., 2018). Therefore, we want to establish if these ATCases have the same stability as CKase. If the ATCase stability is found to be similar to CKase, then we may be able to couple the CKase and ATCase for converting ammonia to CA. CA is valuable product as it could potentially use as nitrogen fertiliser or could convert to other valuable product such as UTP and CTP very important bases in DNA formation. The works presented here on catalytic trimer of

ATCase hyperthermophile Thermococcales provide one of the first investigations into how these enzymes behave at different pH and temperatures in the cell-free experiment and out of their natural habitats.

2.3. Materials and Methods

2.3.1. Material

Polyacrylamide gels were cast using acrylamide and bis-acrylamide solutions from Bio-Rad and were run on a Mini-PROTEAN® Tetra system. Bio-safe™ Coomassie Blue was used for staining. Protein markers, restriction enzymes and the Quick DNA Ligation™ Kit were purchased from New England Biolabs (NEB) Inc. Alkaline phosphatase was from Roche Diagnostics GmbH. Agarose for electrophoresis was purchased from Invitrogen. Nutrients for broth were purchased from Bacto Laboratories Pty Ltd. L-Aspartate, ampicillin and N--chloromethylphthalimide were obtained from Sigma-Aldrich®. HPLC analysis was performed on a Hewlett-Packard Series 1100 separation system operated by Chemstation from Agilent Technologies. The sequencing was carried out by the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University. The cells were lysed by Thermo Fisher Scientific's Omni Sonic Ruptor 400.

2.3.2. Strains and Plasmids

The native genes of the catalytic subunits of ATCase (PyrB) from *P. furiosus* (UniProt number Q8U373), *T. barophilus* (UniProt number F0LI09), *Thermococcus sibiricus* (UniProt number A0A124FFJ7) and *E. faecalis* (UniProt number Q9L4T8) were taken from UniProt and constructed by GeneArt Thermo Fisher Scientific flanked by *Nde*I and *Eco*RI restriction sites and provided in pMK plasmids. The pETMCSIII plasmid (Neylon et al., 2000) (Figure 2.7) is not a commercial vector; however, it has already been used in research and described in the literature. pETMCSIII is a His-tag expression vector (contains ampicillin resistance gene (*ampR*)) allowing leaky expression of recombinant proteins due to the lack of the *Lac*I gene in the plasmid (Love, Lilley, & Dixon, 1996; Neylon et al., 2000) and *E. coli* strain BL21 (DE3) were used for expression of the enzymes. Amino acid sequence similarity and UniProt alignment were performed using Clustal Omega and SnapGene Viewer 4.2.6.

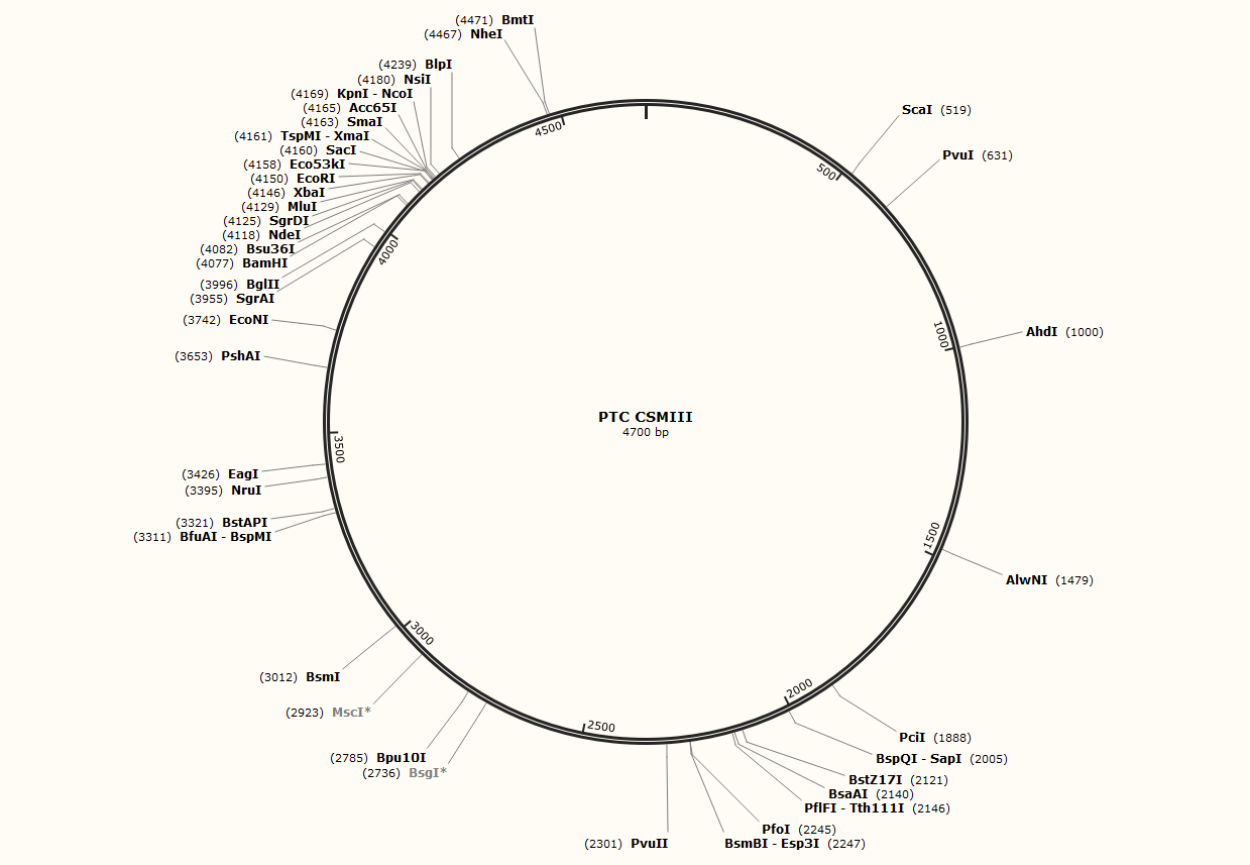


Figure 2.7: pETMCSIII expression vectors allowing leaky expression of recombinant proteins. It lacks the *lac*I gene and includes His-tag in N-terminal and contains ampicillin resistance gene (*ampR*). (Love et al., 1996). Promotor: T710, Ori: ColE1, Primers pET 3 (forward) (5'-CGA CTC ACT ATA GGG AGA CCA CAA C-3'); pET4 (reverse) (5'-CCT TTC GGG CTT TGT TAG CAG-3').

2.3.3. Cloning and Expression of ATCase

Plasmids with the *P. furiosus, T. barophilus, T. sibiricus* and *E. faecalis pyrB* genes were transformed into *E. coli* (*AN1459*) cells. using electroporation and amplified. *E. Coli* AN1459 is a suitable host for plasmid preparation, selection and long-term storage during molecular cloning procedure (Vasudevan et al., 1991). The genes and pETMCSIII plasmid was digested by *Nde*I and *EcoR*I restriction enzymes using an NEB CutSmart® Kit as follows:

- 10X CutSmart buffer 10 µL
- restriction enzyme *Nde*I 2 µL
- restriction enzyme *EcoR*I 2 µL
- Mq water 56 µL
- DNA template (ATCase or pETMCSШ) 30µL

They were heated at 37 °C for a total of two hours. After one hour, 1 µL of alkaline phosphatase and 20 µL de-phosphorylation buffer were added to the vector solution. After digestion, the ATCase genes were cleaned using the gene QIAquick® Gel Extraction Kit (QIAprep) protocol (following the manufacturer's guidelines), and the vector was run on 1.2% agarose gel. The vector band was detected using a UV lamp based on their base pair kDa, and then the band was cut and cleaned using the cleaning kit. The ATCase genes and pETMCSШ plasmid were then ligated at room temperature for 15 min using *E. coli* AN1459 as follows:

- vector 1 µL
- DNA 3 µL
- water 6 µL
- 2X buffer 10 µL
- ligase 0.5 µL

The pETMCSШ allows the protein to be expressed with an N-terminal hexahistidine tag. The new constructs were then transformed into *E. coli* AN1459 cells and plated onto a Lysogeny Broth (LB) agar plate containing ampicillin (0.5 mg/mL ampicillin), pepton (5 g/L), yeast extract (5 g/L) and NaCl (10 g/L) and left at 37 °C overnight. Plasmids from individual colonies were isolated using a QIAprep Spin Miniprep Kit and screened by Sanger sequencing. The sequencing was carried out by the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University. After confirming the sequence, the pETMCSШ, containing the gene of interest was transformed into *E. coli* BL21 (DE3) cells for expression. The cells containing the plasmid were grown in LB liquid medium containing 0.5 mg/mL ampicillin for 16 hours at 37 °C with shaking at 200 rpm.

2.3.4. Purification of ATCase

After culturing *E. coli* BL21 (DE3) in LB for 16 hours, the *E. coli* cells were then collected from 50 mL culture by centrifugation (6000 × *g* for 10 min), resuspended in 20 mL binding buffer containing (20 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7.4). They were lysed over ice by Thermo Fisher Scientific Omni Sonic Ruptor 400, equipped with an ORT-375 processing tip set at 50% power and 50% pulser for 6 min. The lysate was centrifuged at 20,000 × *g* for 60 min at 4 °C, and the enzymes were purified from the supernatant using His GraviTrap™ columns from GE Healthcare using the conditions recommended by the manufacturer (load 10 mL binding buffer two times, add sample, add binding, another 10 mL buffer two times, collect the enzyme by 3 mL elution buffer). The protein was collected in a 3 mL elution buffer containing 0.02 M phosphate buffer

(Na_2HPO_4/NaH_2PO_4), pH 8.8, 0.5 M NaCl and 0.5 M imidazole. Then the enzyme solution was changed to 50 mM Tris-HCl pH 8.5 and kept at 4 °C for three months without losing any activity.

Protein concentration was measured using either the NanoDrop™ ND-1000 Spectrophotometer from Thermo Fisher Scientific or the Shimadzu UV-Visible Spectrophotometer at 280 nm. The extinction coefficients needed for calculation were generated from the online ProtParam tool based on the protein sequence. The catalytic subunit was analysed for purity by electrophoresis on 20% SDS-PAGE gel.

2.3.5. ATCase Activity Assay

ATCase enzyme activity was measured through the consumption of aspartate and the simultaneous production of CA by either HPLC or HPLC/MS. The enzyme solution was pre-incubated at 37 °C for 5 min before adding CP. A typical reaction mixture (500 μl) contained 50 mM Tris-HCl, pH 8.5 (as it was the pH for most ATCases, yet we did not know the optimal pH for my project's ATCases), 23 mM aspartate (to use more than 20 mM, being the k_m for a few ATCases from other organisms) 20 mM CP (high excess of CP as is labile) and one of the purified ATCases (0.1 μM for *T. barophilus, T. sibiricus, E. faecalis* and *P. furiosus*) were incubated for a further 5 min at 37 ºC.

An aliquot of 50 μL was then withdrawn at each time point and mixed with 50 μL of 1 M HCl, followed by the addition of 50 μL of 1 M NaOH for neutralising followed by HPLC or LC/MS measurement.

2.3.6. Derivatisation of Aspartate

The consumption of aspartate was determined by a reverse-phase HPLC (Agilent 1100) analysis using a Waters AccQ-Tag™ Ultra Derivatisation Kit and AccQ-Tag™ C18 column.

For derivatisation, 1 mL of acetonitrile was added to AccQ-Fluor Reagent Powder (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate), mixed and dissolved by heating at 55 ºC for 10 min. After that, 40 μL of AccQ Borate Buffer was added to a 10 μL sample (which had already been neutralised and diluted) and finally, 10 μL of AccQ-Fluor Reagent Solution was added. The solution was heated for 10 min at 55 ºC and run by HPLC.

2.3.7. HPLC Detection of Aspartate

The reverse-phase separation of L-aspartic acid was performed on an Agilent 1100 HPLC system fitted with a PDA detector (also referred to as a diode array detector [DAD]), using an AccQ-Tag™ column (3.9 × 150 mm, 4 μm, 60 Å) separating the hydrolysate amino acid derivatives produced in the reaction. Samples were eluted (1 mL/min) at 37 ºC with a gradient of 10% AccQ Eluent (v/v Eluent/water) (Solvent A) and 100% acetonitrile (Solvent B), according to the following program: 0 min 100% B, 0–5 min 99% B, 5–18 min 95%B, 18–25 min 60% B, 25–45 min 100% B, with linear transitions between mixtures. The observed retention time for aspartic acid was around 15 min.

2.3.8. HPLC Detection of Carbamoyl Aspartate

Since, CA couldnot be detected by AccQ-tag, tTo detect CA by HPLC, the reagent N-chloromethylphthalimide was used as the derivatisation reagent for HPLC. N-chloromethylphthalimide is a UV-absorbing reagent for the formation of derivatives for HPLC.

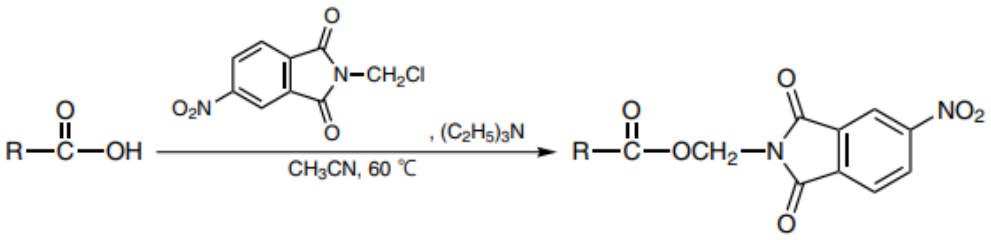


Figure 2.8: N-chloromethylphthalimide reaction with a carboxylic group.

To label the sample with N-chloromethylphthalimide, the following protocol was followed (Figure 2.8). A 3 mg sample was dissolved in 1 mL of acetonitrile. 1 mL of labelling reagent N-chloromethylphthalimide/acetonitrile (10 mg/mL) and 1 mL triethylamine/acetonitrile solution (5 mg/mL) were added. Then the sample was incubated at 60 ºC for one hour and cooled to room temperature. The sample can be detected by reverse-phase HPLC at 254 nm (Lindner & Santi, 1979).

2.3.9. Detection of Aspartate and Carbamoyl Aspartate by HPLC and HPLC/MS

The amount of residual aspartate was measured by a reverse-phase HPLC analysis of a 10 μL aliquot of the resultant solution derivatised with AccQ-Tag™ reagent, following manufacturer guidelines. Also, the amount of CA produced was measured by HPLC/MS analysis on a Waters Alliance 2695 separation module coupled to a Waters TQD detector. HPLC separation was performed using a Phenomenex Develosil RPAQUEOUS-AR 5 μ C30 (250 × 4.6 mm) column heated at 35 °C, eluting with 0.1% formic acid in H_2O (0–1 min), followed by a linear gradient to 0.1% formic acid in ACN/H2O 30/70 (1–7 min), at a flow rate of 0.7 mL/min (15:1 flow splitter). CA (retention time 5.5 min) was identified and quantified using integrated multiple reaction monitoring (MRM) and a calibration curve developed with authentic commercial material. The MRM parameters used were CA (parent (m/z): 176.9475); channel 1: daughter (m/z): 45.9169, dwell (s): 0.025, cone (V): 20, collision (V): 24; channel 2: daughter (m/z): 69.9541, dwell (s):0.025, cone (V): 20, collision (V): 26; channel 3: daughter (m/z): 73.917, dwell (s): 0.025,cone (V): 20, collision (V): 24; channel 4: daughter (m/z): 87.9496, dwell (s): 0.025, cone (V): 20, collision (V): 18; and channel 5: daughter (m/z): 45.9169, dwell (s): 0.025, cone (V):20, collision (V): 24. The CA parent was used for quantification (Figure 2.9).

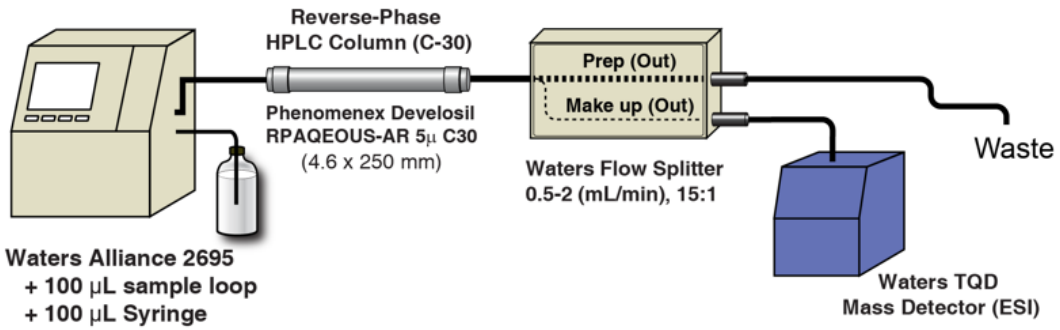


Figure 2.9: LC/MS analysis scheme.

2.3.10. Substrate Saturation Assay to Determine Apparent *Km* Values

Aspartate saturation experiments were performed at 40–60 mM CP, 50 mM Tris-HCl, pH 8.5 and 1 unit (unit defined as the amount of enzyme necessary to turn over 1 mM aspartate per min) purified ATCase in a total volume of 0.5 mL.

CP saturation experiments were performed at 23–40 mM aspartate and 50 mM Tris-HCl pH 8.5 and 1 unit ATCase from *T. barophilus*, *T. sibiricus*, *E. faecalis* and *P. furiosus* in a total volume of 0.5 mL. They were pre-incubated at 37 ºC for 10 min and initiated by adding CP and assayed for 5 min. The kinetic parameters were calculated on the average of each enzyme activity by using GraphPad Prism 8.0.0 using Michaelis-Menton and allosteric curve fitting curve to find the best fit.

2.3.11. Effects of Temperature

The thermal stabilities of the catalytic subunit of enzymes (1 unit purified enzyme ATCases) were measured using different assays and compared. In the first assay, the enzymes solutions (in 50 mM Tris- HCl, pH 8.5) were pre-incubated at 40, 60, 80 and 100 ºC for 10 min, chilled on ice for 10 min and assayed at 37 ºC by adding aspartate and CP, respectively. The assays were initiated by adding CP 5 min after adding aspartate and run for 5 min (since CP binds first to ATCase, therefore, the assay was initiated by adding CP to be able to measure the initial velocity of the enzyme). In the second assay, the thermostability of highly stable catalytic subunits from *T. barophilus* or *P. furiosus* was studied at a high temperature for a longer time. They were pre-incubated at 80 ºC without substrates for up to 24 hours and assayed at 37 ºC. Further, in the third assay, the ATCases were assayed at 37 and 40 ºC and in the fourth assay, the ATCases were pre-incubated at 40 ºC without substrates for up to 36 days, and samples were assayed at 37 ºC as a function of time. To determine the optimal temperature, the reaction solutions containing 50 mM Tris-HCl, pH 8.5, 20 mM CP, 23 mM aspartate and a catalytic subunit of enzymes (1 unit purified enzyme ATCase) were separately pre-incubated at different temperatures 10, 25 and 37 ºC for 10 min and assayed at 10, 25 and 37 ºC for another 5 min and not above, as CP is unstable at elevated temperature.

2.3.12. Effects of pH

To determine the optimal pH, two buffers were used over a broad pH range, 50 mM Tris-HCl for pH range 6.0 to 8.8 and 50 mM bicarbonate for pH range 8.8 to 11. The reaction solution contained 1 unit of catalytic subunits of enzymes ATCases, 50 mM of either buffer, 20 mM

CP and 23 mM aspartate. The reaction mixture was pre-incubated at 37 ºC for 10 min and then initiated by adding CP and assayed for another 5 min.

2.4. Results and Discussion

Most studies on the ATCase have only focused on *E. coli*; therefore, our knowledge about ATCase from other organisms is either very limited or unavailable. Moreover, the ATCase from *E. coli* has its unique properties, and, therefore, it makes it hard to expand *E. coli* properties to other species. For example, ATCase from a human is a part of multienzymatic protein that has been attached to two enzymes from the pyrimidine pathway. Whereas for *E. coli*, the ATCase is a separated enzyme that interacts with other enzymes from the pyrimidine pathway. Therefore, it is important for a wide range of scientific and industrial processes to identify and characterise the ATCase from other organisms or microorganisms. For instance, the ATCase from hyperthermophiles has a critical role in protecting CP from decomposition at high temperatures. We have already shown in our published paper that ATCase from the hyperthermophile *T. barophilus* is able to couple with CKase *T. barophilus.* This combination of two enzymes not only sequestrates the pollutant ammonia (from wastewater) and carbon dioxide (from greenhouse gases) but also converts them to a value-added product (Hennessy et al., 2018). Moreover, we have also demonstrated in our published work that CKase from hyperthermophiles and one mesophile could be expressed in *E. coli* in high yield. In addition, the data from CKase characterisation indicated good pH, temperature stability and robustness (Hennessy et al., 2018).

Therefore, the aim of this chapter was the cloning, expression and characterisation of catalytic subunits (C3) of ATCase from hyperthermophilic Archaea P. furiosus, T. barophilus and T. sibiricus and their isozyme mesophile, E. faecalis.Moreover, to investigate ATCases stability in different pH and temperature range. If the ATCases exhibit the same stability as CKase (from previous study), ATCases and CKases could combine together to produce CA from ammonia and carbon dioxide (the aim of next chapter).

2.4.1. Sequence Analysis and Identification of ATCase

The catalytic subunits of ATCase (from the *pyrB* gene) sequences from three hyperthermophiles and one mesophile were investigated with regard to their amino acid sequence identity (Figure 2.10). The ATCase genus, *T. barophilusa* and *T. sibiricus* are hyperthermophilic Archaea from the *Thermococcus* family, while *P. furiosus* is from the *Pyrococcus* family. However, all three are from the Thermococcales order. The mesophile *E. faecalis* is from the Enterococcus family like *E. coli*. Therefore, it is expected to see more

identity between *T. barophilus* and *T. sibiricus*, followed by *T. barophilus* and *T. sibiricus* and *P. furiosus*. The results from the amino acid alignment from *T. barophilus* and *T. sibiricus* presented 75 per cent amino acid identity between *T. barophilus* and *T. sibiricus.* Also, the identity between ATCase *P. furiosus* and *T. barophilus* was 90 per cent, and ATCase *P. furiosus* and *T. sibiricus* exhibited 74 per cent amino acid sequence identity (Table 2.1). Surprisingly, the identity between *T. barophilus* and *P. furiosus* was the highest identity among hyperthermophiles, demonstrating more structural similarity between *T. barophilus* and *P. furiosus* compared to *T. barophilus* and *T. sibiricus.*

Concerning the sequence identity that was investigated above, some studies have shown that proteins with more than 50 per cent sequence identity have very similar backbones and only differ by less than 1 Å root mean square (average distance between backbone atoms of superimposed proteins) deviation (Doolittle, 1986 Gerstein, 1998). The sequence identity for hyperthermophiles was higher than 50% indicating a similar backbone among them.

Conversely, the sequence identity study of ATCase from mesophiles and hyperthermophiles demonstrated that the ATCase from one mesophile *E. faecalis* shared between 36 to 38 per cent amino acid sequence identity with other hyperthermophiles. it is not likely that their structure is similar (Koehl & Levitt, 2002). Further, all three hyperthermophiles and one mesophile showed the same conserved sequence, indicating the importance of the conserved amino acid sequence for ATCase activity. However, the conserved amino acids for the active site were the same in all ATCases except for ATCase *E. faecalis*, in which amino acid number 265 was alanine instead of leucine for the active site. An alignment of the amino acid sequences of the four ATCase investigations is presented in Table 2.1 and Figure 2.10.

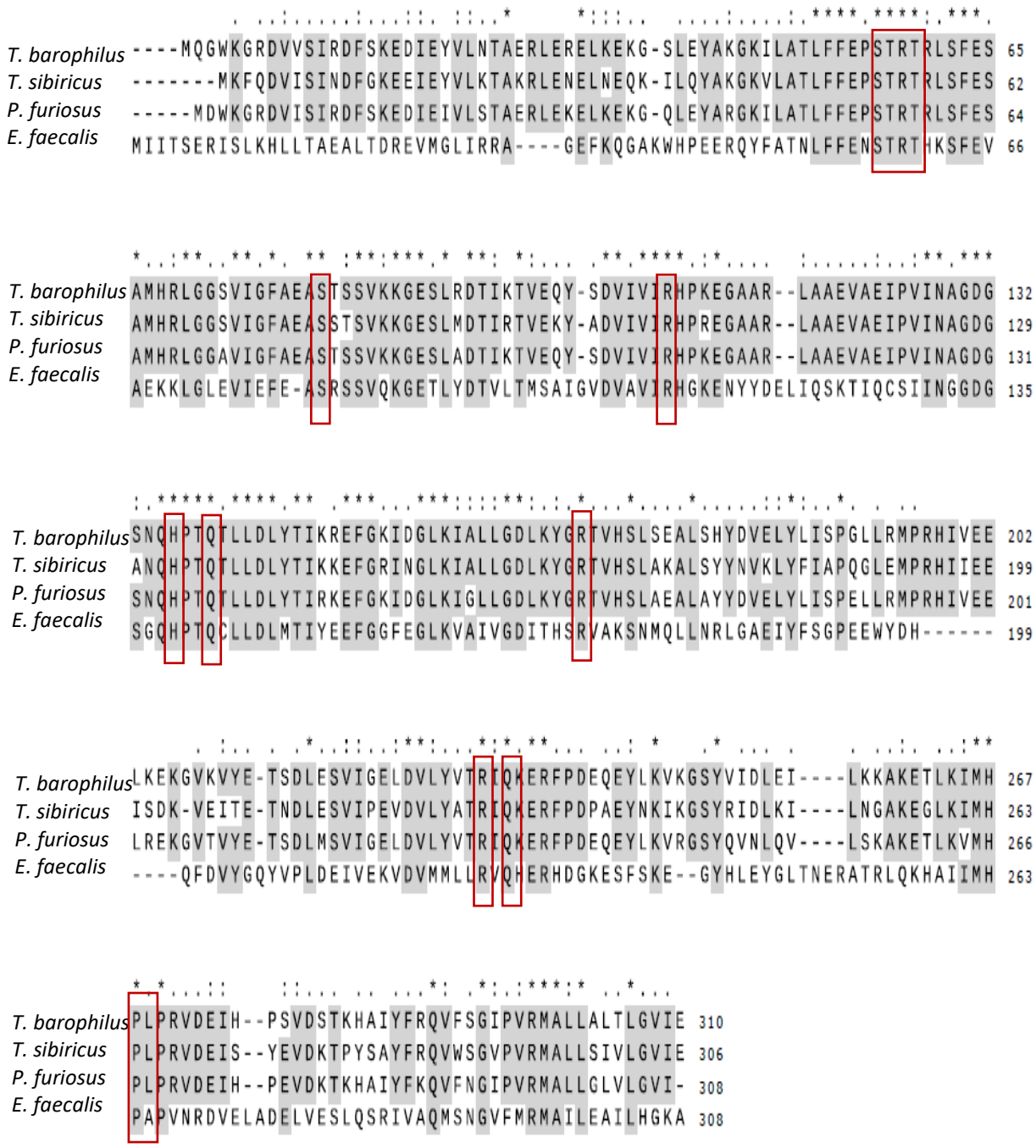


Figure 2.10: Clustal Omega alignment of catalytic subunits sequences of ATCase from *T. sibiricus*, *T. barophilus* and *P. furiosus* with the mesophile *E. faecalis.* The highlight shows the identity in different organisms. The red box shows the conserved amino acids in the active site.

Table 2.1: Comparison of Catalytic Subunits Sequences of ATCase from *T. sibiricus*, *T. barophilus* and *P. furiosus* with *E. faecalis*

Microorganism	Amino acid number	*T. sibiricus*	*P. furiosus*	*E. faecalis*
T. barophilus	310	77%	90%	38%
T. sibiricus	306	-	75%	37%
P. furiosus	308	-	-	36%
E. faecalis	308	-	-	-

2.4.2. Cloning, Expression and Purification of ATCase

The cloning, expression, and purification of ATCase were investigated. This study focuses on the catalytic subunits and catalytic activity of ATCase from the hyperthermophiles and the mesophile. No regulatory subunit was used in this study since regulatory subunits are solely responsible for allosteric effects and have no role in catalytic activity (the regulatory subunits increase or decrease the activity rate). Therefore, the regulatory subunits were not used for this project as it unnecessarily makes the project more complicated. Having catalytic subunits as a functional enzyme for industrial biocatalyst applications allows us to have an active enzyme and a less complex system for our investigation.

Accordingly, the ATCase genes for catalytic chains of the mentioned ATCases enzymes were synthesised based on the sequences available on the UniProt database and cloned into pETMCSШ vectors under the control of the T7 promoter, encoding a hexahistidine tag at the N-terminus of the protein.

The constructs were then transformed into *E. coli* BL21 (DE3) cells for expression, and the cells were cultured overnight in LB medium. The results from SDS-PAGE demonstrated that good overexpression of the catalytic subunits of enzymes from *T. sibiricus*, *T. barophilus, P. furiosus* and *E. faecalis* was achieved (Figure 2.11). This result agrees with the previous studies in which archaeal genes could be expressed in *E. coli* under the control of powerful promoters, such as plac and T7 RNA polymerase (Dong, Vieille, Savchenko, & Zeikus, 1997; Dong, Vieille, & Zeikus, 1997; Uemori, Ishino, Toh, Asada, & Kato, 1993). After cell lysis, the enzymes from *T. barophilus*, *P. furiosus* and *E. faecalis* were mostly soluble, with small amounts of the overexpressed protein precipitating in the insoluble fraction. In

contrast, the ATCase from *T. sibiricus* was found in equal concentrations in insoluble and soluble fractions. To obtain more soluble fractions from *T. sibiricus*, the cells were grown at different temperatures (27, 30 and 37 °C) for either 6 hours or overnight in the absence of IPTG and in addition to IPTG (0.1 and 1 mM). However, the results were similar, with equal amounts of protein in the insoluble fraction. However, there was enough protein in a soluble fraction for purification. The soluble fractions were purified through Ni (II) ion affinity chromatography to give pure enzymes.

The results from purification demonstrated that the expression yield for all ATCases was very high, even for the less soluble enzyme *T. sibiricus* (about 4 mg enzyme yield from only 100 mL culture), and there was enough enzyme for our investigation. Therefore, there was no need for optimisation.

The determinations of the approximate molecular mass and purity of the recombinant proteins were done by 20% SDS-polyacrylamide gel electrophoresis. The results exhibited a molecular mass of about 37 kDa, including 3 kDa His-tag segment, appended for catalytic subunits. Also, the SDS-PAGE showed a high purity from ATCase after purification. All four species' catalytic subunits resemble the catalytic chains of ATCase from other organisms in size and sequence (Figure 2.11).

Further, the pure enzyme concentration was measured by spectrophotometry at 280 nm and calculated in mg from 100 mL cell culture, as shown in Table 2.2. Finally, the ATCase catalytic trimer must be confirmed by their activity since three catalytic subunits must be associated with each other to create an active site for ATCase catalysis activity.

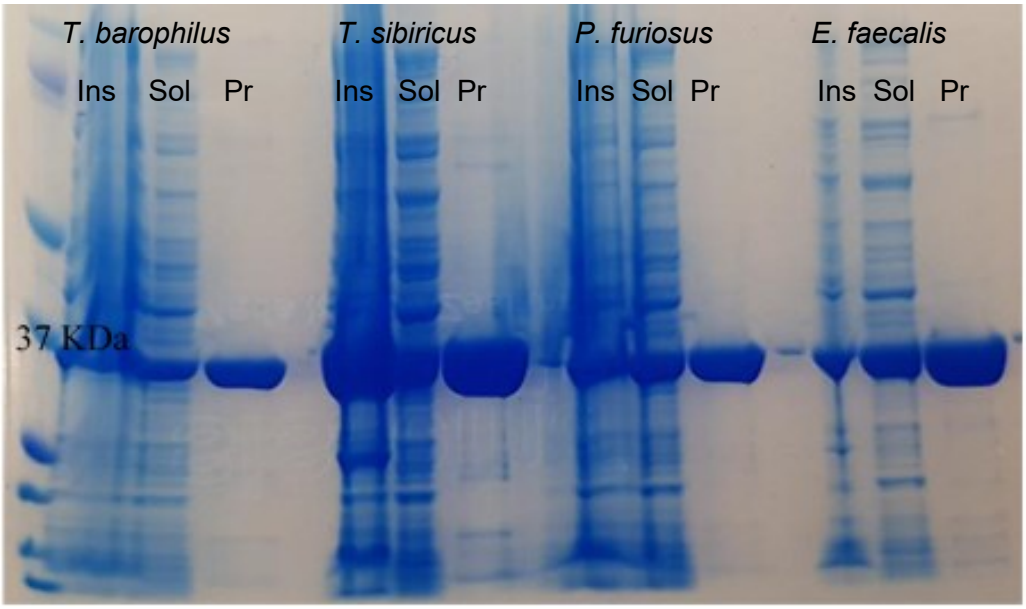


Figure 2.11: SDS-PAGE analysis of the expression of catalytic subunits of ATCase from *T. sibiricus*, *T. barophilus* and *P. furiosus*. *E. faecalis*. Ins = Insoluble fraction; Sol = soluble fraction; Pr = pure protein after Ni-column. *E. faecalis* pure protein has been diluted two times to run by SDS.

Table 2.2: Mass of Enzyme Isolated from 100 mL of Cell Culture, Measured by Spectrophotometry at 280 nm.

Organism	Yield (mg/100 mL culture)	Extinction coefficient
T. barophilus	2.97	23380
T. sibiricus	3.59	26360
P. furiosus	1.84	23380
E. faecalis	6.90	27390

2.4.3. Determination of ATCase Catalytic Subunit and Activity

In this section, the formation of ATCase catalytic trimer was investigated as ATCase catalytic subunits must form trimer to be active. The ATCase catalytic site is positioned at the interface between two neighbouring catalytic chains in the same trimer. The catalytic amino acid chains are incorporated into the catalytic site and activity. The catalytic trimer of three

catalytic subunits comprises three 34 kDa subunits. Therefore, the total molecular mass of the three catalytic subunits is 102 kDa (or 111 kDa, including 3 kDa His tag for every subunit). The results from size exclusion chromatography confirmed the formation of trimer catalytic structure with molecular mass of 111 kDa (Figure 2.12).

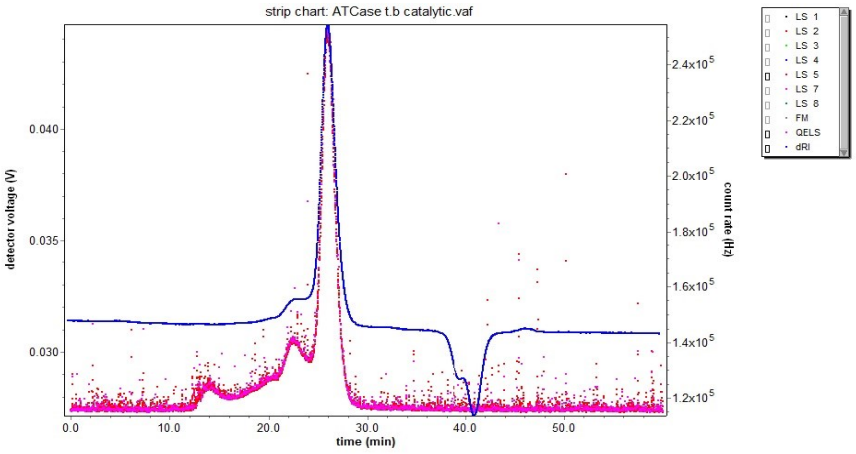
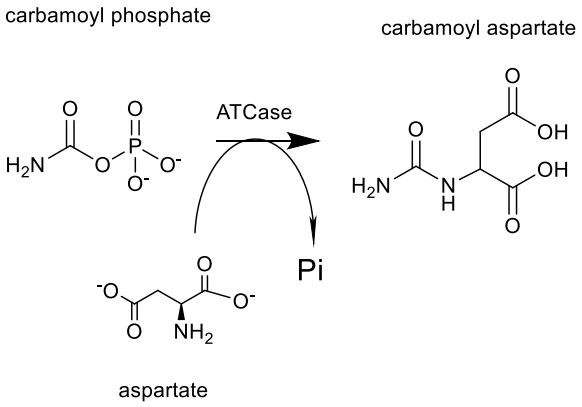


Figure 2.12: Size exclusion chromatography confirming molecular mass of the ATCase catalytic trimer (111 kDa consisting of three subunits of 34 kDa with 3 kDa His-tag).

Another approach to confirm the formation of the catalytic trimer is to examine the ATCase activity, as it would only be active if ATCase trimer catalytic subunits associate with each other to create an active site for catalysis (Allewell, 1989).

2.4.4. Carbamoyl Aspartate Detection by HPLC and HPLC/MS

In this section, the detection of ATCase activity was investigated by using HPLC or HPLC/MS. To measure ATCase activity, aspartate consumption or CA production should be measured. Therefore, in this section, the measurement of CA production was investigated as an indication of ATCase activity (Scheme 2.3). The aspartate concentration can be measured by AccQ tagging technique; however, there is no HPLC technique to measure CA using AccQ.



Scheme 2.3: Aspartate transcarbamoylase (ATCase) reaction. ATCase catalyses the transfer of the carbamoyl moiety from CP to the α-amino group of L-aspartate to yield CA and orthophosphate.

Therefore, to detect CA by HPLC, N-chloromethylphthalimide was used as the derivatisation reagent suitable for detecting dicarboxylic acids and fatty acids (Lindner & Santi, 1979). The chlorine in the N-methyl group is highly reactive and reacts with alkali metal or ammonium salts CH-, OH- and NH- acid compounds quantitatively (Figure 2.13). By adding trimethylamine to the reaction mixture, the salt of the free acid can be formed. Carboxylic acids are good examples for detection by this reagent since carboxyl groups can be derivatised to easily detectable ester. Moreover, the polarity of acids declines, which gives better chromatographic properties. Therefore, in this study, succinic acid, fumaric acid and capric acid (Figure 2.14) were chosen to be detected by HPLC to ensure that derivatisation occurs, as demonstrated in the reference paper (Lindner & Santi, 1979). Then the detection of CA was performed. To conduct the detection, each analyte was dissolved in either acetonitrile or water, except CA, which was dissolved in 100 mM NaOH. N-chloromethylphthalimide and triethylamine (which had already been dissolved in acetonitrile) were then added to the solution. Finally, the solutions were heated to 60 ºC for 1 hour and analysed by HPLC. The results demonstrated that only capric acid could be detected and measured by HPLC and CA, fumaric acid and succinic acid could not be quantified by HPLC using this method.

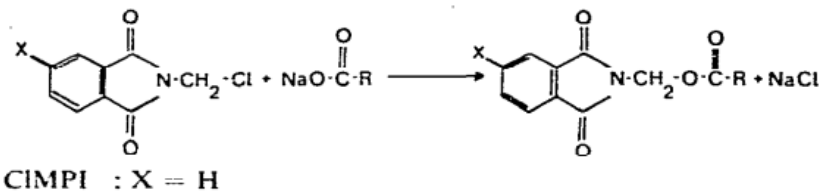


Figure 2.13: N-chloromethylphthalimide reaction with alkali metal salts.

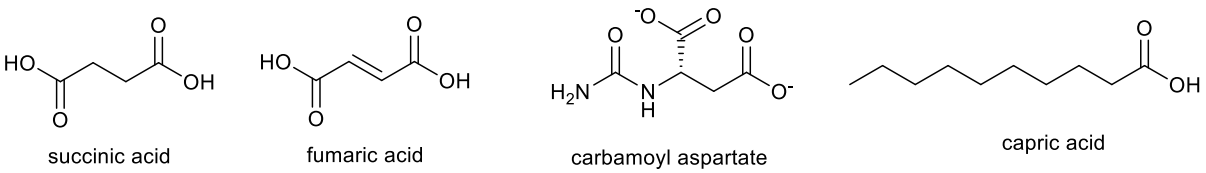


Figure 2.14: Carbamoyl aspartate, succinic acid and fumaric structure.

Being unable to detect CA with HPLC, the detection of CA was pursued by using HPLC/MS as described in Materials and Methods. The results revealed that CA could be detected and quantified by HPLC/MS, and a standard curve for CA could be drawn. (Figure 2.15).

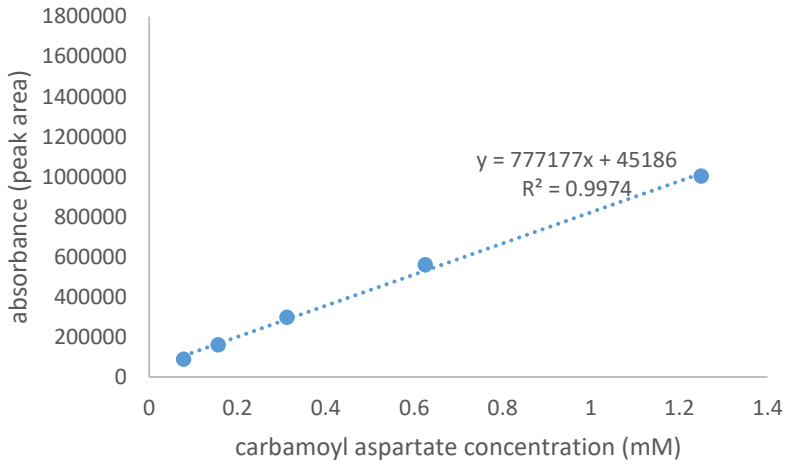


Figure 2.15: Carbamoyl aspartate standard curve.

2.4.5. Determination of ATCase Catalytic Activity

From the previous section, it was confirmed that CA could be detected by LC/MS. Therefore, in this section, the activity of ATCase was investigated using LC/MS. The activity of ATCase depends on the formation of the ATCase catalytic trimer. Therefore, the formation of CA corresponds to the activity of ATCase and the formation of ATCase catalytic trimer. The activities of ATCases from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* were determined by measuring CA production.

The results from the measurement of CA production by LC/MS as an indication of ATCase activity showed that all ATCase from different microorganisms are active and produce CA. Therefore, CA production demonstrated that ATCases have formed the trimeric structure in proper folding, resulting in active enzymes. It also revealed that the mesophilic ATCase *E.*

faecalis represented the highest CA production yield, followed by *T. sibiricus, T. barophilus* and *P. furiosus* (Figure 2.16). It is important to mention that all assays have been conducted at 37 °C, which is the optimal temperature for the mesophile *E. faecalis.* Since the temperature is not hyperthermophile optimal temperature, such production yield is likely seen for hyperthermophilic and mesophilic ATCases.

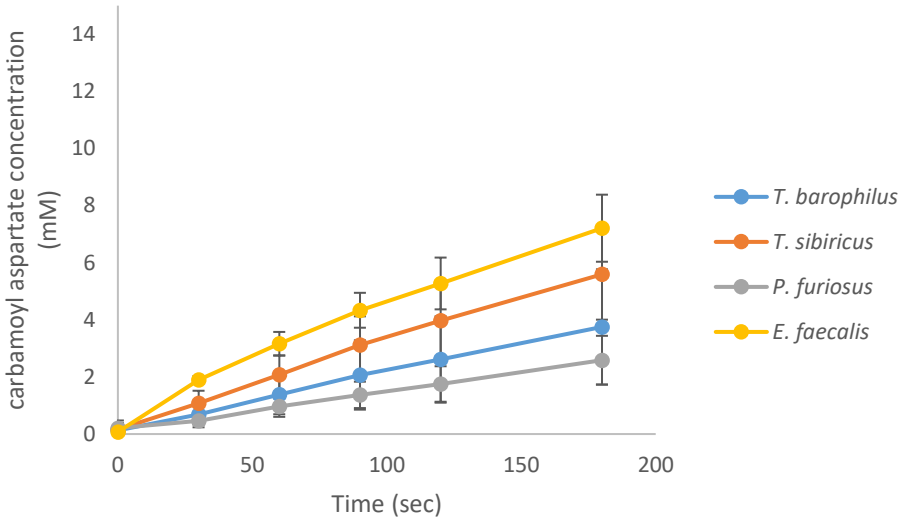
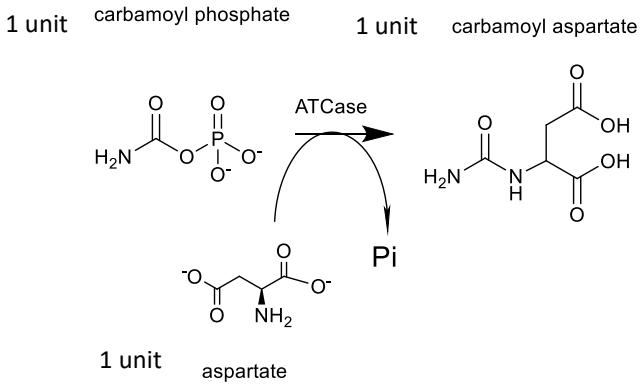


Figure 2.16: ATCase activity by measuring the production of CA by HPLC/MS. They assayed at 37 ºC in 50 mM Tris-HCl pH 8.5, 23 mM aspartate and 20 mM CP using 0.1 µM *T. barophilus, T. sibiricus, E. faecalis* and 1 µM *P. furiosus.* Values are the average of two replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

To confirm the production of CA is correlated with aspartate consumption (Scheme 2.4), another assay was run for hyperthermophile microorganisms as an indication for all organisms. Aspartate consumption was measured by HPLC, and CA production was measured by HPLC/MS. The measurements showed that CA production corresponded to aspartate consumption. The results indicated that aspartate consumption is proportionally related to CA production and has nearly 100% efficiency (Figure 2.17).



Scheme 2.4: ATCase reaction. 1 unit CP and 1 unit Asp produce 1 unit CA.

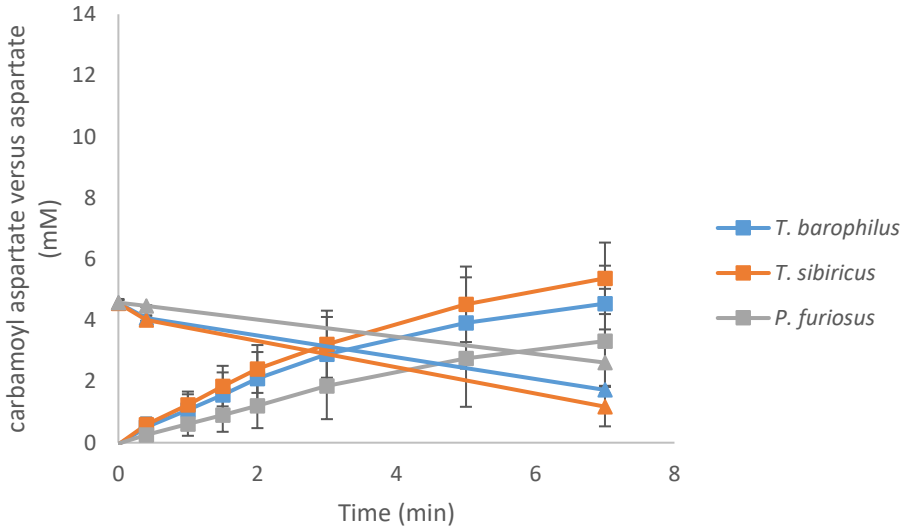


Figure 2.17: ATCases activity by measuring the consumption of aspartate versus the production of CA by HPLC and HPLC-MS, respectively. They assayed at 37 ºC in 50 mM Tris-HCl pH 8.5, 5 mM aspartate and 20 mM CP. The triangle represents aspartate consumption, and the square represents CA production. Values are the average of two replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.**Effects of Substrate Concentration**

In this section, the kinetic properties of ATCase were investigated. To optimise enzyme performance concerning the determination of the true substrates for the enzyme and the affinity of the enzyme for its substrate, it is important to measure kinetic properties such as K_m and V_{max} for the enzyme. V_{max} is the maximal velocity in which the maximal activity from the saturation curve by aspartate consumption and CA production is observed, and K_m is the concentration of substrate at half of V_{max}. A variety of K_m has been reported from ATCase from different microorganisms such as 4, 7.9, 8.4, 12.4 and 44.7 mM for aspartate and

0.028 mM for CP from *E. Coli,* depending on different conditions, and 0.11 and 7 mM for CP and aspartate, respectively from *Bacillus subtilis*. Therefore, saturation experiments for aspartate and CP for ATCases were performed in 50 mM Tris-HCl pH 8.5 at 37 ºC. The temperature was kept at 37 ºC due to the high thermolability of CP with a half-life of ~10 min at 37 ºC, as suggested by other published works (Q. Wang et al., 2008). CP concentration was kept at 40 mM to determine the aspartate saturation curve, which is much higher than its K_m (in other organisms) due to its lability. Saturation experiments for CP were measured in the presence of 25–40 mM aspartate.

Further, the previous studies have shown that ATCase binds to substrates in ordering behaviour, ATCase firstly binds to CP (EA), and then ATCase CP complex binds to aspartate (EAB). Then the complex (EAB-EPQ) first releases the phosphate (P), followed by CA (Q) (Scheme 2.5) (Wedler 1974).

Scheme 2.5: Schematic view of ordered bi-substrate binding to the enzyme. E = enzyme; EA = enzyme binding to the first substrate; B = the second substrate; EAB-EPQ = the enzyme complex with both substrates and enzyme complex with both products; P = the first product; EQ = enzyme complex with the second product; Q = the second product.

Therefore, the reaction rate equation is more complex for the ATCase bi-substrate. To analyse the reaction, one substrate should be kept constant while varying the second substrate concentration, so the enzyme behaves like a uni-substrate enzyme. The ordered bi-substrate reaction rate equation is as follows:

Ordered bi-substrate reaction rate equation.

$$\left(\frac{1}{V}\right) = \frac{1}{Vmax} + \frac{Km^A}{(Vmax[A])} + \frac{Km^B}{(Vmax[B])} + \frac{(Km^A Km^B)}{(Vmax[A][B])}$$

Where Km^A and Km^B are the [A] and [B] required for half maximal activity.

The results from kinetic studies showed that the saturation curve for catalytic subunits of all four species exhibited the hyperbolic curve for aspartate and CP following the Michaelis-Menten model (Figure 2.18)). These findings are contrary to *E. coli* aspartate saturation curve, that is, the sigmoidal curve (with and without regulatory subunit) (Porter, Modebe, & Stark, 1969). The reason for the sigmoidal curve in *E. coli* is the binding of aspartate to one active site that changes the entire enzyme to an R state. This results in an increase in the activity of the other active sites. This change in the active site is called cooperativity (Porter et al., 1969) (Allewell, 1989; W. N. Lipscomb, 1994; William N. Lipscomb & Kantrowitz, 2012; Stebbins et al., 1989).

The study of kinetic properties of ATCase demonstrated that in contrast to earlier findings from *E. coli*, no evidence of cooperativity was detected in catalytic trimer of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis.* The ATCase catalytic trimer saturation curve for both aspartate and CP fitting curves was found hyperbolic and not sigmoidal; therefore, the catalytic trimer of ATCase from hyperthermophiles and the mesophile has no homotropic or heterotropic interaction towards CP and aspartate in the absence of regulatory subunits. These results are consistent with the results obtained from catalytic subunits of hyperthermophilic ATCase *S. acidocaldarius* and *P. abyssi* (V. Durbecq et al., 1999; Purcarea et al., 1994). Hence, the catalytic subunits of hyperthermophilic ATCase show no cooperativity behaviour. Also, the results from ATCase *E. faecalis* showed different behaviour compared to *E. coli* (although both are mesophiles). It might be due to having a different amino acid sequence compared to *E. coli.*

The highest catalytic rate was observed in the hyperthermophile *T. sibiricus,* while the lowest catalytic rate was observed in the hyperthermophile *P. furiosus* (Table 2.3).

Overall*, T. sibiricus* from hyperthermophiles and *E. faecalis* from mesophiles have the highest catalytic activity (Table 2.3). Therefore, they might be good candidates for further usage in my projects as *T. sibiricus* represents the hyperthermophile and *E. faecalis* represents the mesophile. Therefore, we have one representative from each family unless the stability studies show different results, and hence, we have to change our candidate ATCase for the next chapter.

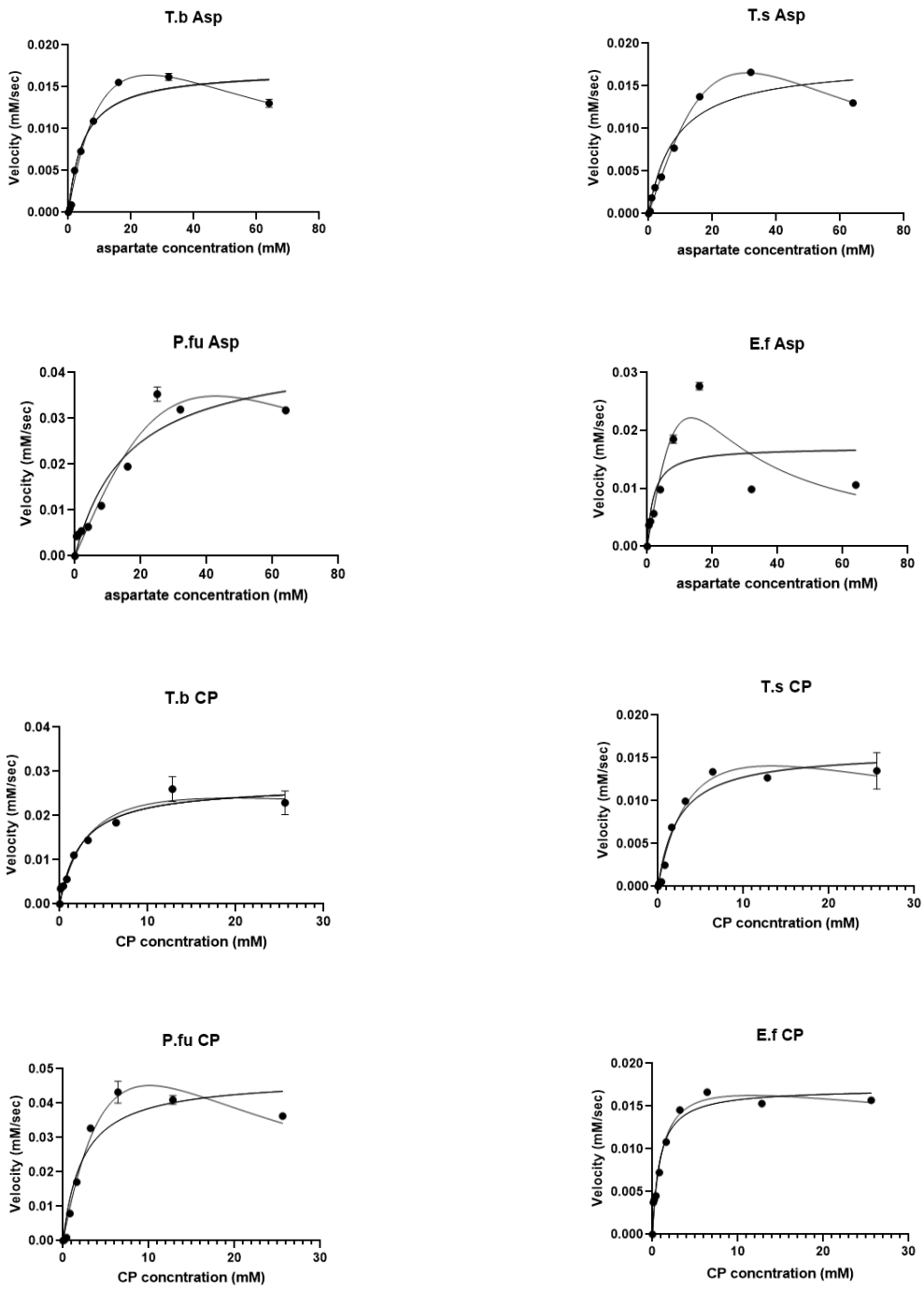


Figure 2.18: Aspartate and Carbamoyl phosphate hyperbolic Saturation Curve (top 4) and Carbamoyl Phosphate (bottom 4) of *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* ATCase Catalytic Subunits.

Table 2.3: Apparent and Approximate Kinetic Parameters for Aspartate Saturation Curve (top) and Carbamoyl Phosphate (bottom) of *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* ATCase Catalytic Subunits

Organism	Aspartate		
	Apparent K_m (mM)	Approximate V_{max} (mM/sec)	Approximate K_{cat}/K_m (1/sec. mM)
T. barophilus	4.85±0.086	0.0170±0.0004	8.79±0.930
T. sibiricus	8.51±0.250	0.0178±0.0001	21.40±1.537
P. furiosus	16.33±0.462	0.045±0.0008	1.77±0.537
E. faecalis	1.95±0.035	0.0171±0.0001	32.9±0.937

Organism	Carbamoyl phosphate		
	Apparent K_m (mM)	Approximate V_{max} (mM/sec)	Approximate K_{cat}/K_m (1/sec.mM)
T. barophilus	2.438±0.0362	0.0270±0.0011	27.60±0.957
T. sibiricus	2.378±0.566	0.0150±0.0002	49.38±1.953
P. furiosus	2.306±0.086	0.0473±0.0009	12.44±0.674
E. faecalis	0.843±0.062	0.0170±0.0002	61.08±0.882

Note. Michaelis-Menten model for calculating apparent K_m for aspartate in ATCase *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* using GraphPad Prism. Approximate V_{max} is maximal velocity, the maximal observed activity from saturation curve by aspartate consumption and CA production. Apparent K_m is the concentration of substrate at half of V_{max}. Approximate K_{cat} is the rate of enzyme converting the substrate to product per second. The assays were done in 50 mM Tris-HCl pH 8.5, 40–60 mM CP and 23 to 40 mM aspartate for the aspartate and CP saturation curves, respectively. The results are the average of duplicate experiments.

2.4.7. Promiscuity Towards Acetyl Phosphate

Enzyme promiscuity is the capability of an enzyme to either catalyse a side reaction in addition to its main reaction or utilise a substrate other than its main substrate (O'Brien & Herschlag, 1999a). Promiscuity has great importance in biotechnology (Ferrer et al., 2016; Nobeli, Favia, & Thornton, 2009), evolution (R. Huang et al., 2012; Price & Wilson, 2014; Yip & Matsumura, 2013) and the environment (i.e., the enzyme from plants and their effects on plants and, therefore, the environment) (H. Huang et al., 2015). For example, it can increase the usage of biocatalysts in biotechnology. However, conversely, promiscuity can also be a drawback for an enzyme as it decreases the main product yield by producing by-products through its promiscuity towards other substrates.

Some scientists consider the promiscuity of an enzyme the initial point for the divergence of enzyme evolution. New protein functions can be exploited from existing structural scaffolds during the evolution of enzyme promiscuity (James & Tawfik, 2003; O'Loughlin et al., 2006). In the event of a single point mutation, the substrate- specific enzymes may show a promiscuous activity towards an unnatural substrate, thereby making the enzyme more efficient or even enabling the organism to survive. After gene duplication, the promiscuous enzyme can be subjected to more mutations to optimize or gain new function (O'Brien (Aharoni et al., 2005; Olga Khersonsky et al., 2006; O'Boyle et al., 2007; O'Brien & Herschlag, 1999a) The new promiscuous activity of an intermediate evolution enzyme can evolve sufficient “native” function to give the organism a survival advantage. This may lead to the production and optimization of a new “specific” enzyme after gene duplication and mutation (Khersonsky, (Olga Khersonsky et al., 2006; O. Khersonsky & Tawfik, 2010).

In this section, we aimed to study the ATCase promiscuity towards acetyl phosphate. It is one of the important chemicals in the next chapters and has a similar size and structure to CP having acetyl instead of carbamoyl in CP. Therefore, ATCase might be able to use acetyl phosphate instead of CP as a substrate to produce acetyl aspartate. Hence, the focus of this section is to study the affinity of ATCase towards AP as a substitute for CP.

The results demonstrated that all ATCases, particularly hyperthermophiles, can utilise AP as a substrate in addition to CP to yield acetyl aspartate. However, the affinity of ATCase towards CP is much higher than AP. Interestingly, hyperthermophiles have higher promiscuity towards AP compared with the mesophile., hyperthermophiles enzymes convert AP to acetyl phosphate at higher rate compared to mesophile based on the higher turnover number (K_{cat}) (data not shown) and K_{cat}/K_m values (Table 2.4). It might be due to one amino acid difference in the conserves active site in the mesophile *E. faecalis* compared to

hyperthermophiles. The *E. faecalis* amino acid number 265 in the active site is alanine instead of leucine for the hyperthermophile. Moreover, the promiscuity may result from the evolutionary pathway in which the more developed organisms (bacteria and Eukaryotes) show more specificity towards one substrate or reaction (R. Huang et al., 2012; Price & Wilson, 2014; Yip & Matsumura, 2013).Therefore, hyperthermophiles *T. barophilus*, *T. sibiricus* and *P. furiosus* showed more promiscuity towards AP compared to mesophiles. Since, hyperthermophiles belongs to a group of Archaea which are the oldest organisms on the earth and the source of promiscuity. Archaea enzymes have many disordered proteins that can be seen in “hub proteins” in the metabolic regulation of many organisms. Therefore, promiscuity is one of the evolutionary tools for enzymes to assume specialised catalytic roles (Arora, Mukherjee et al. 2014). It is probably because hyperthermophiles are not specialised in using one substrate due to the environmental condition at the early stages of life on Earth. Due to the restricted availability of chemicals in the early stage of life on Earth, hyperthermophiles adapted to use other substrates rather than their specific substrate to survive in harsh conditions. While the mesophile is more developed, organisms have been more specialised in using their specific substrate to produce high-yield products rather than by-products. Although these findings rather restrict my project goal to gain a high-yield main product, CA, they may be useful for other industrial applications such as peptide synthesis using ATCase promiscuity.

Consequently, to be able to use ATCase in the reaction mixture containing AP, the AP concentration should be kept lower than each ATCase K_m for AP to minimise the by-product.

Table 2.4: Kinetic parameters of *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* ATCase

Organism	Acetyl phosphate		
	Km (mM)	Vmax (mM/sec)	Approximate K_{cat}/K_m (1/sec.mM)
T. barophilus	5.89±0.246	0.011±0.0017	5.12 ±0.601
T. sibiricus	9.36±0.621	0.0052±0.0001	2.14±0.629
P. furiosus	8.24±0.362	0.089±0.0081	1.82±0.415
E. faecalis	5.69±0.553	0.0025±0.0008	0.84±0.199

Note. Michaelis-Menten model for calculating Km for acetyl phosphate in ATCase *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* using GraphPad Prism. Vmax is the maximal velocity, being the maximal observed activity from the saturation curve by aspartate consumption and CA production. Km is a concentration of substrate at half of Vmax. Kcat is the rate of enzyme converting the substrate to a product per second. The assays were done in 50 mM Tris-HCl pH 8.5, 23–40 mM aspartate and different AP amounts. The results are the average of duplicate experiments, and error bars corresponding to standard deviations of duplicate experiments. Values are the average of duplicate replicates of each enzyme.

2.4.8. Effect of Temperature

In this section, the thermostability of ATCase was investigated. One of the most important factors to be considered in the expression of hyperthermophiles Aarchaea in *E. coli* is that recombinant enzymes could maintain all their native enzymatic and biochemical properties, such as proper folding, thermostability and optimal activity at high temperature (Andra, Frey, Jaenicke, & Stetter, 1998; Arnone et al., 1997; Auerbach et al., 1997).

As discussed in the previous section, *T. sibiricus* from hyperthermophiles and *E. faecalis* from mesophiles have the highest catalytic activity, making them suitable candidates for their usage in the next chapter (combination of CKase and ATCase). However, their stability should be examined before deciding to choose the ideal ATCase candidate, especially from hyperthermophiles (as we only have one mesophile for our study).

Accordingly, one of the factors that demonstrate enzyme stability is the thermostability of the enzyme. The more stable enzyme presents higher thermostability. Therefore, to examine the stability of ATCase from hyperthermophiles and the mesophile, their thermostability was

investigated. In addition, further research should be undertaken to investigate the optimised condition (pH or temperature) for the catalytic activity of ATCase.

Conversely, concerning wastewater treatment, thermostability is one of the major problems. As temperature sometimes varies in wastewater treatment, it affects microorganism activity to decline or lose activity. For example, microorganism in the bioreactor in the wastewater treatment system can lose their activity if the temperature exceeds 38 °C (Grady, Daigger, & Lim, 1999). In addition, it has been shown that the effect of temperature in wastewater treatment is crucial for solubility of oxygen, nitrification and the rate of biological activity in aerobic systems (Howland, 1953; Ksentini, Hmidi, Hajlaoui, & Mansour, 2015). However, debates continue about the best temperature for the wastewater treatment system.For example, it has been shown that elevating the temperature helped the removal of suspended solids and COD in wastewater treatment. Thus, depending on different wastewater treatment methods, the temperature can vary in the system. The temperature of raw sewage varies significantly depending on external environmental factors, from 10 ºC to 40 ºC (Gerardi, Arthur, Water Environment, & Task Force on Wastewater Biology: the Life, 1994). Consequently, an implication of these studies raises the possibility of ATCase usage in wastewater treatment. Therefore, an enzyme with high thermostability is optimal for guaranteed reliable enzyme activity in wastewater as the more stable enzyme shows higher thermostability. Furthermore, thermostability in microorganism and enzymes have attracted a variety of industries other than wastewater treatment plant. The enzymes enhanced stability makes them suitable for usage in harsh conditions such as their application in industries that require enzyme processing in high chemical materials. Therefore, different temperatures have been measured in this study to investigate the stability of the enzymes for the purpose of wastewater treatment or other industry purposes.

To study the stability of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis*, the catalytic subunits were pre-incubated at various temperatures for 10 min, cooled on ice and assayed at 37 °C. The hyperthermophilic ATCase from *T. barophilus* and *P. furiosus* were found stable up to 100 °C. While *T. sibiricus* was very stable up to 60 °C, it showed a significant decline in activity at 80 °C and was almost inactive at 100 °C (Figure 2.19). This may be because *T. sibiricus* exists at a lower temperature (60–84 °C) in nature (Mardanov et al., 2009; Miroshnichenko et al., 2001) in comparison with *P. furiosus* and *T. barophilus*, which live at 85 to 100 ºC (Fiala & Stetter, 1986; Marteinsson et al., 1999).

Notwithstanding, the activity of all ATCase hyperthermophiles declined as temperature increased. It is important to mention that the thermostability could not be associated with CP

decomposition in these assays as the enzymes pre-incubated at different temperatures without the substrates. They all assayed at 37 °C, in which the substrates (CP and aspartate) were added to the reaction mixture at high concentrations. Therefore, if the CP decomposes at 37 °C after a few minutes of assay, it will happen in all enzyme reaction solution and affects all enzyme activity for thermostability at the same level due to a shortage of CP. However, the results showed that thermostability is varied in ATCases at the range of temperatures, which shows that different temperatures have affected the enzymes and not the substrates or reaction mixture ingredients. The decrease in hyperthermophilic ATCases activity at elevated temperatures may be result from:

1. a lack of regulatory subunits to protect the enzyme and substrates from higher temperatures (V. Durbecq et al., 1999; Evans et al., 1987; Peterson, Zhou, Hsieh, Creager, & Schachman, 1994; Purcarea, 2001; Purcarea et al., 1994; Van Boxstael et al., 2003b; Van Boxstael et al., 2005).
2. a heterologous expression of hyperthermophilic ATCase in *E.coli* may result in lower thermostability (Purcarea et al., 1997).

In contrast to the three hyperthermophiles discussed above, mesophile *E. faecalis* was stable up to 40 °C and was almost completely inactive at 6 °C. In conclusion, these results demonstrated that the catalytic subunits of hyperthermophilic ATCase from *T. barophilus, T. sibiricus* and *P. furiosus* have higher thermostability when compared with mesophile *E. faecalis.*

Moreover, since *T. barophilus* and *P. furiosus* were active at higher temperatures, their thermostability was studied at 80 °C for up to 24 hours. Aliquots were taken at different time intervals, and their activity assayed at 37 °C. Under these conditions of up to one hour, *T. barophilus* and *P. furiosus* retained more than 84% and 73% of their activity, respectively. *T. barophilus* lost 95% of its activity at 24 hours while *P. furiosus* was very stable, having more than 54% activity (Figure 2.19, top left). These results were consistent with the study of ATCase from *P. abyssi* incubating at 90 ° for 6 hours (Purcarea, 2001; Purcarea, Simon, Prieur, & Hervé, 1996).

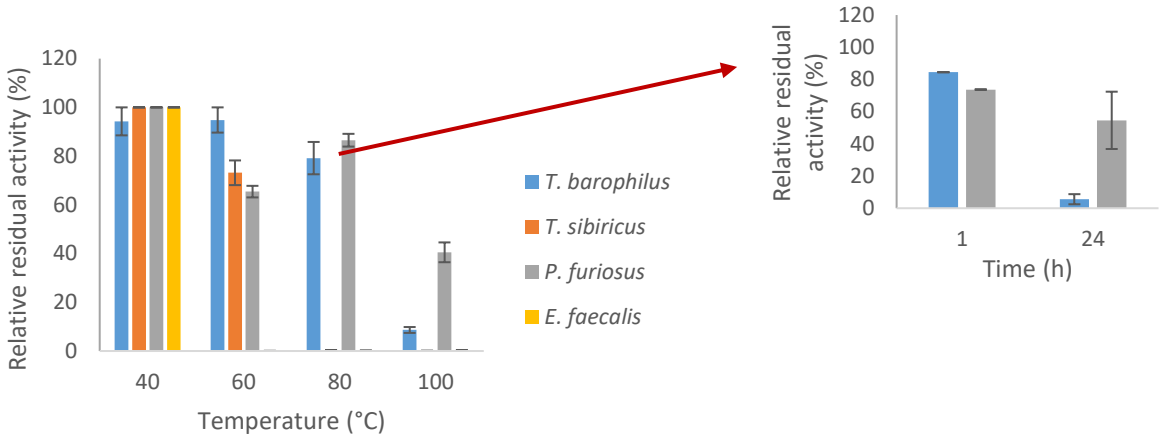


Figure 2.19: Thermostability study of ATCase from *T. barophilus*, *T. sibiricus*, *P. furiosus* and *E. faecalis* in different temperatures. The ATCases were pre-incubated for 10 min at 37 °C, 40 ºC, 60 ºC, 80 ºC and 100 ºC in 50 mM Tris-HCl buffer pH 8.5 and 23 mM aspartate. Top right: *T. barophilus* and *P. furiosus* at 80 °C for 24 hours. The assay was run at 37 °C and initiated by adding 20 mM CP. Values are the average of duplicate replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

So far, all experiments have been conducted at 37 °C, which is the standard temperature for the ATCase assay. However, the optimal temperature for CKase assays (in the next chapter) is 40 °C. Therefore, an experiment was run at 37 and 40 °C to examine the ATCase activity at 37 and 40 °C (pre-incubated at 37°C and assayed at 37°C and 40 °C). The results showed that ATCase activity for hyperthermophiles increased at 40 °C, and the reaction was slower at 37 °C probably due to the nature of enzymes and being hyperthermophiles. While mesophile ATCase activity slightly decreased at 40 °C. As explained in the previous section, the mesophile optimal temperature is much lower than the hyperthermophile optimal temperature. Therefore, by increasing the temperature, the mesophile activity slightly decreased (Figure 2.20).

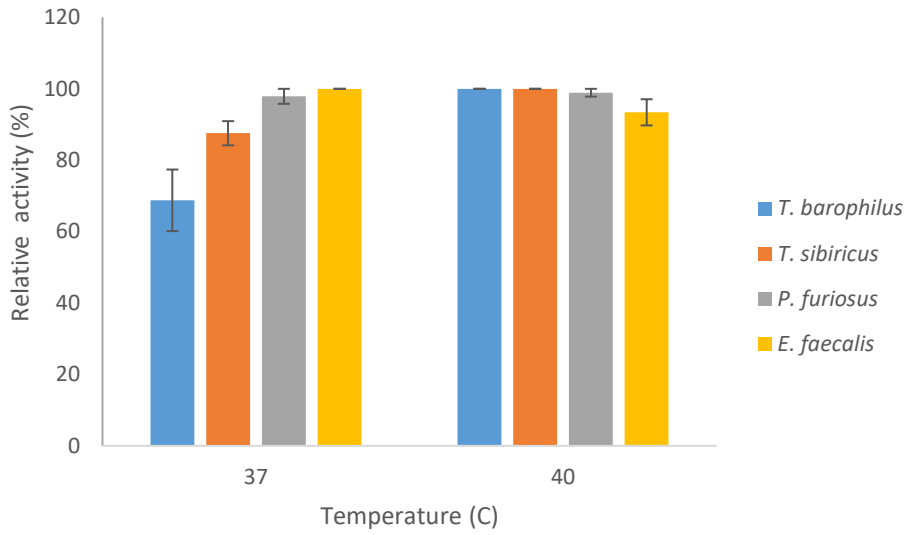


Figure 2.20: Thermostability study of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* at 37 and 40 ºC in 50 mM Tris-HCl buffer pH 8.5 and 23 mM aspartate. The assays were initiated by adding 20 mM CP. The enzyme unit adjusts to 1 unit (1 unit is the amount of enzyme that converts 1 mM of aspartate to CA in 1 min) at 37 °C for all enzymes. Values are the average of duplicate replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

Having already examined the ATCases at different temperatures, the following assay aims to explore the long-term thermostability of ATCases. As discussed on the effect of temperature on ATCase stability, hyperthermophiles exhibited higher stability at higher temperatures compared to mesophiles. Therefore, the purpose of this assay is to explore the ATCase stability at a certain temperature (40 ºC, in this case) in long-term incubation.

Hence, ATCases from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* were examined for thermostability at 40 °C (the reason for choosing 40 °C for thermostability experiments is 40 °C being the CKase optimal temperature and ideal temperature for coupling CKase and ATCase). The ATCases were incubated at 40 ˚C without substrates for 36 days, and samples were withdrawn at the due time point and assayed at 37 ˚C. The results demonstrated a slow exponential inactivation in ATCases from *T. barophilus, T. sibiricus* and *P. furiosus* from Day 0 to 36. At Day 36, *T. barophilus, T. sibiricus and P. furiosus* maintained 12%, 22%, and 40% of their activity, respectively. Whereas *E. faecalis* gradually lost its activity and only maintained around 5% of its activity at Day 10 and was completely inactive at Day 21. Hence, the hyperthermophiles were found to be more stable in long-term thermostability assay than mesophiles (Figure 2.21).

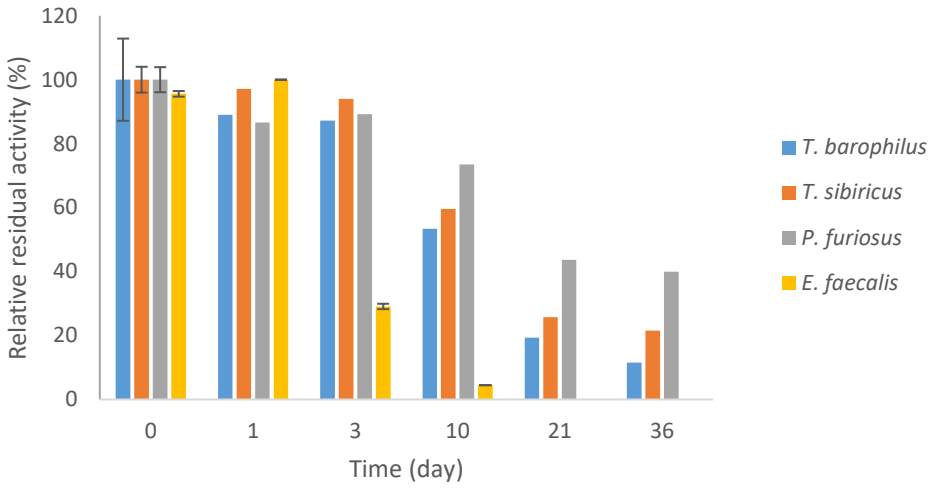


Figure 2.21: Thermostability study of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* at 40 ºC. The ATCase were pre-incubated at 40 ºC for several days in 50 mM Tris-HCl buffer pH 8.5. The assay was run at 37 ºC and initiated by adding 23 mM aspartate and 20 mM CP.

Finally, the enzyme stability was evaluated at different temperatures while assaying.

This assay analyses the impact of the correlation between initial velocity (reaction rate) and temperature. The results of this study indicated that the initial velocity of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* were increased in elevating the temperature up to 37 ºC. Surprisingly, ATCases from hyperthermophiles were even active at low temperatures, demonstrating a broad range of activity even at the mesophile range and lower (10 ºC-46.1 ºC) (Figure 2.22). Combining these results with previous results on the effect of temperature on ATCase, we can conclude that all hyperthermophile ATCase activity increased to 40 ºC, and then gradually activity started to decrease (there is an urgent need to address that hyperthermophiles contained only catalytic subunits and it might be the reason that they gradually lost some of their residual activity at high temperature, as discussed in details in the previous section). In contrast, mesophile ATCase activity increased to 37 °C and then decreased at higher temperatures. Therefore, the optimal temperature for ATCase from hyperthermophiles and the mesophile in these experiments is 37 and 40 °C for mesophile and hyperthermophile, respectively.

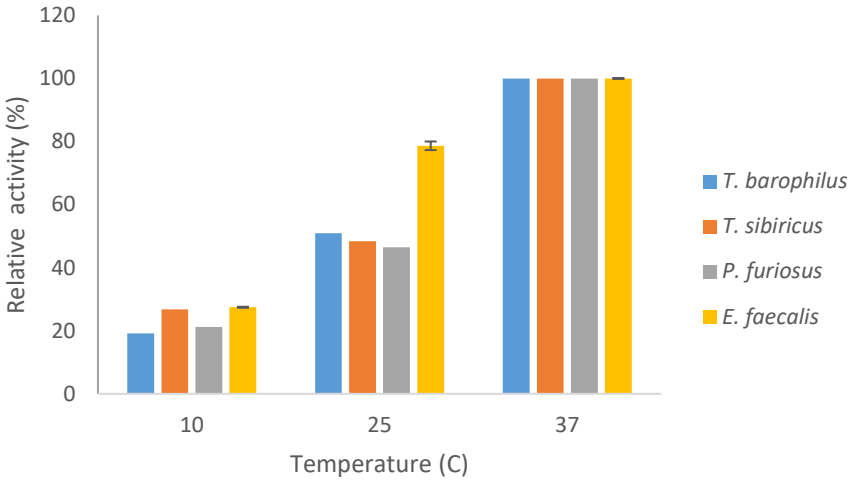


Figure 2.22: Study of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* enzyme activity at different temperatures. The ATCase were pre-incubated at 10, 25 and 37 ºC for 10 min and assayed at 10, 25 and 37 ºC for another 5 min in 50 mM Tris-HCl buffer pH 8.5 and 23 mM aspartate. The assays were initiated by adding 20 mM CP. The enzyme unit adjusts to 1 unit (1 unit is the amount of enzyme that converts 1 mM of aspartate to CA in 1 min) at 37 °C for all enzymes.

2.4.9. Effects of pH

In this section, the effect of pH was investigated. As discussed in the previous sections, the effect of temperature and pH is very crucial for wastewater treatment. The previous results showed that hyperthermophiles have high thermostability compared to mesophile ATCase. Therefore, in this section, the focus of the study is on the pH effect on ATCases.

It has been shown that one of the factors that creates difficult conditions in wastewater treatment systems is variation in pH. The variation in pH affects the solubility of materials (such as heavy and toxic metals) and enzyme activity in biological systems in wastewater treatment. Therefore, using a novel stable enzyme over a broad pH range offers great potential in wastewater treatment.

The pH effect on ATCases catalytic subunits from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis* were studied from a range of 6 to 11 by using two buffers: Tris-HCl (6, 7, 8 and 8.8) and bicarbonate (8.8, 9.4, 10.3 and 11). In terms of pH range, ATCase activity was measured at pH 8.8 in both buffers (as it was the pH in the effective pH range of both buffers) to evaluate the effect of buffer change on ATCase activity. The results demonstrated that the residual activity of the ATCase was affected by the change in buffer. The findings

showed that the residual activity of *T. barophilus* and *P. furiosus* activity decreased in the bicarbonate buffer while *E. faecalis* and *T. sibiricus* residual activity increased (Figure 2.23). The changes in ATCase activity with buffer change might be because the concentration and type of buffer affects the enzyme activity per following the factors: the electrostatic association of protein ion, the oppositely charged buffer ions, the near isoelectric point and the interaction of proteins with buffer ions, especially with the anions (Dyson & Noltmann, 1968).

Further, the results obtained from the effect of pH (in the range of our interest 6–11 and pH values that would be used in the next section) on ATCase activity revealed that all ATCases except *P. furiosus* revealed maximum activity at pH 8.8. At the same time, *P. furiosus* showed the highest activity around pH 8 (Figure 2.23). In addition, ATCase *T. barophilus* graph showed that there had been a gradual increase in residual activity from pH 6 to pH 8.8 and then is a sharp decline in residual activity of ATCase to pH 11. ATCase *T. sibiricus* showed a similar pH pattern to *T. barophilus*; however, its residual activity was always higher than *T. barophilus*, especially at a pH higher than 8.8. Moreover, ATCase *P. furiosus* exhibited sharp residual activity from pH 6 to 8, after which it showed a sharp decrease to pH 8.8. It then showed a slight increase at pH 9.4; again, the activity decreased at pH 10.3, followed by pH 11. The results from the hyperthermophiles pH study indicated that ATCases from hyperthermophiles are not as stable as CKases from hyperthermophiles (from our published work). Firstly, it might be due to a lack of regulatory subunit, which is an important factor in the stability of ATCase (Gavrilov et al., 2016). Secondly, all hyperthermophile enzymes do not necessarily have inherent pH stability when assayed out of the cell (probably the enzyme stability is mostly associated with intracellular matrix and the fact that enzymes' optimal activity would be achieved in their natural habitat (Niehaus, Bertoldo, Kähler, & Antranikian, 1999)).

Accordingly, the pH profile for *E. faecalis* was similar to *T. barophilus* and *T. sibiricus*; the residual activity elevated by increasing the pH to 8.8. After that, it slowly decreased to pH 11.

Next, the residual activity of ATCase *T. sibiricus* was maintained at over 13% over the broad pH range of 6 to 11. At the same time, ATCase *P. furiosus* showed a dramatic decline in activity at a pH range higher than 8, leading to a 6% residual activity at pH 11. In contrast with the hyperthermophilic ATCase *P. furiosus* and *T. barophilus*, where their activity declines at higher pH, *E. faecalis* exhibits more than 67% residual activity at pH higher than 8.8. Overall, the ATCase from hyperthermophiles and the mesophile showed different pH profiles. It could be due to the big difference between the mesophile *E. faecalis* and the

hyperthermophilic family, as well as ATCase amino acid sequence. For example, the similarity among hyperthermophilic ATCase amino acid sequences is between 75 to 90%, while between ATCase *E. faecalis* and hyperthermophilic enzymes is about 37%. Further, ATCases, like other enzymes, are composed of many basic and acidic groups of amino acid side chains, containing at least one amino and one carboxyl group. The pK value of each reactive group is designated as pK_1 and pK_2, in which pK_1 is around 2, and pK_2 is around 9 to 11. At an isoelectric point, the zwitterion form of ionisation exists, in which carboxyl and amino groups are presented in ionic form. However, a third ionisation group in the R chain can change the net molecular charge by its form of ionisation as an instance, aspartic acid (pK_R = 3.86), glutamic acid (pK_R = 4.25), histidine (pK_R = 6.0), cysteine (pK_R, = 8.33), tyrosine (pK_R = 10.07), lysine (pK_R = 10.53) and arginine (pK_R, = 12.48). Therefore, ATCases are subjected to diverse states of ionisation that depend on the various reactive group pH and ionisation constants. The investigation of the isoelectric point (*pI*) active site (amino acid sequence HQSTKRTRRSER) showed a similar *pI* (12.28) for all ATCase. However, the total isoelectric points for different ATCases were different (Table 2.5). ATCase *E. faecalis* showed lower total isoelectric points than three other hyperthermophilic enzymes. Also, they revealed more acidic and hydrophilic amino acids compared to three other hyperthermophilic ATCase demonstrating being a more hydrophilic enzyme. Therefore, it assumes that total *pI* influences pH profile and active catalytic groups of enzymes.

Also, the effect of pH on substrate ionisation should be considered. Aspartate has two carboxylic acid groups and one amino group. At acidic pH, the amino group becomes protonated and, therefore, the aspartate presents one positive charge ion. In contrast, at alkali pH, both carboxylic acid groups and amino groups become deprotonated and, therefore, present two negative charge ions. Amino and carboxylic groups all become protonated at neutral pH and exhibit one negative charge ion. Hence, concerning the ATCase active site charge at different pH, the ionisation of aspartate can affect the pH profile.

Taken together, to gain the optimal level of function for enzymes, the catalytic and binding sites should be in their proper state of ionisation that could be affected by pH. Therefore, the function of enzymes depends on pH due to the change in the activity, structure stability, substrates and solubility of the enzyme (Dyson & Noltmann, 1968; Frankenberger & Johanson, 1982; Segel, 1975, 1993; M. Wagner, 1976).

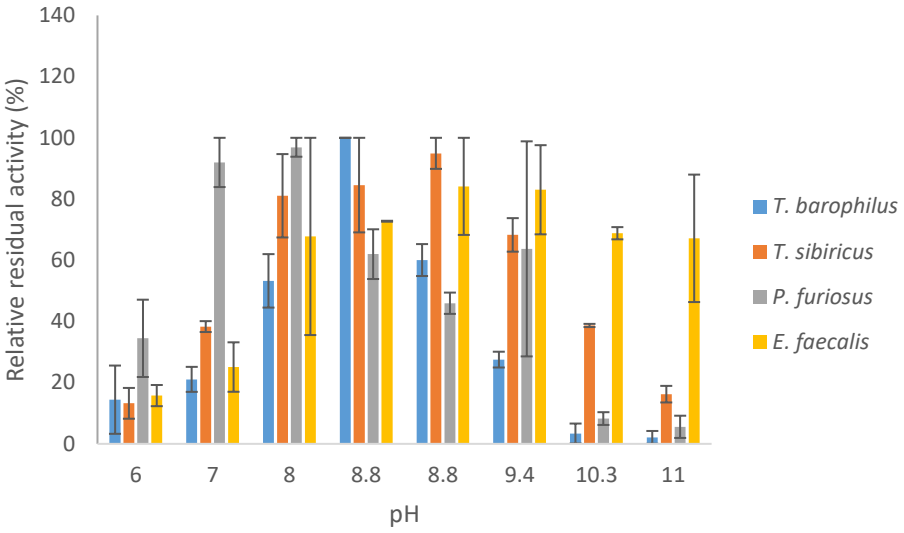


Figure 2.23: pH study of ATCase from *T. barophilus, T. sibiricus, P. furiosus* and *E. faecalis.* The ATCase enzymes were pre-incubated for 10 min at 37 ºC at different pH and assayed at 37 ºC for 5 min. The assays were run in either 50 mM Tris-HCl buffer (pH 6–8.8) or 50 mM sodium carbonate/sodium bicarbonate buffer (pH 8.8–11), 23 mM aspartate and 20 mM CP. Values are the average of duplicate replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

Table 2.5: The Percentage of Different Amino Acid Types in Hyperthermophile *T. barophilus, T. sibiricus, P. furiosus* and Mesophile *E. faecalis* that May Affect the Enzyme Activity at Different pH

Organism/amino acid percentage in protein	pI	Basic	Acidic	Hydrophilic	Hydrophobic
T. barophilus	6.62	17.32	15.03	22.23	45.42
T. sibiricus	6.99	15.45	13.49	23.02	48.04
P. furiosus	6.21	16.29	14.98	22.14	46.59
E. faecalis	5.53	14.87	15.2	26.35	43.58

2.5. Conclusion

In summary, all hyperthermophilic ATCases *T. barophilus, T. sibiricus, P. furiosus* and mesophile *E. faecalis* were all expressed in high yield in *E. coli*.

Hyperthermophilic ATCase *T. barophilus, T. sibiricus, P. furiosus* displayed higher residual activity at elevated temperatures compared to the mesophilic ATCase.

Moreover, hyperthermophilic ATCase *T. barophilus, T. sibiricus and P. furiosus* demonstrated higher thermostability compared to the mesophilic ATCase at 40 ºC, similar to CKase from those hyperthermophiles.

Concerning pH activity, hyperthermophilic ATCase from *T. sibiricus* exhibited higher residual activity over a broad pH range, followed by mesophilic ATCase *E. faecalis* (at pH higher than 8.8). Also, the enzyme efficiency was higher in *T. sibiricus* and *E. faecalis.* Therefore, among hyperthermophilic ATCase *T. sibiricus* is an ideal candidate for enzyme efficiency and pH stability for industry applications where pH is an important factor (i.e., wastewater treatment). Otherwise, all three hyperthermophiles are good candidates with respect to temperature stability.

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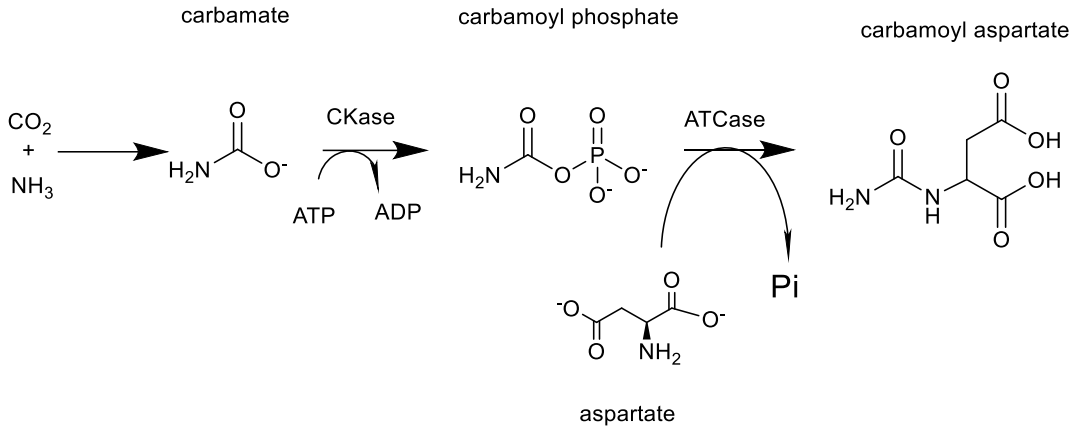
Chapter 3: Combination of Carbamate Kinase and Aspartate Transcarbamoylase

3.1. Background

The previous chapter and the published paper explained the importance of CKase and ATCase in sequestrating ammonia and carbon dioxide from wastewater and the environment (Hennessy et al., 2018). Also, it was presented that CKase was able to convert ammonia and carbon dioxide to CP. CP is very labile and decomposes easily in aqueous solution with a half-life of ~5 min at 37 °C (Allen & Jones, 1964), making it not feasible for industrial application. Therefore, to prevent CP from decomposition in aqueous solution, ATCase was introduced as a good alternative to avoid CP decomposition and produce a value-added product. Hence, Chapter 2 studied the ATCase from three hyperthermophiles (as they showed good stability in CKase in our published paper) and one isoenzyme mesophile. It was demonstrated that ATCase from hyperthermophilic ATCases *T. barophilus, T. sibiricus, P. furiosus* and mesophile *E. faecalis* could successfully be cloned in *E. coli* and had been characterised. The results from ATCase characterisation in cell-free enzyme reaction exhibited that hyperthermophilic ATCases presented higher residual activity at elevated temperatures and a higher thermostability at 40 °C compared to the mesophilic ATCase. Further, hyperthermophilic ATCase from *T. sibiricus* exhibited higher residual activity over a broad pH range, followed by mesophilic ATCase *E. faecalis* (at pH Higher than 8.8). The conclusion from characterisation was that all three hyperthermophiles are good candidates with respect to temperature stability. However, hyperthermophilic ATCase, *T. sibiricus*, was identified as an ideal candidate with respect to enzyme efficiency and pH stability.

Having confirmed the similar stability of hyperthermophile ATCase and CKase in the previous chapter and published paper, respectively, it is important to investigate the combination of CKase and ATCase in the cell-free enzymatic reaction. The combination of CKase and ATCase is promising to not only sequestrate ammonia and carbon dioxide, but also reuse and convert them to CA. Subsequently, many factors should be explored in a combination of CKase and ATCase, such as the combination of CKase and ATCase from different sources, rate-limiting reagents or enzymes, detection of substrates or product, the efficiency of pathway with regards to ATP requirement, the efficiency of the combination concerning cost and time and reagent degradation. Therefore, this chapter focuses on the combination of CKase and ATCase (Scheme 3.1) from the hyperthermophiles and the mesophile. The ultimate aim of this chapter is to provide a cell-free, single-pot,

multienzymatic process to convert ammonia, carbon dioxide and aspartate to CA (an important precursor in the pyrimidine pathway) by using recombinant *E. coli* lysate from one single culture while ATP is recycling.

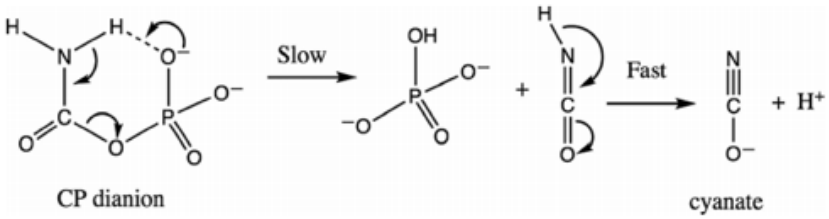


Scheme 3.1: Combination of CKase and ATCase to convert ammonia and carbon dioxide to CA.

3.2. Introduction

As discussed, a growing body of literature recognised the importance of CP as a key metabolic intermediate in the urea cycle and pyrimidine and arginine pathways, where CP can be readily transformed into valuable compounds such as pyrimidine, arginine and urea. However, one of the greatest challenges is that CP is very thermolabile and decomposes easily in aqueous solution (Allen & Jones, 1964). Therefore, the decomposition of CP wastes a significant amount of energy (since the production of each CP requires two ATP molecules) and produces cyanate, which is toxic to the cell (Scheme 3.2) (Q. Wang et al., 2008). Hence, a mechanism or mechanisms must exist in the cell to protect against the thermal decomposition of CP or other thermolabile compounds (Legrain et al., 1995). As such, some organisms have evolved several strategies to protect thermolabile compounds from thermal decomposition; for example, micro-environmental compartmentation, increasing catalytic efficiency, removing or substituting thermolabile compounds and metabolic channelling (the metabolite transfers directly through a channel from the first enzyme active site to the second without diffusing into solution) (al-Habori, 1995; Daniel &

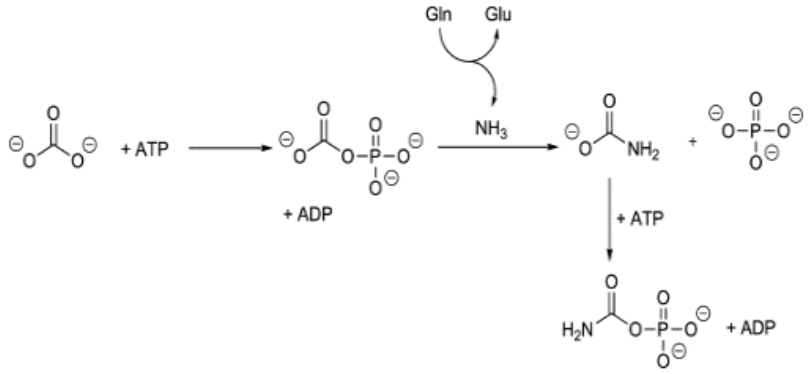
Cowan, 2000; Legrain et al., 1995; J. Massant & Glansdorff, 2004; Jan Massant & Glansdorff, 2005; Jan Massant et al., 2002; Purcarea, Evans, & Herve, 1999; Sterner et al., 1996; Tetas & Lowenstein, 1963; VandeCasteele, Legrain, et al., 1997)



Scheme 3.2: Carbamoyl phosphate thermal decomposition to cyanate (Q. Wang et al., 2008).

3.2.1. CP Production

C P can be produced synthetically or enzymatically. CP can be synthetically formed in a dilute solution of cyanate and orthophosphate, which is an energy-intensive process (Vieyra, Gueiros-Filho, Meyer-Fernandes, Costa-Sarmento, & DeSouza-Barros, 1995). In the cell, CP can be produced by CKase or CPSase, depending on the different microorganisms. CP is classically produced by CPSase in an anabolic pathway by consuming two ATP molecules, carbamate and a different source of ammonia, such as glutamine (Scheme 3.3) (Matxalen Uriarte et al., 2001).



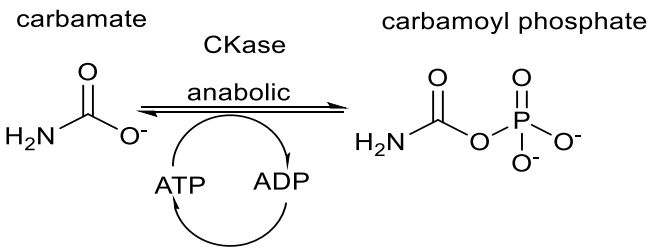
Scheme 3.3: Carbamoyl phosphate formation by CPSase using two molecules ATP and one carbamate and one ammonia. In this example, ammonia comes from glutamine.

3.2.1.1. CPSase

CPSase is a heterodimeric enzyme consisting of two subunits. In *E. coli*, CPSase has an inner channel between the active sites that transfers the intermediates directly from one active site to another without being released into the solution (Thoden, Holden, Wesenberg, Raushel, & Rayment, 1997). CPSase is present in all prokaryote and eukaryote organisms and is classified into three categories based on the specificity of their nitrogen source from either ammonia or glutamine and their requirement for N-acetyl-L-glutamate as a cofactor (Meister, 1989; Rubio, 1993; Rubio & Cervera, 1995; Rubio & Grisolia, 1981; Rubio, Ramponi, & Grisolia, 1981; Thoden et al., 1997; Thoden, Wesenberg, Raushel, & Holden, 1999).

3.2.1.2. CKase

CP can be degraded in a catabolic pathway by CKase generating one ATP molecule and carbamate (Scheme 3.4) (Matxalen Uriarte et al., 2001).



Scheme 3.4: Anabolic and catabolic direction of carbamate kinase. In the catalytic direction, CP can be degraded, generating one ATP molecule and carbamate, and in the anabolic direction, CP is produced from carbamate by using one molecule of ATP.

CKase is the homodimeric enzyme and is relatively small, being between 31 and 37 kDa and exists in many prokaryotes (Figure 3.1). The CKase was isolated from *E. faecalis* as a participant of the arginine dihydrolase fermentation pathway, where it catalyses ATP production (Barcelona-Andres et al., 2002). After that, the CKase gene was identified in many other organisms that use energy from arginine degradation (Barcelona-Andres et al., 2002; Chantratita et al., 2012; Novák et al., 2016). In the hyperthermophilic Archaea such as *P.* and *P. abyssi*, an enzyme initially defined as a 'CKase-like carbamoyl phosphate synthetase' was categorised as a CKase. It was suggested that CKase catalyses anabolic CP formation from carbamate and ATP (Virginie Durbecq et al., 1997; M. Uriarte et al., 1999; Matxalen Uriarte et al., 2001). CKase from *E. faecalis* and hyperthermophiles can naturally

form CP when supplied with carbamate and ATP (Alcantara et al., 2000; Ramón-Maiques, Marina, Uriarte, Fita, & Rubio, 2000). CKase uses one molecule of ATP compared to CPSase, using two molecules of ATP to produce the same amount of product, one molecule of CP (the use of CKase is more beneficial for this project as it uses one molecule ATP which could be recycled by the method that has been explained in the following sections). It is not clear why the preference of most organisms is to use CPSase instead of CKase. One reason might be the advantages of using different sources of nitrogen in CPSases.

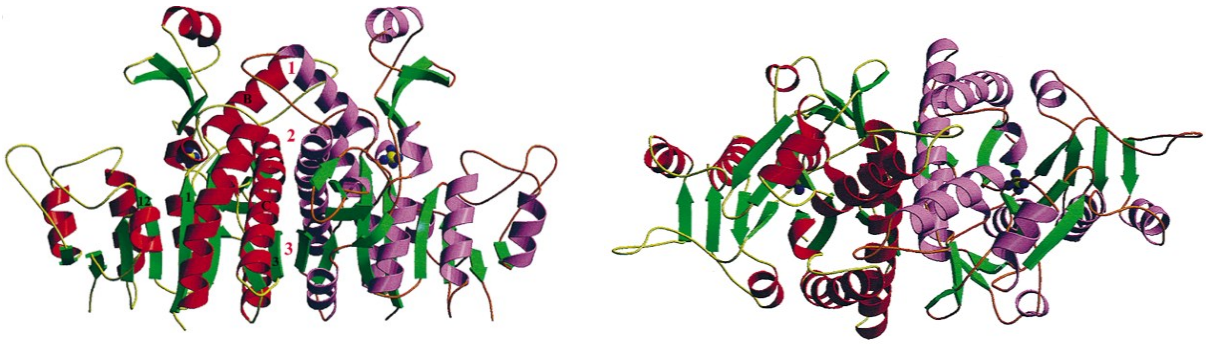
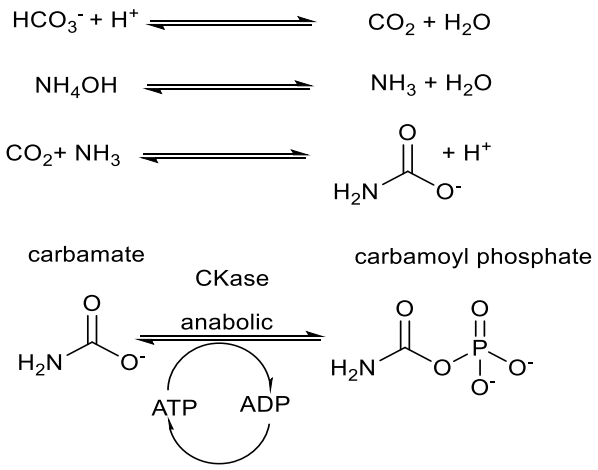


Figure 3.1: Carbamate kinase structure. Left down view right: perpendicular view to the molecular twofold axis. The purple represents the helices, green represents the strands and red the other subunit. The numbers and letters show monomer β strands and α helices (Marina et al., 1998).

3.2.2. CKase Studies

Recent works have established that CKase activity is reversible and can produce CP in an anabolic direction. It has shown that anabolic CKase catalyses the synthesis of CP from one molecule of bicarbonate, one molecule of ammonia and one molecule of ATP (Scheme 3.5). The following steps occur in the production of CP: initially, carbon dioxide chemically reacts with ammonia in solution and forms carbamate, then the carbamate is phosphorylated by CKase using ATP to yield CP (Scheme 3.5) (Mani et al., 2006; Rubio & Cervera, 1995; Ryall, Nguyen, Bendayan, & Shore, 1985; Summar et al., 2003).



Scheme 3.5: CKase anabolic direction produces CP from carbamate, formed in aqueous solution in equilibria between CO_2/HCO_3^- and NH_4OH/NH_3.

It is important to mention that carbamate production is affected by pH due to the ammonium to ammonia ratio, which plays an important role in carbamate formation (Figure 3.2).

The equilibrium constant (*K*) of the reactions that involve carbamate formation can be calculated by the following equation:

$K(dm^3ml^{-1}) = [carbamate] / ([NH_4OH + NH_4OH]. [CO_2 + HCO^{3-}])$

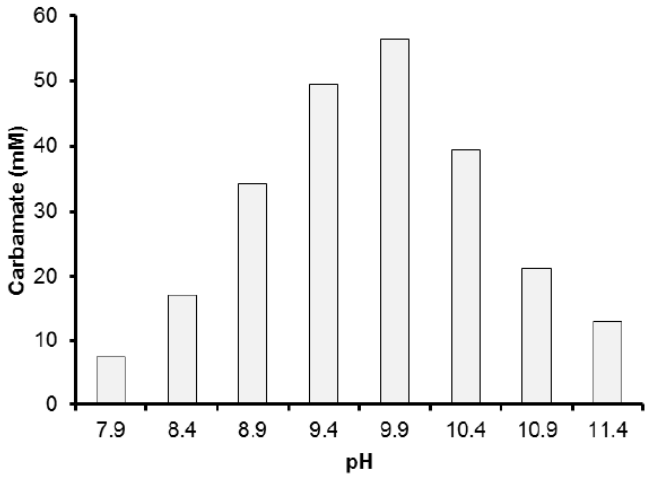


Figure 3.2: Carbamate formation concentration at different pH using 400 mM ammonia and 400 mM 13C-labelled bicarbonate. The carbamate formation was measured by 13C NMR spectroscopy (Hennessy et al., 2018).

Despite the importance of CKase in the production of CP in an anabolic direction, so far there has been little discussion about anabolic CKase, and little attention has been paid to it.

Therefore, in the published paper, It was aimed to to obtain data that help to address this research gap. In this paper, seven CKases from the hyperthermophiles *P. furiosus, T. barophilus* and *T. sibiricus*, the thermophiles *Fervidobacterium nodosum* and *Thermosipho melanesiensis* and the mesophiles *C. tetani* and *E. faecalis* were characterised. Characterisation of CKases provided new insights into hyperthermophilic Archaea. It was revealed that CKases from hyperthermophilic Archaea were robust catalysts for biotransformations due to their outstanding stability and high catalytic activity across a broad range of temperatures and pH (Hennessy et al., 2018). The study showed that all CKases from our study could be cloned and expressed recombinantly in high yield (Figure 3.3).

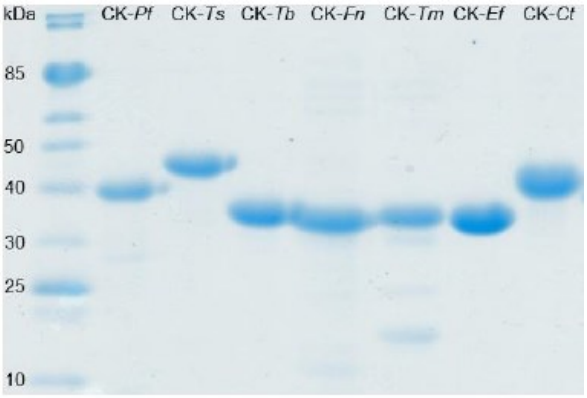


Figure 3.3: SDS-PAGE from CKase from different microorganisms. CK-Pf = CKase *P. furiosus*; CK-Ts = CKase *T. sibiricus*, CK-Tb = CKase *T. barophilus*, CK-Fn = CKase *F. nodosum*, CK-Tm = CKase *T. melanesiensis*, CK-Ef = CKase *E. faecalis*, CK-Ct = CKase *C. tetani* (Hennessy et al., 2018).

The temperature study demonstrated that CKases from hyperthermophiles displayed higher specific activities at elevated temperatures (Figure 3.4). Moreover, the hyperthermophilic CKases revealed greater stability and substrate turnover at alkaline pH than mesophilic CKases (Figure 3.5). Therefore, the stability and robustness of CKases from hyperthermophiles made them good candidates for biotechnological applications, such as wastewater treatment to remove aqueous ammonia.

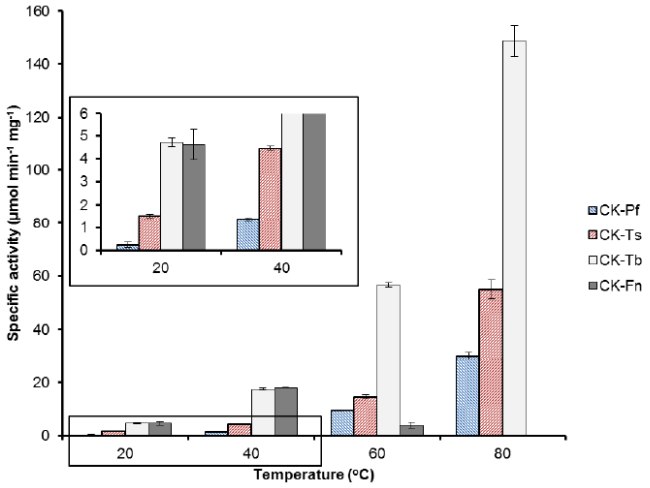


Figure 3.4: CKase temperature study from different microorganisms. CK-Pf = CKase *P. furiosus*, CK-Ts = CKase *T. sibiricus*, CK-Tb = CKase *T. barophilus*, CK-Fn = CKase *F. nodosum* (Hennessy et al., 2018).

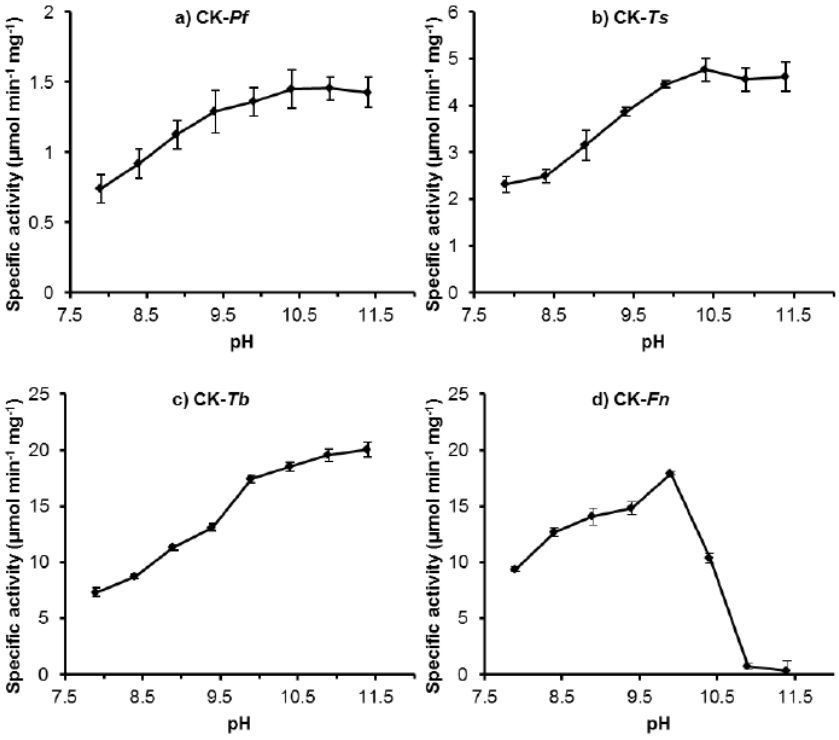


Figure 3.5: CKase pH study from different microorganisms. CK-Pf = CKase *P. furiosus*, CK-Ts = CKase *T. sibiricus*, CK-Tb = CKase *T. barophilus*, CK-Fn = CKase *F. nodosum* (Hennessy et al., 2018).

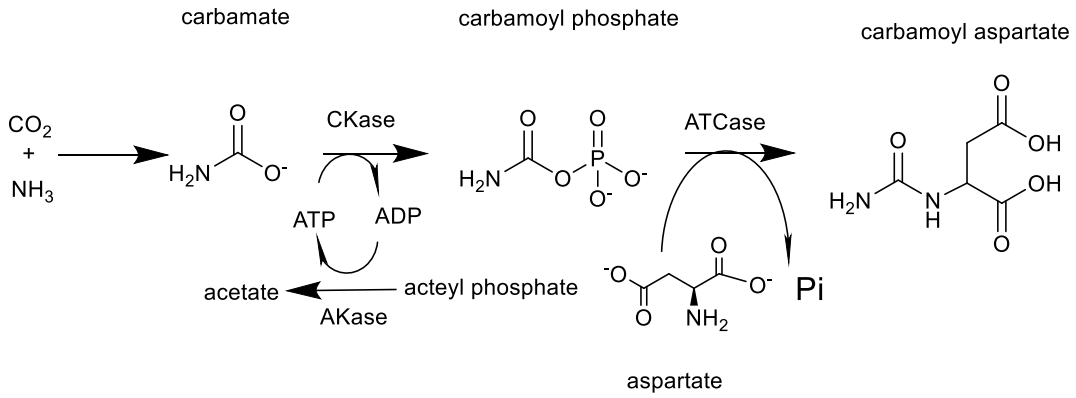
Since CKase uses ammonia and carbon dioxide to produce CP via carbamate, it has the potential to sequester ammonia (from wastewater) and carbon dioxide (the greenhouse gas). As already explained in the previous chapter, removing ammonia from wastewater is a big challenge today. As most of the nitrogen in animal waste and fertiliser is converted to ammonia in water run-off, it increases the ammonia concentration in wastewater (between 100 mg to 1 g/L) (Malovanyy, Sakalova, Yatchyshyn, Plaza, & Malovanyy, 2013). A high amount of ammonia results in eutrophication that is undesirable for aqueous life in the environment and, therefore, must be removed from wastewater to avoid downstream pollution (Du, Li, Yu, Feng, & Li, 2016; Luo et al., 2015; Malovanyy et al., 2013; Yamashita & Yamamoto-Ikemoto, 2014). Consequently, the use of hyperthermophilic CKase may provide a useful route for the sequestration of ammonia and CO_2 greenhouse gas.

However, the whole process is not beneficial without utilising the CP as CP decomposes in aqueous solution. Therefore, the combination of CKase with another enzyme(s) to prevent CP from decomposition is necessary.

As explained above and in the previous chapter, ATCase is a good alternative enzyme to be combined with CKase to prevent CP decomposition and convert it to a value-added compound. In the previous chapter, ATCases from three hyperthermophiles *T. sibiricus, T. barophilus* and *P. furiosus* and one mesophile, *E. faecalis*, were characterised. CKases from the same three hyperthermophiles, *T. sibiricus, T. barophilus* and *P. furiosus* and one mesophile, *E. faecalis,* have already been characterised in theprevious work (Hennessy et al., 2018). It was confirmed that CKases and ATCases stability and activity are similar, and therefore, the in vitro combination of the two enzymes in a one-pot might be possible.

Hence, the focus of this chapter is to investigate the combination of CKase and ATCase in a cost- and time-efficient manner. ATCase and CKase enzyme purification are time-consuming and costly, plus the CKase requires ATP (as a phosphorylation cofactor) to form CP, which is also costly. Hence, it would be beneficial to develop a process or method in which protein purification could be avoided, and ATP could be recycled using cheap materials.

Previously, in the published paper (Hennessy et al., 2018), it was confirmed that ATP can be recycled by using ADP, AP and endogenous *E. coli* lysate enzyme acetate kinase using cheap and readily prepared AP (Scheme 3.6).AP is stable over hours at neural pH and 30 °C for 10 to 15 hours. AP can phosphorylate ADP to ATP at 50°C over several hours without significantly decompose (A. Whicher, E. Camprubi, S. Pinna, B. Herschy, & N. Lane, 2018).



Scheme 3.6: Combination of CKase and ATCase while ATP is recycling using endogenous acetate kinase from *E. coli* lysate.

Also, it is appealing to mention that the price of ATP is much higherthan ADP AMP. Therefore, using ADP and AMP instead of ATP decreases the cost substantially.

Further, AP could be synthesised easily in the lab using H_3PO_4 and acetic anhydride, costing \$0.00011/mg acetyl phosphate.

Finally, we have shown that by adding AP and AMP or ADP to *E. coli* lysate that contains acetate kinase, ATP turns over many times if provided the phosphate needed for CKase activity.

Overall, since CKase requires one ATP molecule to convert carbamate to CP, it would be cost-efficient to recycle ATP. Therefore, applying this process to utilise the combination of CKase and ATCase may be very beneficial by avoiding using expensive ATP.

3.3. Aims

The ultimate aim of this chapter is to provide a cell-free, single-pot multienzymatic process to convert ammonia, carbon dioxide and aspartate to CA, using recombinant *E. coli* lysate from one single culture while ATP is recycling.

The following steps have to be carried out to achieve the ultimate aim:

1. develop a one-pot combination of pure enzyme CKase and the catalytic subunits of ATCase from the hyperthermophilic strains *T. sibiricus, T. barophilus* or *P. furiosus* and the isoenzyme mesophilic strain *E. faecalis* to produce CA from ammonia and carbon dioxide (in the form of bicarbonate).

2. find the best combination of CKase and ATCase from different source organisms to maximise the CA production rate.
3. incorporate ATP recycling system from *E. coli* lysate to the one-pot combination of CKase and ATCase for the synthesis of CA.
4. optimise the conditions of the CKase and ATCase combination to achieve the highest CA yield while ATP is being recycled.
5. co-express the best combination of CKase and ATCase in a vector, transform it to *E. coli*, lyse the cell and use the lysate to minimise the limitations associated with protein production and purification.

3.4. Material and Methods

3.4.1. Materials

Polyacrylamide gels were cast using acrylamide and bis-acrylamide solutions from Bio-Rad and run on a Mini-PROTEAN® Tetra System; Bio-safe®. Coomassie Blue was used for staining. Protein markers, restriction enzymes and a Quick DNA Ligation™ Kit were purchased from NEB. Alkaline phosphatase was from Roche Diagnostics GmbH. Agarose for electrophoresis was purchased from Invitrogen. Nutrients for broth were purchased from Bacto Laboratories Pty Ltd. (L)-Aspartate, ampicillin and N-chloromethylphthalimide were obtained from Sigma-Aldrich®. HPLC analysis was performed on a Hewlett-Packard Series 1100 separation system operated by Chemstation from Agilent Technologies.

3.4.2. Expression of CKase and ATCase

The cloning of ATCases was done as was described in the previous chapter. Eight different vectors were constructed by using a pETMCSШ vector containing CKase (these were provided by Professor Chris Easton from the Research School of Chemistry at the Australian National University) and ATCase from *T. barophilus*, *T. sibiricus*, *P. furiosus* and *E. faecalis.* They were individually transformed into *E. coli* (BL21 (DE3)) cells for expression as the procedure explained in the previous chapter. The cells containing the plasmid were grown in an LB medium containing 50 μg/mL ampicillin for 16 hours at 37 °C with shaking at 180 rpm. The cells were then collected from a 50 mL culture by centrifugation (6000 × *g* for 10 min) and resuspended in 20 mL binding buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 8.8). The pellets were then lysed over ice using a Thermo Fisher Scientific Omni Sonic Ruptor 400, equipped with an ORT-375 processing tip set at 50% power and 50% pulser for 6 min. The lysate was centrifuged at 30,000 × *g* for 60 min at 4 °C. The enzymes were purified from the supernatant using His GraviTrap™ columns from

GE Healthcare using the conditions recommended by the manufacturer (load 10 mL binding buffer two times, the sample, wash with binding buffer two times, collect the enzyme eluting with 3 mL elution buffer). The protein was collected in 3 mL elution buffer solution containing 0.02 M phosphate buffer (Na_2HPO_4/NaH_2PO_4) pH 7.4, 0.5 M NaCl and 0.5 M imidazole and kept at 4 °C. The protein concentration was measured by either a NanoDrop™ ND-1000 Spectrophotometer from Thermo Fisher Scientific or a Shimadzu UV-Visible Spectrophotometer at 280 nm. The extinction coefficients were generated from the online ProtParam tool based on the protein sequence. The catalytic subunits were determined for purity by electrophoresis on 20% SDS-PAGE gel.

3.4.3. Preparation of S30 Cell-Free Lysate

To produce the cell-free S30 lysate, *E. coli* (BL21 (DE3)) were grown in LB medium for 16 hours at 37 °C with shaking at 180 rpm. The cells were then collected from 50 mL culture by centrifugation (4000 × *g* for 10 min), resuspended (1 mL/g wet cell pellet) in Tris-acetate buffer (10 mM, pH 8.3) containing 16 mM KOAc, 14 mM Mg $(OAc)_2$, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. They were lysed on ice in a Thermo Fisher Scientific Omni Sonic Ruptor 400, equipped with an ORT-375 processing tip set at 50% power and 50% pulser for 6 min. The lysate was centrifuged at 20,000 × *g* for 60 min at 4 °C. The supernatant was aliquoted in an Eppendorf tube located on dry ice for snap-freezing and was then stored at –80 °C.

3.4.4. Preparation of CKase Lysate

The pETMCSШ plasmids containing CKase from *T. barophilus*, *T. sibiricus*, *P. furiosus* and *E. faecalis* were transformed into *E. coli* (BL21 (DE3)) cells for expression. The transformed cells were grown in an LB medium containing 50 μg/mL ampicillin for 16 h at 37 °C with shaking at 180 rpm. The cells were then collected from 50 mL culture by centrifugation (4000 × *g* for 10 min), resuspended (1 mL/g wet cell pellet) in Tris-acetate buffer (10 mM, pH 8.3) containing 16 mM KOAc, 14 mM $Mg(OAc)_2$, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. They were lysed on ice in a Thermo Fisher Scientific Omni Sonic Ruptor 400, equipped with an ORT-375 processing tip set at 50% power and 50% pulser for 6 min. The lysate was centrifuged at 20,000 × *g* for 60 min at 4 °C. The supernatant was aliquoted in an Eppendorf tube while being snap-frozen on dry ice and then stored at -80 °C.

3.4.5. Derivatisation of Aspartate

The consumption of aspartate was determined by a reverse-phase HPLC (Agilent 1100) analysis using AccQ-Tag™ Ultra Derivatisation Kit and Waters AccQ-Tag™ C18 column.

For derivatisation, 1 mL of acetonitrile was added to AccQ-Fluor Reagent Powder (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate), mixed and dissolved by heating at 55 ºC for 10 min. After that, 40 μL of AccQ Borate Buffer was added to a 10 μL sample (which had already been neutralised and diluted) and finally, 10 μL of AccQ-Fluor Reagent Solution was added. The solution was heated for 10 min at 55 ºC and run by HPLC.

3.4.6. HPLC Detection of Aspartate

The reverse-phase separation of L-L-aspartic acid was performed on an Agilent 1100 HPLC system fitted with a PDA detector using an AccQ-TagTM column (3.9 × 150 mm, 4 μm, 60 Å) separating the hydrolysate amino acid derivatives produced in the reaction. Samples were eluted (1 mL/min) at 37 ºC with a gradient of 10% AccQ Eluent (v/v Eluent/water) (Solvent A) and 100% acetonitrile (Solvent B), according to the following program: 0 min 100% B, 0–5 min 99% B, 5–18 min 95% B, 18–25 min 60% and 25–45 min 100% B with linear transitions between mixtures. The observed retention time for L-L-aspartic acid was around 15 min.

3.4.7. HPLC/MS Detection of Carbamoyl Aspartate

CA concentration was determined by HPLC-MS on a Waters Alliance 2695 separation module coupled to a Waters TQD detector. HPLC separation was performed using a Phenomenex Develosil® RP-Aqueous-AR 5 μ C30 (250 × 4.6 mm) column heated at 35 °C, eluted with 0.1% formic acid in H_2O (0–1 min) followed by a linear gradient to 0.1% formic acid in ACN/H2O 30/70 min (1–7 min) at a flow rate of 0.7 mL (15:1 flow splitter). With a retention time of 5.5 min, CA was identified and quantified using integrated MRM and a calibration curve developed with authentic commercial material from Sigma. The MRM parameters used were CA (parent (m/z): 176.9475); channel 1: daughter (m/z): 45.9169, dwell (s): 0.025, cone (V): 20, collision (V): 24; channel 2: daughter (m/z): 69.9541, dwell (s):0.025, cone (V): 20, collision (V): 26; channel 3: daughter (m/z): 73.917, dwell (s): 0.025,cone (V): 20, collision (V): 24; channel 4: daughter (m/z): 87.9496, dwell (s): 0.025, cone (V): 20, collision (V): 18; and channel 5: daughter (m/z): 45.9169, dwell (s): 0.025, cone (V):20, collision (V): 24. The CA parent was used for quantification.

3.4.8. HPLC Detection of ADP, ATP and AMP

Conversion of ATP to ADP in the assay samples was measured using reverse-phase HPLC with absorbance detection at 259 nm. The tetrabutyl ammonium cation was used as an ion-pairing agent, allowing separation of the nucleoside phosphates based on the number of

phosphates. In this way, ATP with three ionised phosphates (buffered at pH 5) was retained longer by the tetrabutyl 'ammonium coated' stationary phase than was ADP with two phosphates. More specifically, ion-pair HPLC separation of ADP and ATP was carried out on an Agilent 1100 HPLC system fitted with a PDA detector, using an Alltech Alltima HP C18 column (5 µ, 250 × 4.6 mm, with a 7.5 × 4.6 mm guard column). Samples were eluted (1 mL/min) with a gradient of 60 mM ammonium dihydrogen phosphate and 5 mM tetrabutyl ammonium phosphate in H_2O (solvent A), and 5 mM tetrabutyl ammonium phosphate in methanol (Solvent B), according to the following program: 0–18 min 87% A, 19–23.2 min 70% A, 24–27 min 87% A, with linear transitions between mixtures. Observed retention times were AMP – 6.9 min, ADP – 12.4 min and ATP – 23.9 min. The dwell volume of the analytical system is approximately 1.2 mL, so ATP elutes with 70% Solvent A before the initial solvent mixture is restored for the next analysis. Higher analytical throughput was obtained through monitoring four consecutive sample injections (1 min delay) in a single 27-min analysis window, without co-elution of peaks from different samples.

The concentrations of ADP in assay samples were quantified from the integrated chromatogram peak area (Agilent Chemstation) using a standard calibration curve.

3.4.9. CKase and ATCase Individual Activity Assay

ATCase enzyme activity was measured by monitoring the consumption of aspartate and the simultaneous production of CA. The enzyme solution was pre-incubated at 40 °C for 5 min before adding CP. The final reaction mixture (500 µL) containing 200 mM $NaHCO_3$ and NH_4OH (pH 9.4), 23 mM aspartate, 40 mM CP and one of the purified ATCase (0.1 µM for *T. barophilus, T. sibiricus, E. faecali,* and 1 µM *P. furiosus*) were incubated for a further 5 min at 40 ºC. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water before measurement. The amount of residual aspartate was measured either by a reverse-phase HPLC using AccQ-Tag™ or HPLC/MS. As earlier described, the CA amount was measured by HPLC/MS.

CKase enzyme activity was measured by monitoring the catalytic conversion of ATP to ADP and AMP accompanying the conversion of carbamate to CP. The enzyme solution was pre-incubated at 40 °C for 5 min before adding ATP. The final reaction mixture (500 µL) containing 200 mM $NaHCO_3$/NH_4OH (pH 9.4), 10 mM ATP, 10 mM $MgSO_4$ and one of the purified CKase (0.05 µM for *T. barophilus, T. sibiricus, E. faecalis*, *P. furiosus* and *E. faecalis*) was incubated for a further 5 min at 40 ºC. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH

to neutralise and then diluted by a factor of 10 with water before measurement. The amount of ADP, ATP and AMP was measured by a reverse-phase HPLC as described above.

3.4.10. Combined CKase and ATCase Activity Assay

CKase and ATCase combination assays were performed at pH 9.4 and 40 °C in 0.5 mL water containing 200 mM $NaHCO_3/NH_4OH$ (pH 9.4), 10 mM ATP, 10 mM L-aspartic acid, 10 mM $MgSO_4$, 1 enzyme unit (U) of CKase (where 1 U is the amount of enzyme necessary to turn over 1 mmol of substrate per min) and 1 to 10 U (mM/min) of ATCase. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water before the measurement. The amounts of CA and L-aspartic acid were measured by HPLC/MS.

3.4.11. Combining CKase and ATCase Pure Enzyme with ATP Recycling System

CKase and ATCase combination assays were performed at pH 9.4 because this was the pH between the optimal pH for CKase and the optimal pH for ATCase. The assays were performed at 40 °C (which is the CKase optimal temperature) in 0.5 mL water containing 200 mM $NaHCO_3/NH_4OH$ (pH 9.4), 10 mM L-L-aspartic acid, 10 mM $MgSO_4$, 0.1 mM ADP, 0.1 mM AMP, 1 to 10 µL S30 lysate, 0–20 mM acetyl phosphate, 1 U (mM/min) of CKase and 16 U (mM/min) ATCase. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water before measurement. The amounts of CA and L-aspartic acid were measured by HPLC/MS.

3.4.12. CKase Lysate Activity Assay

CKase lysate activity was measured by monitoring the catalytic conversion of ATP to ADP and AMP accompanying the conversion of carbamate to CP. The lysate was pre-incubated at 40 °C for 5 min before adding AP. The final reaction mixture (0.5 mL) containing 200 mM $NaHCO_3/NH_4OH$ (pH 9.4), 20 mM acetyl phosphate, 10 mM $MgSO_4$ and 10 µL of one of the CKase lysates *(T. barophilus, T. sibiricus, E. faecalis* and *P. furiosus*) was incubated for a further 5 min at 40 °C. An aliquot of 50°µL was then withdrawn at each time point and mixed with 50°µL of 1°M HCl, followed by adding 50°µL of 1°M NaOH to neutralise and then diluted by a factor of 10 with water before measurement. The amount of ADP, ATP and AMP was measured by a reverse-phase HPLC analysis as described in Section 3.4.8.

3.4.13. Combining CKase Lysate with ATCase Pure Enzyme

The assays for the combined CKase lysate and pure ATCase were done similarly to the assay for the combined pure enzyme of CKase and ATCase but using 10 µL CKase lysate instead of pure CKase. The combination assays were performed at pH 9.4 and 40 °C in 0.5 mL of water containing 200 mM $NaHCO_3/NH_4OH$ (pH 9.4), 10 mM L-aspartic acid, 10 mM $MgSO_4$, 10 mM ATP, 10 µL CKase lysate and 16 U ATCase. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water before measurement. The amount of CA and L-aspartic acid were measured by HPLC/MS.

3.4.14. Combining CKase Lysate and ATCase Pure Enzyme with ATP Recycling System

The assays of combined CKase lysate and pure ATCase while ATP is recycling were done similarly to the assay for combined CKase and ATCase pure enzyme. However, instead of adding pure CKase and *E. coli* S30 lysate, 10 µL lysate was added to the reaction solution. The assays for the combination were performed at pH 9.4 and 40 °C in 0.5 mL of water containing 200 mM $NaHCO_3/NH_4OH$ (pH 9.4), 10 mM L-L-aspartic acid, 10 mM $MgSO_4$, 20 mM acetyl phosphate, 10 µL CKase lysate and 16 U ATCase. An aliquot of 50 µL was then withdrawn at each time point and mixed with 5 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water before measurement. The amount of CA and L-aspartic acid were measured by HPLC/MS.

3.4.15. Co-Expression of CKase and ATCase

Co-expression of CKase *T. sibiricus* and *E. faecalis* was enabled by using the pRSFDuet-1 vector (Novagen) (Figure 3.6). This vector contains two multiple cloning sites (MCS). Each cloning site has a T7 promoter and a ribosome binding site. The vector also has a kanamycin resistance gene. The primers for MSC1 are the DuetUP1 primer and the DuetDOWN1 primer, and for MSC2 are the DuetUP2 primer and the T7 Terminator primer for sequencing the inserted gene. Moreover, the vector has a His-tag and an S-Tag (a special sequence for detection and purification of protein complexes, as shown in Figure 3.6) for detection and purification of the protein. ATCase *T. sibiricus* and *E. faecalis* were inserted into two vectors in MSC1 and into CKase *T. sibiricus* and *E. faecalis* in MCS2. Four different co-expressed enzyme combinations were made: ATCase *T. sibiricus* and CKase *T. sibiricus*; ATCase *T. sibiricus* and CKase *E. faecalis*; ATCase *E. faecalis* and CKase *T. sibiricus*; ATCase *E. faecalis* and CKase *E. faecalis*.

The restriction enzymes *Sac*I and *Hind*III were used for ATCase and *Nde*I and *Pac*I for CKase.

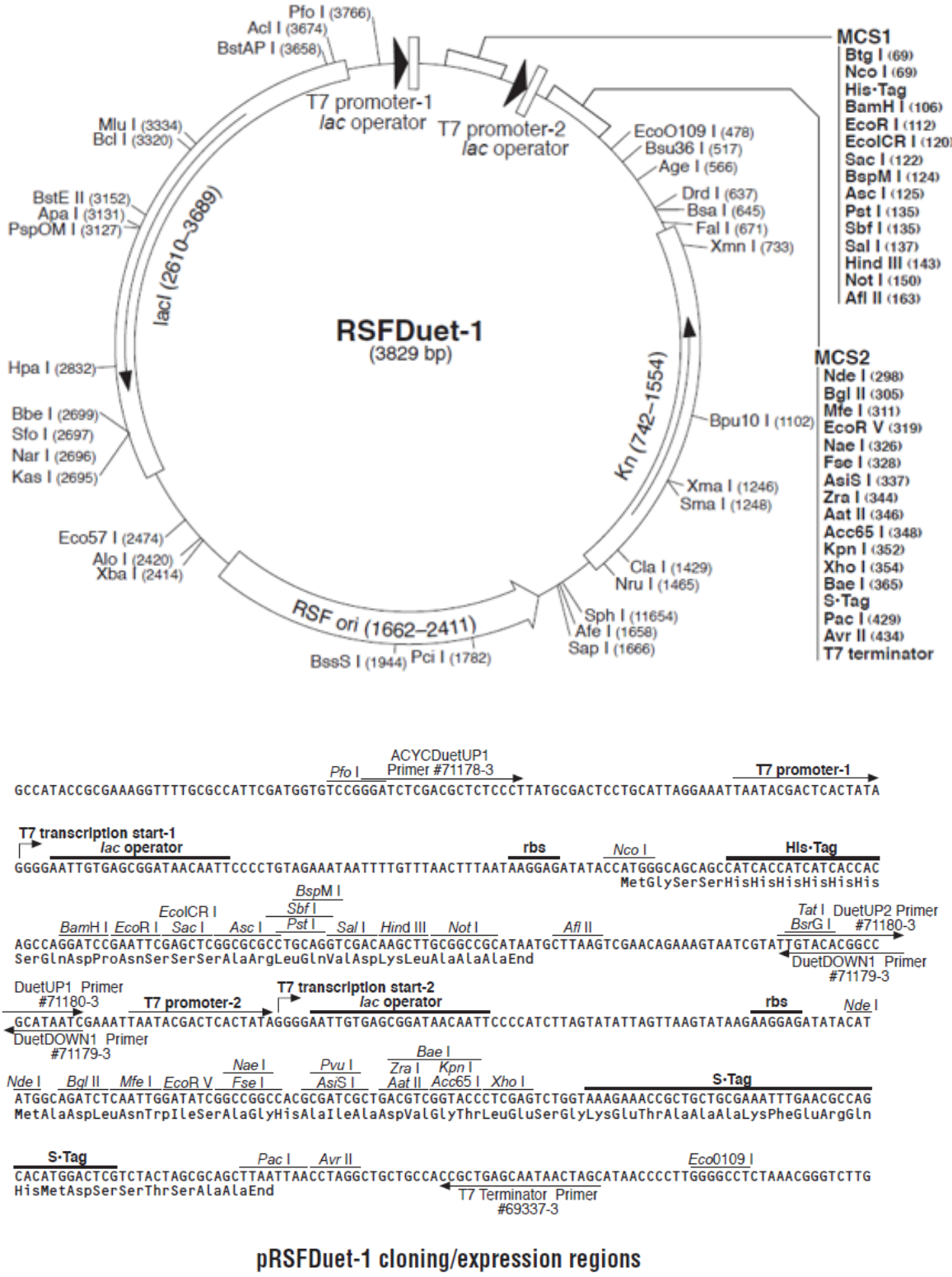


Figure 3.6: pRFS1Duet Plasmid map and sequence.

i. Gene Amplification

The ATCase and CKase genes were commercially synthesised in the pMK vector (GeneArt) and have been subcloned into pETMCSIII as described in the previous chapter using NEB Kits. To clone the genes in the pRSF1 vector, firstly, the genes were amplified by PCR as follows:

*dNTP (10 mM)	0.5 µL
**5X High fidelity buffer	5 µL
Phusion® DNA polymerase	0.25 µL
Primer forward (10 µM)	1 µL
Primer backward (10 µM)	1 µL
Water	15.5 µL
Vector	1 µL
DMSO	0.75 µL

* Represents deoxyribose nucleoside triphosphate that contains all necessary dNTP for DNA polymerisation. ** High fidelity buffer includes high fidelity buffer and 1.5 mM $MgCl_2$.

The PCR protocol:

- 98 ºC preheat
- 98 ºC 2 min
- 98 ºC 10 sec
- 60 ºC 30 sec — for 35 cycles
- 72 ºC 30 sec
- 72 ºC 10 min
- 4 ºC ∞

New primers containing the restriction endonuclease recognition sites were used for amplification. The high fidelity kit was used for high-yield amplification and less mismatching.

ATCase *E. faecalis*

Forward 5′ GGGAAAAGAGCTCATGATTATTACCAGCGAACGTATTAGC 3′

Backward 5′ AAAAGGGGAAGCTTTTATTAGGCTTTACCATGCAGAATTG 3′

ATCase *T. sibiricus*

Forward 5′ GGGGGAAAAGAGCTCATGAAATTCCAGGATGTGATCAGCATC 3′

Backward 5′ AAAGGGAAGCTTTTATTATTCAACAACACCCAGAACAATACC 3′

CKase *E. faecalis*

Forward 5′ CCCGGGGAAAACATATGATGGGCAAAAAAATGGTTGTTGC 3′

Backward 5′ AAAGGGGGTTAATTAATTATCATTTGGTCACAACGGTGCC 3′

CKase *T. sibiricus*

Forward 5′ AAAAAAGGGGGCATATGATGCGTAAACGTGTTGTTATTGC 3′

Backward 5′ AAAGGGGGTTAATTAATTATTAACGGGTAATCTGGGTGCC 3′

ii. Digestion and Ligation of ATCase and Vector

After amplifying the CKase and ATCase genes, they were digested by restriction enzymes and the pRSF vector. The ATCase genes and pRSF vector MCS1 were digested by restriction enzymes from *Sac*I and *Hind*III using an NEB CutSmart® Kit as follows:

- 10X CutSmart buffer 10 µL
- restriction enzyme *Hind*III 2 µL
- restriction enzyme *Sac*I 2 µL
- water 56 µL
- DNA template (ATCase or PRSF1) 30 µL

They were incubated at 37 °C for a total of 2 hours. After 1 hour, 1 µL of alkaline phosphatase and 20 µL de-phosphorylation buffer were added to the vector solution. After digestion, the ATCase genes were cleaned using the gene prep kit (QIAprep) protocol (following the manufacturer's guidelines) and the vector was run on 1.2% agarose gel. The

vector band was detected by UV lamp based on their base pair kDa, and then the band was cut and cleaned using the cleaning kit. The ATCase genes and pRSF1 MCS1 were then ligated using *E. coli* AN1549 as follows:

- Vector 1 µL
- DNA 3 µL
- Water 6 µL
- 2X buffer 10 µL
- Ligase 0.5 µL

The cells were cultured in an LB medium containing 50 µg/mL of kanamycin for 16 hours at 37 °C.

iii. PCR Colony and Sequencing

To ensure that ligation had occurred, one colony of each plate was harvested, and a PCR was run as follows using an NEB Taq PCR Kit:

- dNTP (10 mM) 2.5 µL
- Tag buffer (10X) 2.5 µL
- Tag DNA polymerase (5,000 unit/mL) 0.25 µL
- Primer DuetUP 1 (10 mM) 1 µL
- Primer DuetDOWN1 (10 mM) 1 µL
- $MgCl_2$ (25 mM) 0.75 µL
- Mq water 16 µL
- DNA template 1 µL

The PCR protocol:

- 95 ºC 2 min
- 95 ºC 30 sec
- 55 ºC 30sec } for 30 cycles
- 72 ºC 1 min
- 72 ºC 10 min
- 4 ºC ∞

The primers for MCS1 are DuetUP1 primer and DuetDOWN1 primer, and the primer for MCS2 are the DuetUP2 primer and T7 Terminator.

The PCR product was run on agarose gel to confirm the insertion of the ATCase gene in the vector.

After confirming the correct molecular weight for ATCase, the ATCase gene should be sequenced for the next step. The pRSF1 containing either ATCase *E. faecalis* or *T. sibiricus* is amplified by PCR as follows:

- BDT 1 µL
- 5× tag Buffer 3.5 µL
- Primer (10 mM) 0.32 µL (DuetUP1 primer or DuetDOWN1 primer)
- MQ water 10.18 µL
- DNA template 10 µL

PCR protocol:

- 94 ºC 5 min
- 96 ºC 10 sec
- 50 ºC 5 sec — for 50 cycles
- 60 ºC 4 min
- 4 ºC ∞

The gene sequencing was performed by the Sanger method at the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University.

iv. Digestion and ligation of CKase into pRSF1 containing ATCase

After the sequence confirmation of the construct having the ATCase gene in the pRSF1 plasmid, the CKase genes should be inserted into MCSII. Therefore, the pRSF1 and CKase genes were digested by *Nde*I and *Pac*I. After cleaning the CKase gene and pRSF1 as described, they were ligated into *E. coli* AN1549. The cells were cultured in an LB medium containing 50 mg/mL kanamycin for 16 h at 37 ºC.

v. PCR colony and Sequencing

To ensure that ligation has occurred, one colony from each plate was harvested, and PCR was run as explained before. The PCR product gene was run on agarose gel to detect the existence of CKase in the vector.

After confirming the correct molecular weight for CKase, the sequencing of the CKase gene was the next step. The pRSF1 containing the genes for CKase *E. faecalis* or *T. sibiricus* and ATCase *E. faecalis* or *T. sibiricus* was amplified by PCR and sequenced by the Sanger method at the ACRF Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University).

3.4.16. Preparation of Co-Expressed Cell-Free Lysates

One µL of RSFDuet-1 containing the genes of interest (CKase and ATCase) was transformed into 50 µL of *E. coli* (BL21 (DE3)) cells for expression. The cells containing the plasmid were grown in an LB agar plate containing 50 mg/mL kanamycin for 16 hours at 37 °C to make a starter culture. One colony from each plate was transferred to 5 mL of LB medium containing 30 µg/mL kanamycin for 16 h at 37 °C with 180 rpm shaking. The starter culture of *E. coli* was transferred to 500 mL LB containing kanamycin (30 µg/mL) to reach an OD600 of 0.6. The protein expression from the vector was then induced by using a final concentration of 1 mM IPTG and incubating for 5 hours. The cells were then collected from 500 mL of culture by centrifugation (4000 × *g* for 10 min), resuspended (1 mL/g wet cell pellet) in Tris-acetate buffer (10 mM, pH 8.3) containing 16 mM KOAc, 14 mM $Mg(OAc)_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. They were lysed on ice using a Thermo Fisher Scientific Omni Sonic Ruptor 400, equipped with an ORT-375 processing tip set at 50% power and 50% pulser for 6 min. The lysate was centrifuged at 20,000 × *g* for 60 min at 4 °C. The supernatant was aliquoted in an Eppendorf tube while being snap-frozen on dry ice and then stored at –80 °C for further usage.

3.4.17. Co-Expressed Lysate Activity Assay

The co-expressed lysates of CKase and ATCase were assayed similarly to the assay of the combination of CKase lysate and ATCase. The assays were performed at pH 9.4 and 40 °C in 0.5 mL water containing 200 mM $NaHCO_3/NH_4OH$ (pH 9.4), 10 mM L-L-aspartic acid, 10 mM $MgSO_4$, 10 mM ATP, 10 µL co-expressed lysate. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH to neutralise and then diluted with water by a factor of 10 before the analysis. The amounts of CA produced, and L-aspartic acid consumed were measured by HPLC/MS.

3.4.18. Carbamoyl Aspartate Production from Different Co-Expressed Lysate Assay

The characterisation assays were performed at different temperatures (10, 20, 40, 60 and 80 ºC) and different pH levels (8.5, 8.8 and 9.4 and not lower pH as it is beyond the reliable

buffering capacity of ammonia and bicarbonate). The assays contained 200 mM $NaHCO_3/NH_4OH$, 10 mM L-L-aspartic acid, 10 mM $MgSO_4$, 10 mM ATP and 10 µL of co-expressed lysate. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by adding 50 µL of 1 M NaOH for neutralising and then diluted with water before analysis. The amounts of CA produced, and L-aspartic acid consumed were measured by HPLC/MS.

3.4.19. Synthesis of aqueous acetyl phosphate

1 M solution of Acetyl phosphate was prepared from acetic anhydride and H3PO4 as previously described (Crans, 1983). Acetic anhydride was added slowly to H3PO4 (85%) at 0 °C with mixing. After 6 hours the aqueous solution was extracted with ethyl acetate, neutralised with NaOH, and stored at -20 °C until use.

3.5. Results and Discussion

In the previous chapter, ATCases were cloned and characterised. The pH and temperature study showed that all hyperthermophilic ATCases were thermo- and pH stable and have good stability and robustness. Also, in our published paper, CKases were cloned and characterised with respect to temperature and pH. It was confirmed that CKase from hyperthermophiles is more stable than mesophilic microorganisms. Moreover, the previous studies showed that ATCase and CKase from different sources have similar behaviours towards temperature and pH. Therefore, it might be possible to combine CKase and ATCase together to convert ammonia and carbon dioxide to CA. The focus of this chapter is the combination and optimisation of CKase and ATCase in an energy-efficient manner to remove ammonia and carbon dioxide and convert them to CA in high yield.

3.5.1. Expression and Purification of CKase and ATCase

The CKase gene and catalytic subunit genes of ATCase from *T. sibiricus, T. barophilus*, *P. furiosus* and *E. faecalis* were transformed into *E. coli* BL21 (DE3) cells for expression, and the cells were cultured overnight in LB medium. Good overexpression of ATCase was achieved, as explained in the previous chapter. Most CKases were soluble with a small amount in an insoluble fraction (Figure 3.7). The soluble fractions were purified through Ni (II) ion affinity chromatography. The determination of the molecular mass of the recombinant proteins was done by SDS-polyacrylamide gel electrophoresis. The observed molecular mass of CKase from *P. furiosus, T. barophilus, T. sibiricus* and *E. faecalis* agreed with the calculated molecular mass from the UniProt database. Further, the pure enzyme concentration was measured by spectrophotometry at 280 nm and calculated in mg from 100 mL cell culture, as shown in Table 3.1.

Table 3.1: Purified Enzyme Yields of CKase from 100 mL Cell Culture Measured by Spectrophotometry at 280 nm.

Organism	Yield (mg CKase/100 mL culture)	Extinction coefficient
T. barophilus	3.52	34.678
T. sibiricus	3.67	34.561
P. furiosus	1.39	34.429
E. faecalis	0.97	32.927

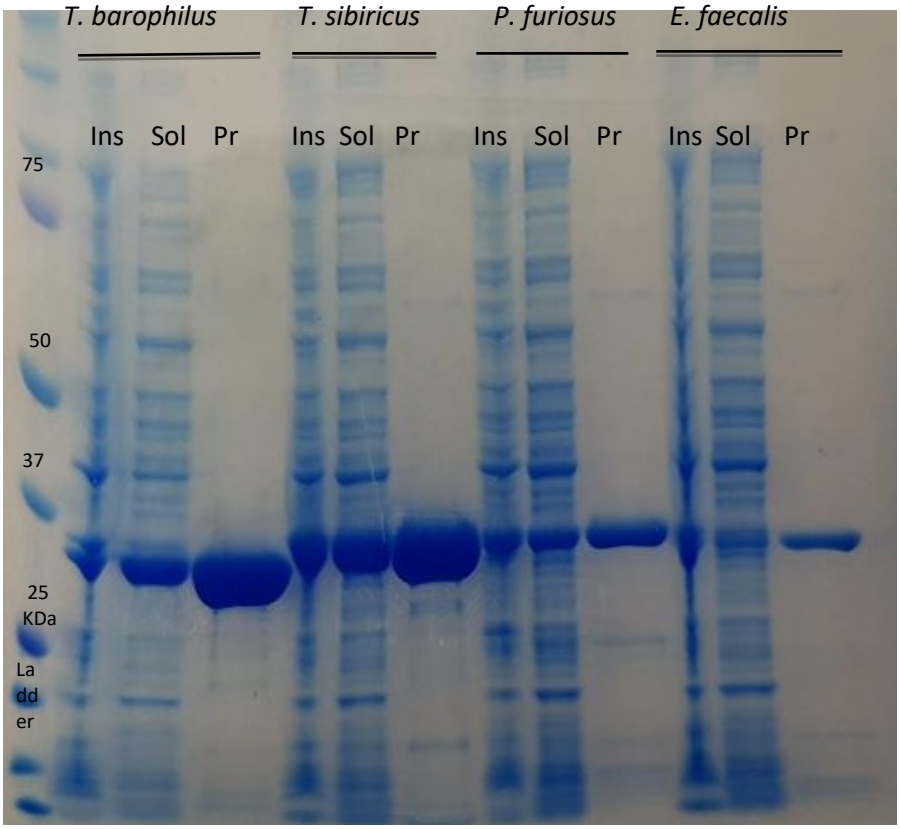


Figure 3.7: SDS-PAGE analysis of the diluted expression of CKase from *T. sibiricus*, *T. barophilus* and *P. furiosus*. *E. faecalis*. Molecular mass markers are the NEB 10-200 kDa unstained protein ladder. Ins = Insoluble fraction; Sol = soluble fraction; Pr = pure protein after Ni-column.

3.5.2. Aspartate Detection by HPLC in the Combination Assay

It has been explained in the previous chapter that aspartate could be easily detected and measured by HPLC using AccQ-Tag™ in the ATCase assay. However, in the combination assay of CKase and ATCase, discrepancies were observed in aspartate quantification at the time zero (the HPLC showed different numbers for the aspartate concentration at time zero, although the same amount of aspartate was used for all experiments). It was assumed that it might result from using different reaction mixtures in the combination of CKase and ATCase assay compared to the ATCase assay. In the combination CKase and ATCase assay, 200 mM bicarbonate and ammonia and 10 mM ATP were used instead of 50 mM Tris-HCl in the ATCase assay. The investigation revealed that less aspartate got tagged by increasing the ammonia and bicarbonate concentration (Figure 3.8). Therefore, it was assumed that aspartate tagging was either inhibited by ammonia, bicarbonate or ATP or AccQ-Tag™ became consumed by ammonia and bicarbonate. Further studies demonstrated that AccQ-Tag™ was consumed by ammonia; therefore, there was insufficient AccQ-Tag™ for aspartate. Hence, to solve this problem, either the AccQ-Tag™ should be increased or ammonia concentration should be decreased before the tagging. Since increasing AccQ-Tag™ concentration would not be cost-effective, the ammonia concentration was diluted by a factor of 40 before the tagging to the final concentration of 5 mM, in which all aspartate was fully tagged.

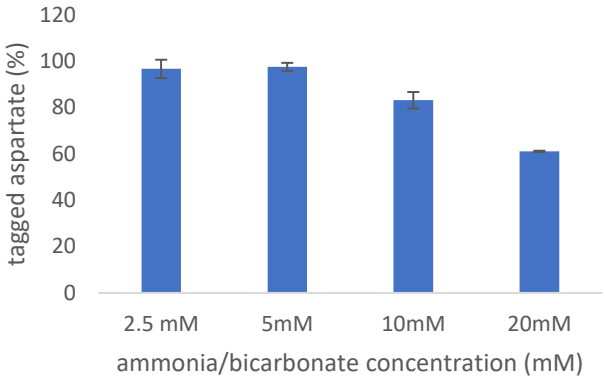
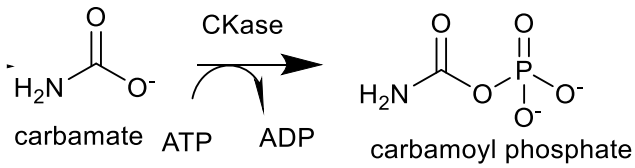


Figure 3.8: HPLC detection of aspartate in different concentrations of ammonia and bicarbonate. They were derivatised by the AccQ-Tag™ Reagent. The data is the average of duplicate experiments, and the standard deviation shows the deviation of duplicate experiments.

3.5.3. Detection of ATP, ADP and AMP by HPLC

To study CKase and ATCase combination activity, it is important to first measure each individual enzyme activity. As explained in the previous chapter, the ATCase enzyme activity was measured by aspartate consumption and CA production. In this chapter, the CKase activity measurement has been described. The activity of CKase was measured by measurement of ADP production (Virginie Durbecq et al., 1997) while producing CP (Scheme 3.7). The ADP formation was measured using the HPLC-UV method at 259 nm absorbance. The retention times for AMP, ADP and ATP were 6.9 min, 12.4 min and 23.9 min, respectively, and the standard curve was calculated for ADP (Figure 3.9).



Scheme 3.7: CP production by using CKase. One ADP molecule is formed for every one CP molecule.

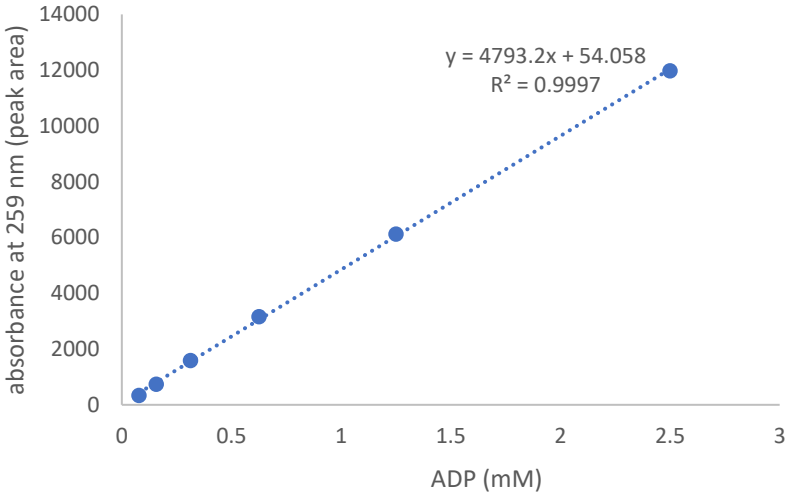
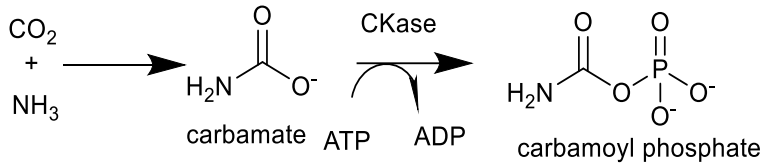


Figure 3.9: ADP standard curve calculated from HPLC results.

3.5.4. Determination of CKase Activity

To conduct CKase and ATCase combination experiment, the CKase and ATCase activity should be confirmed first. Therefore, CKase and ATCase activities were measured individually. ATCase activity has been confirmed in the previous chapter. In this chapter, CKase activity was measured at 40 °C (since all hyperthermophilic and mesophilic CKases

showed the highest activity at this temperature) by HPLC following the formation of ADP. The results showed that all CKases are active; CKase *E. faecalis* has the highest ADP production rate, followed by *T. sibiricus* and *T. barophilus* and *P. furious* has the lowest ADP production rate (Figure 3.10).



Scheme 3.8: CP production by using CKase. One ADP molecule is formed for every one CP molecule.

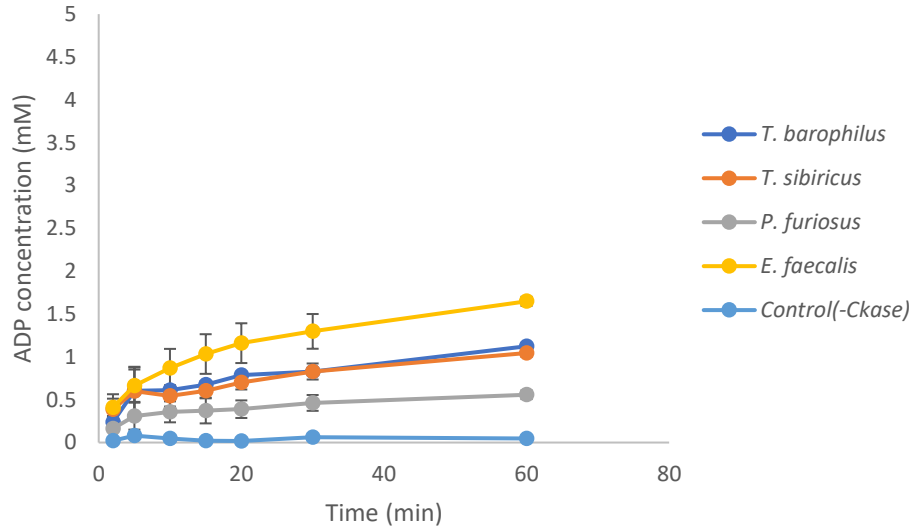


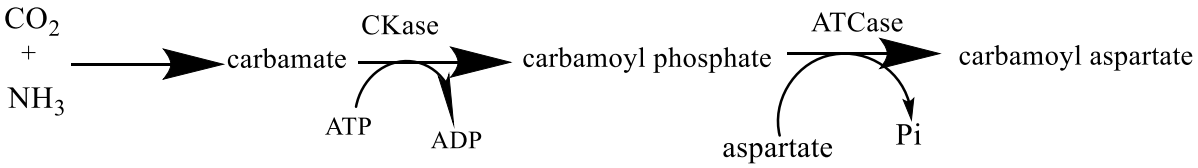
Figure 3.10: CKase activity during ADP formation as measured by reverse-phase HPLC. The reaction mixture contained 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 10 mM ATP, 10 mM $MgSO_4$ and 0.5 µM CKase from one of the enzymes *T. barophilus, T. sibiricus*, *E. faecalis* and *P. furiosus* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.5. Determination of the Activity of the Combination of Pure CKase and Pure ATCase

After confirming CKase and ATCase individual activities from the previous section and chapter, the combination of pure CKase and ATCase was assayed. In the CKase and ATCase combination assay, CKase produces CP from ammonia and bicarbonate using ATP as a phosphorylation cofactor. ATCase produces CA using aspartate and CP produced by

CKase (Scheme 3.9). Therefore, the production of the final product, CA, demonstrates that both pure enzymes are active in a combination reaction mixture and can work with each other. The experiment was performed at pH 9.4, as this was the pH between the optimum pH for ATCase (~8.8) and CKase (~10). Also, at this pH, the second highest carbamate concentration is produced from ammonia and bicarbonate (the highest carbamate production is at pH 9.9) (Hennessy et al., 2018). The ATCase and CKase from the same organism were used in each assay. The ATCase concentration was used in excess of CKase to ensure that all CP converts to CA.

The results indicated that CKase and ATCase can be successfully combined and that P. *furiosus* has the lowest CA production rate. Moreover, the CKase and ATCase combination from *E. faecalis* has the highest CA production rate, followed by *T. sibiricus* and *T. barophilus* as predicted from the individual activities of CKase and ATCase (Figure 3.11).



Scheme 3.9: Carbamoyl aspartate production using the combination of CKase and ATCase enzymes.

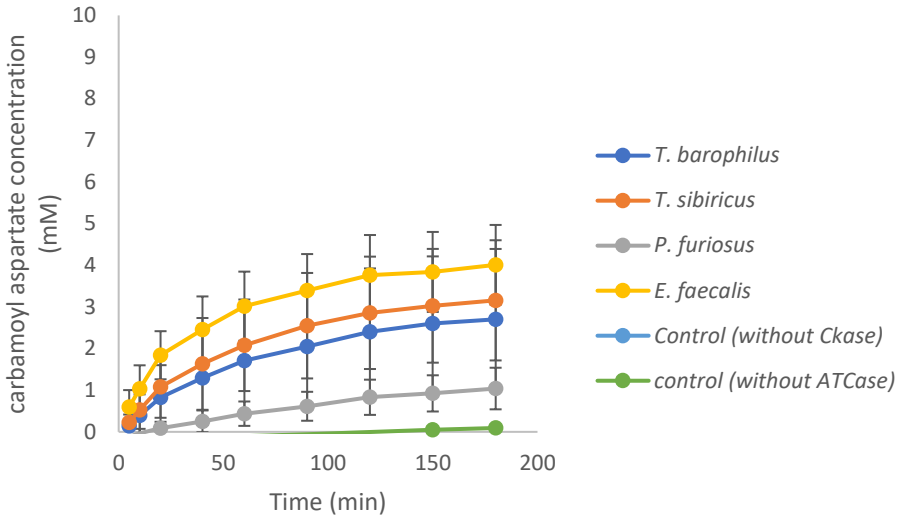


Figure 3.11: Carbamoyl aspartate production using a combination of pure enzyme CKase and ATCase. The CKase and ATCase were sourced from the same microorganism in each assay. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate and CKase: ATCase 1:10 from one of the enzymes *T. barophilus, T. sibiricus*, *E. faecalis* and *P. furiosus* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.6. Determination of the Best Combination of CKase and ATCase

The results obtained from the preliminary analysis of a combination of CKase and ATCase from the previous section confirmed that CKase and ATCase were able to work together. The product of CKase, CP, could be consumed by ATCase to produce the final product, CA.

The focus of this section is to determine the best combination of different sources of CKase and ATCase with respect to CA production rate at 40 °C. Therefore, several cross-combinations between CKase from *T. barophilus, T. sibiricus* and *E. faecalis,* and ATCase from *T. barophilus, T. sibiricus* and *E. faecalis* were conducted.

It should be noted that CKase and ATCase from *P. furiosus* were already excluded from further study because of demonstrating a low production rate for CP and CA in CKase and ATCase assays, respectively. Moreover, the combination of CKase and ATCase from *P. furiosus* also presented a lower CA production rate than the rest of the CKase and ATCase combination.

The results from the combination of CKase and ATCase from *E. faecalis* represented the highest CA production rate (Figure 3.12 bottom), followed by CKase from *T. sibiricus* and ATCase *E. faecalis* (Figure 3.12 middle). However, the production difference between the mentioned two combinations of CKase and ATCase from *E. faecalis* and CKase from *T. sibiricus* and ATCase *E. faecalis* were not significant (p>0.05). Accordingly, the combination of CKase *T. sibiricus* and ATCase *T. sibiricus* (Figure 3.12 middle) and the combination of CKase *E. faecalis* and ATCase *T. sibiricus* (Figure 3.12 bottom) showed the third- and fourth-level highest CA production rates, respectively. Lastly, the lowest CA production rate was observed in the combination of CKase from different sources and ATCase from *T. barophilus* (Figure 3.12 top). The CA production from the combination of CKase *T. barophilus* and ATCase from different sources was not significant (p>0.05).

Therefore, in summary, CKase and ATCase from *T. sibiricus* and *E. faecalis* had the highest CA production rate. Yet, it should be considered that these data were obtained from the experiments conducted at 40 °C, otherwise, the hyperthermophile would probably exhibit better results. Consequently, to pursue our study, the CKase and ATCase from *T. sibiricus* and *E. faecalis* were considered the best enzyme combination with respect to CA production rate. The CKase and ATCase from *T. barophilus* were excluded from the further study. They did not show the competitive CA production rate compared to the rest of the enzyme combinations.

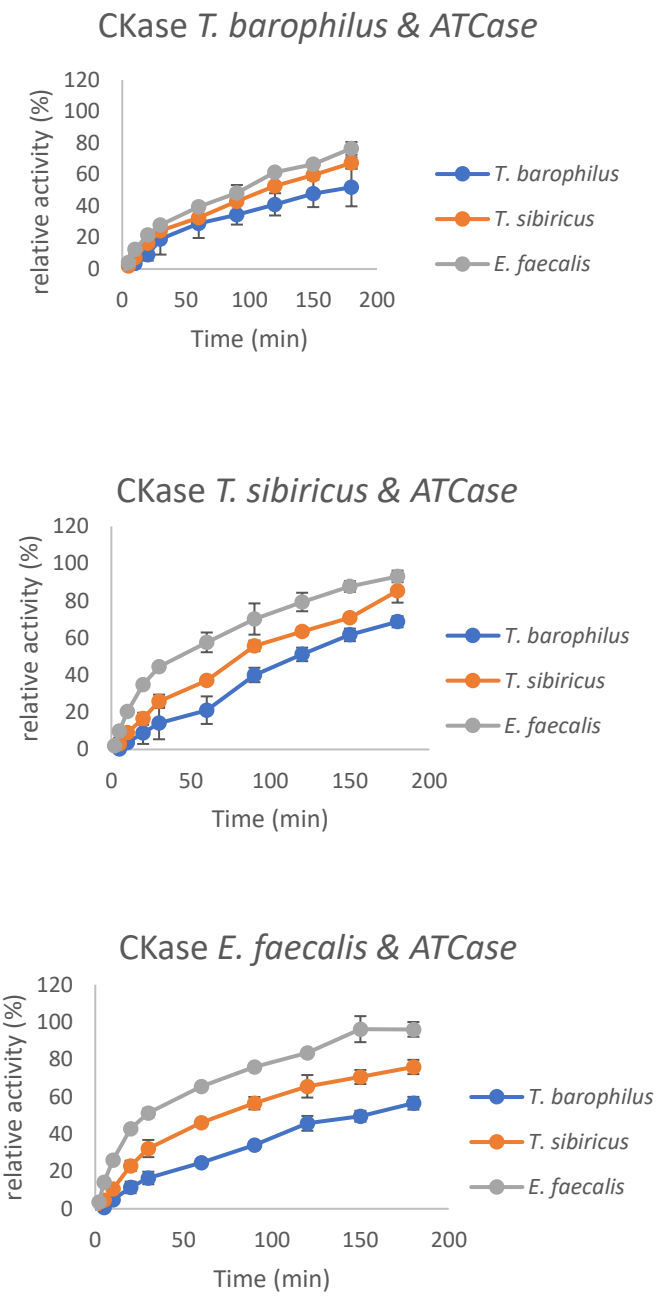


Figure 3.12: Choosing the best combination of CKase and ATCase. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate and 1 unit CKase and 16 unit ATCase from one of the *T. barophilus, T. sibiricus* and *E. faecalis* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.7. Combining CKase with Varying Concentration of ATCase

The previous section illustrated the best CKase and ATCase combination. It concluded that CKase and ATCase *E. faecalis* and *T. sibiricus* were the best enzymes for further study with

respect to the CA production rate. In this section, the ratio of CKase and ATCase was investigated. The enzyme unit used in the assay was calculated with respect to mM/min production of either CA or ADP. The ratios of ATCase to CKase were 1, 2, 4, 8 and 16.

The results demonstrated that the CA production increases with the increase of ATCase (Figure 3.13), However, by increasing the CKase ratio, the CA production did not change (data are not shown). Therefore, ATCase is a rate-limiting enzyme in the combination assay. Further, the combination of CKase and ATCase from *E. faecalis* had the highest production rate of CA (6.17 mM CA production in 3 min), followed by *T. sibiricus* (5.15 mM CA production in 3 min).

Taken together, these results suggest that the combination of CKase and ATCase from mesophiles has a higher CA production rate compared to hyperthermophiles. However, we should not neglect that these results have been obtained from experiments conducted at 40 °C, which is not the hyperthermophile optimal temperature. Therefore, it is important to mention that these results were achieved from the temperature range close to the mesophile optimal temperature rather than for hyperthermophiles. Therefore, it could be expected to observe a better production rate for mesophiles than for hyperthermophiles. However, the hyperthermophile *T. sibiricus* showed only slightly less CA production rate than mesophile *E. faecalis*, indicating high enzyme activity in hyperthermophiles, even at low temperatures.

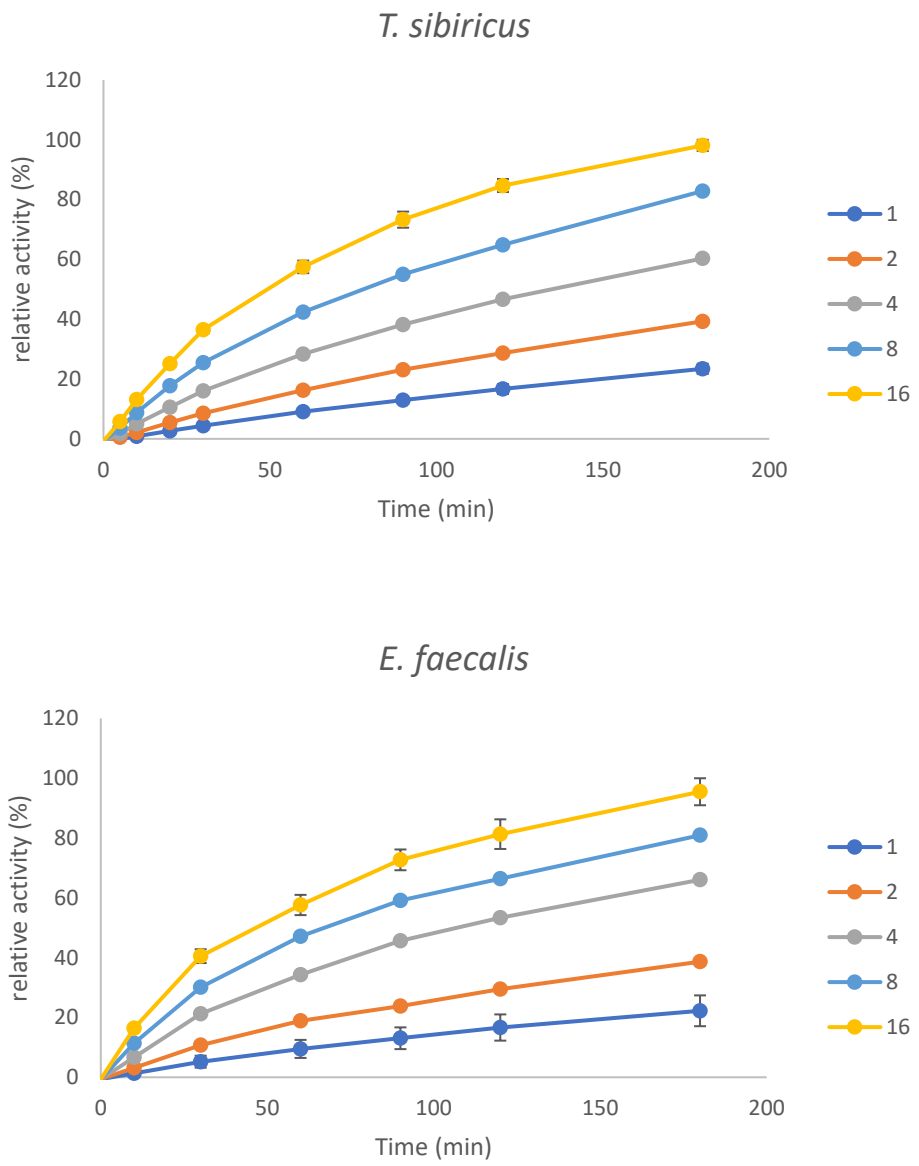
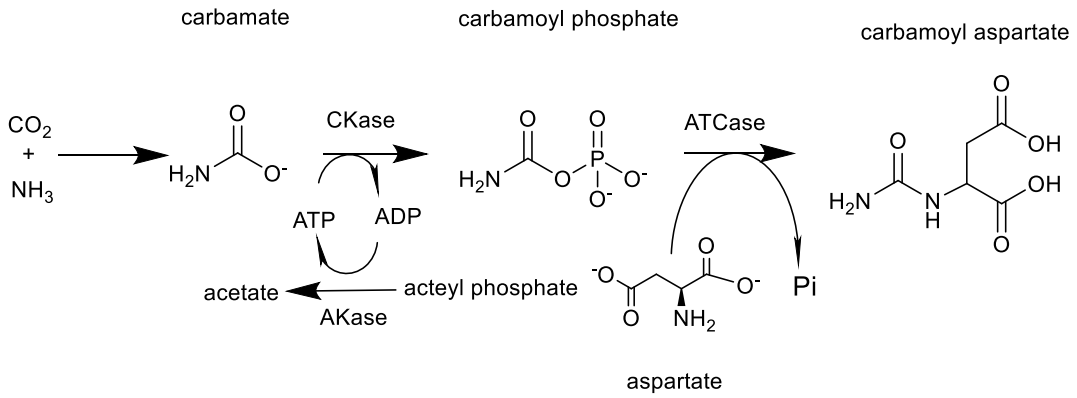


Figure 3.13: Combination of CKase and ATCase *E. faecalis* and *T. sibircus* with varying ratio of ATCase. The ratios of ATCase to CKase are 1, 2, 4, 8 and 16. In each assay, the CKase and ATCase were used from the same microorganism of *T. sibiricus* or *E. faecalis*. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate and 1 unit (mM/min ADP production) CKase and different unit (mM/min CA production) ATCase from one of the microorganism *T. sibiricus* and *E. faecalis* at 40 ºC. Values with standard bar are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.8. Applying the ATP Recycling System to the Best Combination of Pure CKase and ATCase Enzymes

The results obtained from the previous section presented that ATCase is a rate-limiting enzyme. Therefore, its concentration should be kept higher than CKase to ensure that ATCase consumes all CP. The highest CA production rate will be achieved by considering the CKase and ATCase ratio from the previous section.

In the following section, it was presented the application of the ATP recycling system in the combination of CKase and ATCase (Scheme 3.10). CKase uses one molecule ATP to convert carbamate to CP. Therefore, it will be an efficient process if ATP can be recycled. Acetate kinase is an enzyme that efficiently catalyses the conversion of ADP and acetyl phosphate to ATP and acetate to recycle the ATP. It is worth mentioning that in our previous study, we found that *E. coli* lysate in the S30 buffer had abundant endogenous acetate kinase to recycle the ATP (Hennessy et al., 2018; Loan, Easton, & Alissandratos, 2019).



Scheme 3.10: Schematic view of CKase and ATCase combination while ATP is recycling by acetate kinase (AKase) existing in *E. coli* lysate, named S30. Acetyl phosphate was prepared in the lab from acetic anhydride and H_3PO_4 as previously described (Crans and Whitesides1983) and added to the system.

Therefore, the application of the ATP recycling system was examined in this section. Firstly, the acetate kinase activity from *E. coli* S30 lysate was examined by adding ADP and AP to lysate and observing ATP production and ADP consumption.

Then, the correlation between ATP concentration and CKase activity in a combination assay of CKase and ATCase was studied. Finally, the *E. coli* S30 lysate was added to the combination of CKase and ATCase to explore the activity of the ATP recycling system in the combination assay of CKase and ATCase.

The results from *E. coli* S30 lysate showed that acetate kinase was present and active in the lysate and was able to convert ADP and APto ATP and acetate (Figure 3.14). Also, the results showed that a low concentration of AMP was produced from ADP conversion at the beginning of the reaction. The AMP production results from adenylate kinase (another endogenous enzyme in *E. coli* S30 lysate that catalyses the interconversion of adenine nucleotides) that catalyses the reversible conversion of AMP to ADP. However, the ADP from adenylate kinase activity also converts to ATP by acetate kinase.

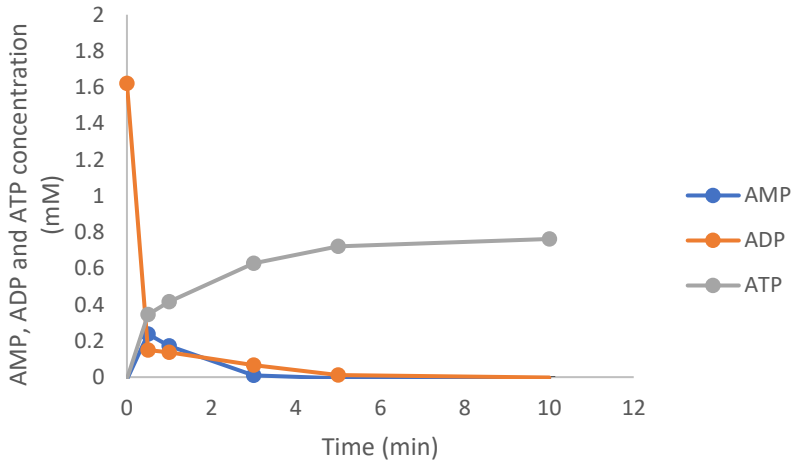


Figure 3.14: Acetate kinase activity measured by measuring ADP consumption and ATP production by reverse-phase HPLC. The 0.5 mL reaction mixture contained 200 mM NH_4OH and $NaHCO_3$ pH 9.4, 1 mM ADP, 5 mM acetyl phosphate, 10 mM $MgSO_4$ and 10 µL *E. coli* lysate S30 at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

Further, the results from the correlation between CKase activity and ATP concentration in a combination assay of CKase and ATCase confirmed that by elevating ATP concentration, the CA concentration proportionally increased. However, at a higher concentration of ATP, the CA production rate slightly decreased compared to a lower ATP concentration (Figure 3.15). This might be due to ATCase acting as the rate-limiting enzyme that probably cannot convert all produced CP to CA at the same speed as CP production.

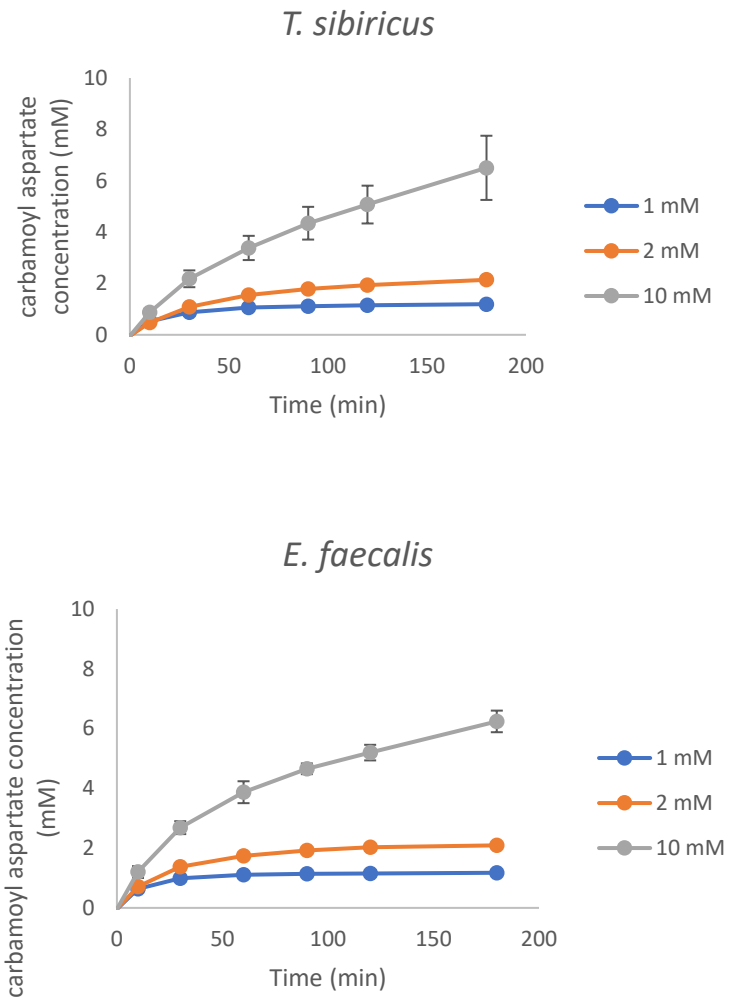


Figure 3.15: CA production formation vs. time using different concentrations of ATP and a combination of CKase and ATCase. In each assay, the CKase and ATCase were used from the same microorganism. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 1, 2 or 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate and 1 unit CKase and 16 unit ATCase from one of the enzymes from *T. sibiricus* and *E. faecalis* at 40 ºC. The trend with the standard bar shows the duplicated experiments, and their values are the average of duplicate experiments.

Finally, the results from coupling the ATP recycling system from *E. coli* lysate S30 with the combination of CKase and ATCase (using ADP instead of ATP in the assay) showed that acetate kinase was active in a combination of CKase and ATCase and was able to recycle ATP from ADP and acetyl phosphate (Figure 3.16). The results demonstrated by using 10 mM acetyl phosphate, ATP turned over around 4 and 40 times using 1 mM and 0.1 mM ADP respectively, for CKase and ATCase *T. sibiricus,*. Also, ATP turned over more than three times and 30 times using 1 mM and 0.1 mM ADP respectively, for CKase and ATCase *E.*

faecalis. Consequently, the results showed that the CA production significantly decreased after almost one hour and yielded between 40% to 50% CA production with respect to aspartate concentration when using the ATP recycling system. The ATP turnover was measured based on the initial ADP and final CA concentrations. This assumes one stoichiometric amount of ATP donates one phosphate to one carbamate to produce one CP by CKase activity.

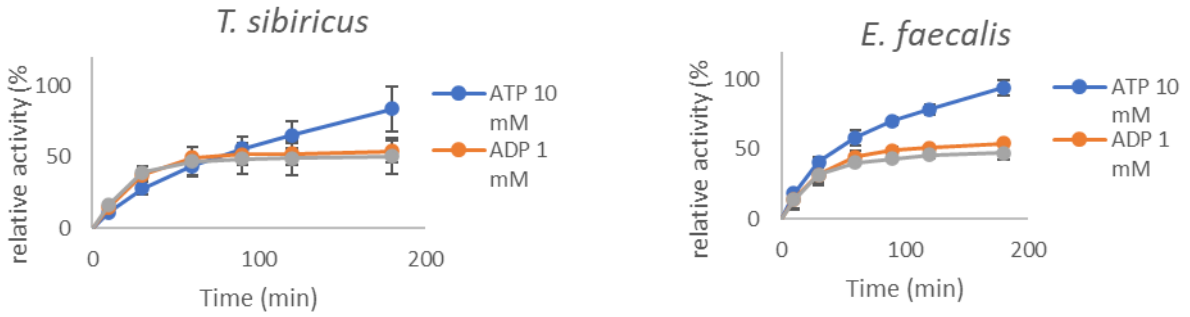


Figure 3.16: Applying ATP recycling system in a combination of CKase and ATCase. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ pH 9.4, 1 mM ADP, 10 mM acetyl phosphate, 10 µL (2% v/v) S30 lysate, 10 mM $MgSO_4$, 10 mM aspartate and 1 unit CKase and 16 unit ATCase from one of the enzymes from *T. sibiricus* and *E. faecalis* at 40 ºC. The trend with the standard bar shows the duplicated experiments, and their values are the average of duplicate experiments.

3.5.9. Varied *E. coli* Lysate S30

The previous section demonstrated that the ATP recycling system was present and active in E. coli lysate. In this section, we aimed to measure the efficiency of the E. coli S30 lysate with respect to acetate kinase concentration. Therefore, different amount of lysate was used in the combination of CKase and ATCase experiments. The results showed that by increasing the lysate amount (2 to 10 µL), CA production increased slightly and not significantly ($p>0.05$). Further, it exhibited that volumes as low as 1 µL (0.2% v/v) of S30 lysate have enough acetate kinase to convert ADP to ATP (Figure 3.17). Overall, these results suggested that the acetate kinase is abundant in the E. coli S30 lysate and can easily convert ADP to ATP using acetate kinase.

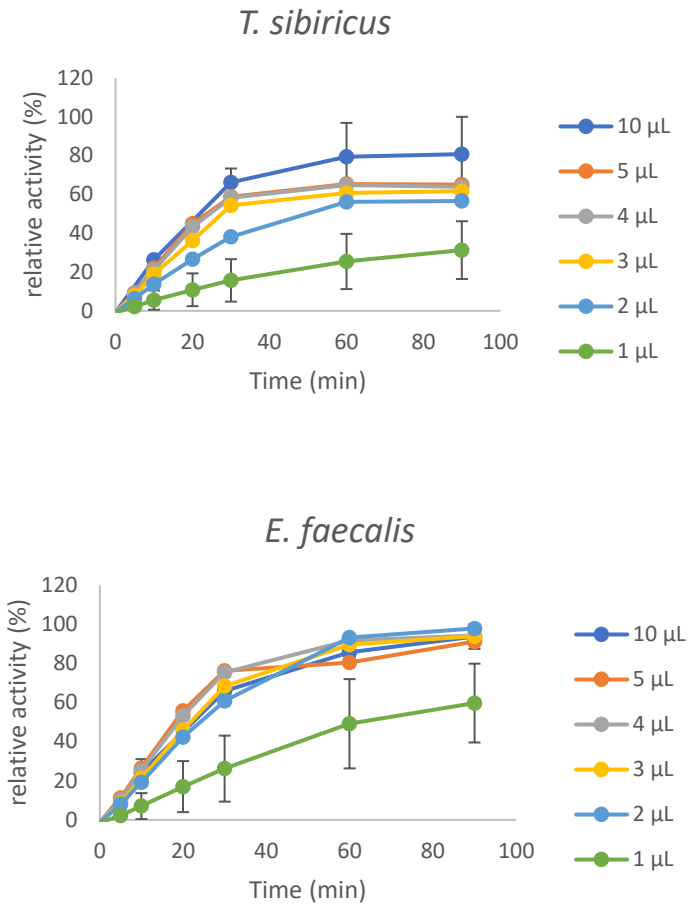


Figure 3.17: CA concentration when using different amounts of S30 lysate with a combination of CKase and ATCase. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 0.1 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate, 10 mM acetyl phosphate, different amount of S30 lysate (2%-0.2% v/v) and 1 unit CKase and 16 unit ATCase from one of the enzymes *T. sibiricus* and *E. faecalis* at 40 ºC. The trend with the standard bar shows the duplicated experiments, and their values are the average of duplicate experiments.

3.5.10. Using AMP as a Cofactor for ATP Recycling System in the Combination of CKase and ATCase Assay

The previous section showed that *E. coli* lysate contains acetate kinase that converts ADP to ATP in the combination of CKase and ATCase experiment. This section examined the possibility of using another cheap and more stable cofactor AMP. Our previous study showed that *E. coli* S30 lysate contains endogenous adenylate kinase in addition to acetate kinase (Hennessy et al., 2018). The adenylate kinase converts the cheaper and more stable cofactor AMP to ATP.

Therefore, to conduct these experiments, AP was used as the source of phosphate. The *E. coli* lysate was used as the acetate kinase and adenylate kinase sources in the assay. If ATP recycles, CA production must be more than the initial cofactor concentration.

The results showed that the ATP recycling system was active, and AMP was converted to ATP at high turnover based on the initial cofactor sub-stoichiometric quantities and the final product (Figure 3.18). The average ATP turnover when using 0.1 mM AMP was more than 30 times in *E. faecalis* and around 40 times in *T. sibiricus,* although a delay of 5 min is observed as ATP forms and the reaction reaches the steady state. Consequently, the results showed that the CA production stopped after almost two hours and yielded between 40 to 50% CA production with respect to aspartate concentration when using the ATP recycling system (ADP and AP). This may be due to the effect of AP concentration on the ATP recycling system. The AP might have been decomposed in aqueous solution and therefore affected the acetate kinase activity (the concentration of AP affects the ATP recycling system because acetate kinase uses acetyl phosphate as a phosphate donor to convert ADP or AMP to ATP). Therefore, an assay must be performed to investigate the relationship between CA production and AP concentration in the reaction mixture.

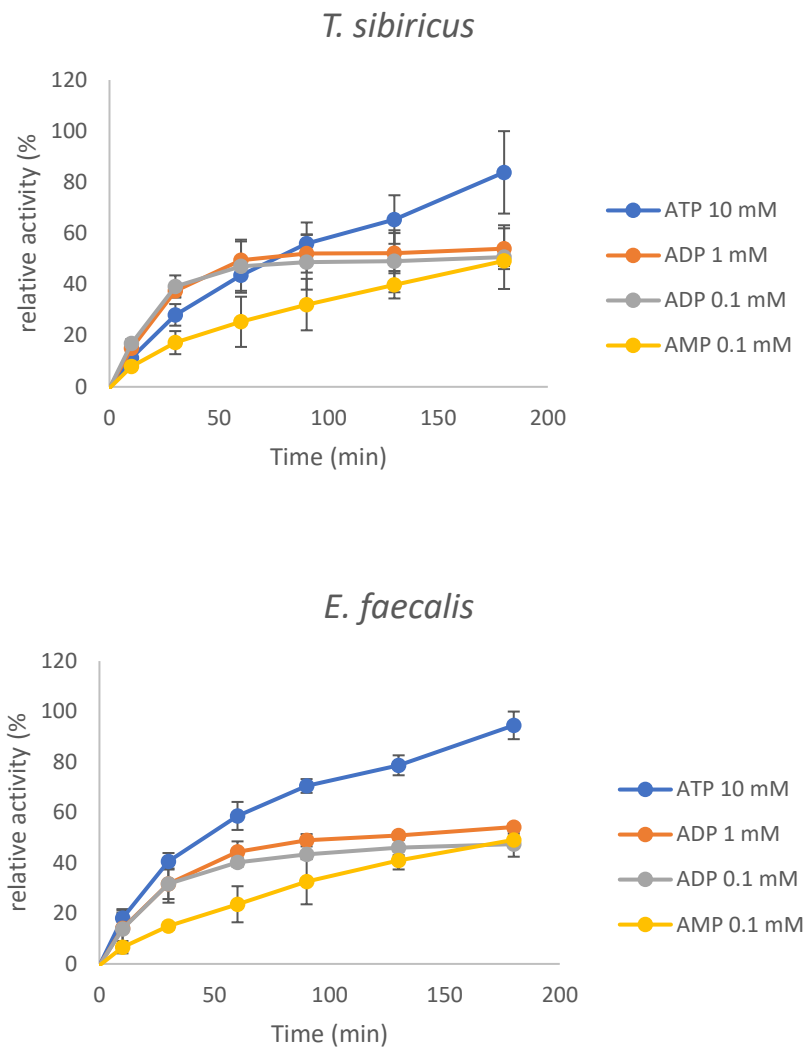


Figure 3.18: The effect of using AMP in CA production when using a combination of CKase and ATCase. The reaction mixture in the ATP recycling system contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 0.1 mM AMP, 10 mM $MgSO_4$, 10 mM aspartate, 10 mM acetyl phosphate, 2% v/v S30 lysate and 1 unit CKase and 16 unit ATCase from one of the enzymes *T. sibiricus* and *E. faecalis* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.11. Varied Acetyl Phosphate

The previous results suggested that the concentration of acetyl phosphate might affect CA production and can be the limiting reagent for ATP recycling system enzymes. Therefore, this section investigated the relationship between AP concentration and CA production rate in a combination assay of CKase and ATCase.

The results suggested that AP (which is made in the lab from acetic anhydride and inorganic phosphate) is a limiting reagent for ATP recycling system in the combination of CKase and ATCase assays (Figure 3.19). At a lower concentration of AP, the CA production rate is around 100% (AP concentration ≤2.5 mM)), and the rate gradually decreases as the AP concentration increases. At concentrations of 10 mM AP, the CA production rate was around 50%.

This might be due to the hydrolysis of AP during the assay. It has been shown in previous studies that AP hydrolysed up to 20% at 20 °C pH range 7 to 11 (Loan, Easton & Alissandrates, 2019). Also, it was demonstrated that temperature affected the AP hydrolysis, and by elevating the temperature, the AP hydrolysis increased. Yet, the pH has little effect on hydrolysis at any temperature (Alexandra Whicher, Eloi Camprubi, Silvana Pinna, Barry Herschy, & Nick Lane, 2018).

The other reason for not achieving a 100% CA production rate with respect to AP could be ATCase promiscuity towards AP. It has been shown in the previous chapter that ATCase from hyperthermophiles at a high concentration of AP showed some degree of promiscuity towards AP. It has been shown that ATCase can convert AP and aspartate to the by-product acetyl aspartate.

Therefore, to overcome these problems, the AP concentration should be kept low in the solution to avoid hydrolysis. It should be added to the solution gradually and portion-wise and at a higher concentration (due to the inevitable decomposition rate) than needed.

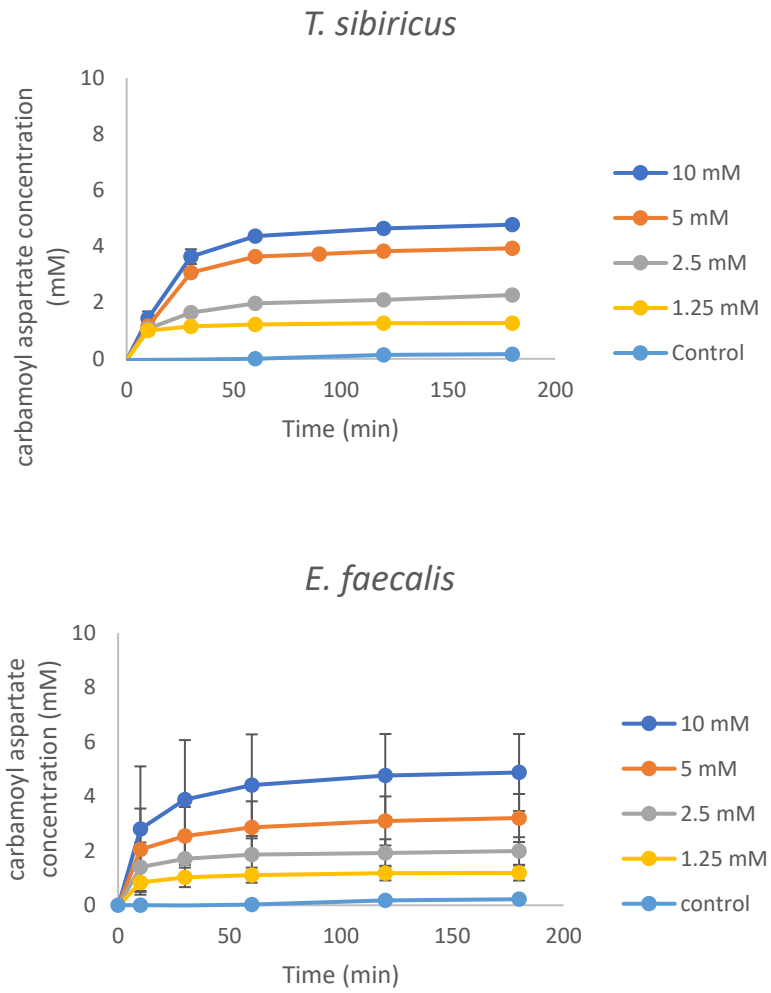


Figure 3.19: The effect on CA production using different acetyl AP concentrations with a combination of CKase and ATCase. The CKase and ATCase were used from the same microorganism in each assay. The reaction mixture in the ATP recycling system contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 0.1 mM AMP, 10 mM $MgSO_4$, 10 mM aspartate, 2% v/v S30 lysate, 1.25–10 mM AP and 1 unit CKase and 16 unit ATCase from one of the microorganisms *T. sibiricus* and *E. faecalis* at 40 ºC. The trend with the standard bar shows the duplicated experiments, and their values are the average of duplicate experiments.

3.5.12. Optimal Combining Conditions of CKase and ATCase with ATP Recycling System

As was demonstrated in the previous sections, CKase and ATCase could be combined successfully while ATP was recycling using endogenous *E. coli* S30 lysate enzymes. Also, it was pointed out that AP is a limiting factor in this assay and should be added at higher concentration and gradually to the solution. This section focuses on optimising the assays to achieve the highest CA production rate. Since AP was the limiting factor and it was observed

that at a concentration of about 10 mM, the CA production rate was less than 50%. Therefore, double the amount of AP was used and added portion-wise (20 mM AP was used to ensure there was always an excess of AP in the reaction mixture and available for acetate kinase activity) to gain 10 mM CA production.

The results indicated that in the combination assays of the CKase and ATCase *E. faecalis* and CKase and ATCase *T. sibiricus*, the CA yields were ~91% and 79% respectively, regarding aspartate by adding 0.1 mM AMP (Figure 3.20).

Next, the CKase and ATCase combination assay was performed similarly to those above, but without the addition of any cofactor. This was done to investigate whether there is enough endogenous cofactor in the *E. coli* S30 lysate to initiate the assay. Interestingly, the results exhibited CA production without adding any cofactor and only by adding AP. The CA yield for a combination of CKase and ATCase *E. faecalis* and CKase and ATCase *T. sibiricus* was around 80% and 60%, respectively. Also, the data showed that the initial rate of CA production in assays without a cofactor was slower than in the assays with added AMP, presumably due to the lower concentration of the cofactor from lysate. However, the *E. coli* lysate had enough endogenous ATP (probably from the breakdown of endogenous adenosine-containing materials) to initiate the acetate kinase activity after adding AP (Figure 3.20) (Loan et al., 2019).

In conclusion, the combination of CKase and ATCase produced a CA production rate up to 80% with respect to aspartate, while ATP is recycling without adding any exogenous ADP, AMP or ATP.

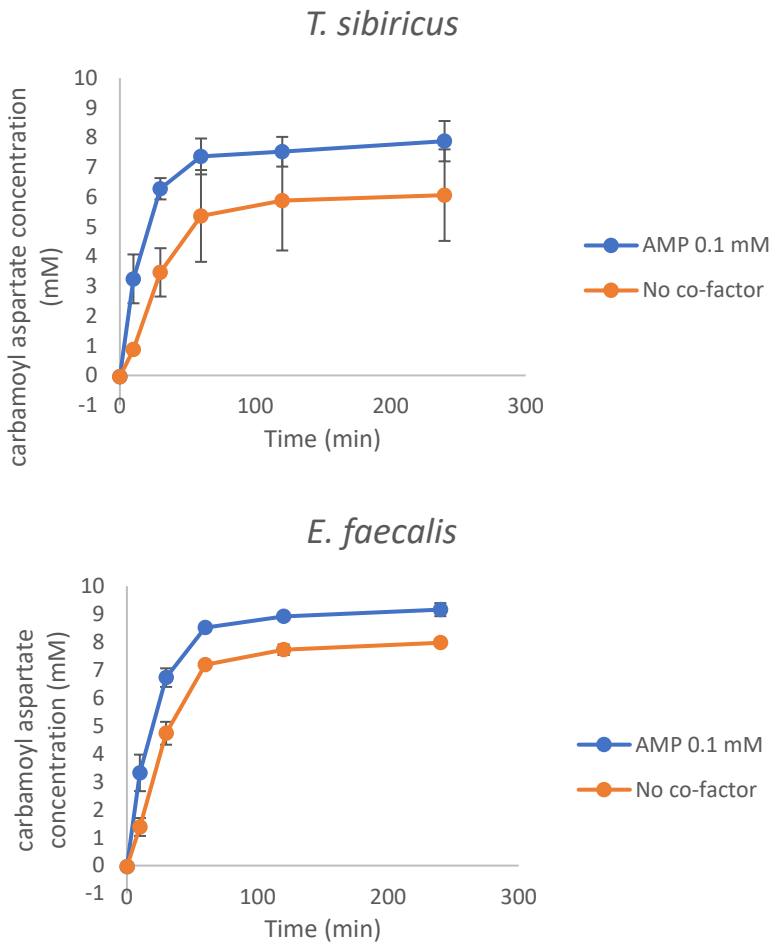


Figure 3.20: Optimisation of the conditions for CA production using a combination of CKase and ATCase. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 0.1 mM AMP or no cofactor (no ADP), 10 mM $MgSO_4$, 10 mM aspartate, 10 µL S30 lysate, 20 mM acetyl phosphate and 1 unit CKase and 16 unit ATCase from one of the enzymes *T. sibiricus* and *E. faecalis* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.13. Characterisation of the CKase and ATCase Combination

As explained earlier, CKase and ATCase could successfully be combined and optimised to gain the highest CA production rate. To better understand CKase and ATCase combination behaviour, the combination of CKase and ATCase should be characterised at different pH values and temperatures. In this part, CKase and ATCase combination of *T. sibiricus* and *E. faecalis* were characterised at different pH values and temperatures.

In the first experiments of this section, CA production was evaluated using a CKase and ATCase combination from *T. sibiricus* and *E. faecalis* at three different pH values 8.5, 8.8 and 9.4 (as this is an important pH range for the next chapter) (Figure 3.21).

The results from the pH study showed that in the combination of CKase and ATCase *T. sibiricus* at different pH values and *E. faecalis* at pH values 8.5 and 8.8 there is no significant difference in CA production. The results also indicated that in the combination of CKase and ATCase *E. faecalis,* the CA production decreased slightly at pH 9.4, probably due to less ATCase activity at higher pH. However, the production decrease was not significant (Figure 3.21).

Therefore, it could be concluded that in the combination of CKase and ATCase, ATCase has a more prominent role at different pH (although not significant) rather than CKase due to ATCase being the rate-limiting enzyme.

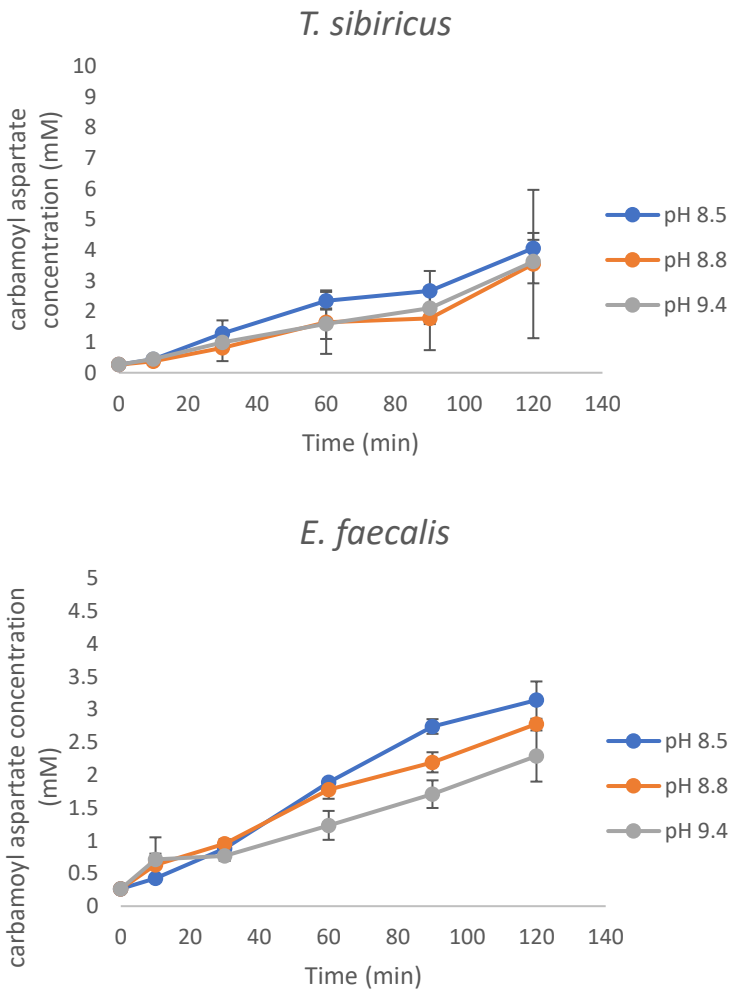


Figure 3.21: Production of CA using CKase and ATCase combinations at different pH values. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 8.5, 8.8 and 9,4), 10 mM $MgSO_4$, 10 mM aspartate, 10 µL S30 lysate, 20 mM AP (adding gradually and portion-wise to the mixture over 120 minutes) and 1 unit CKase and 16 unit ATCase from one of the enzymes *T. sibiricus* and *E. faecalis* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

Conversely, the effects of temperature on CKase and ATCase combination were studied in the second experiments sections. The results from the temperature study indicated that CA production increased by increasing the temperature in a combination of CKase and ATCase *T. sibiricus* (Figure 3.22). This might be because both CKase and ATCase were from hyperthermophilic enzymes that are more stable at high temperatures. Although it was noted in the previous chapter in ATCase kinetic properties studies that ATCase activity from *T.*

sibiricus gradually decreased at higher temperatures, it was explained this could be probably due to the lack of regulatory subunits. Thus, the question that needs to be addressed here is 'why does CA production increase at elevating temperature in the presence of the CKase and ATCase combination'? An answer to this question might be metabolic channelling between CKase and ATCase that transfers CP from CKase directly to ATCase through a channel without diffusion into the solution. Thus, CKase attachment to ATCase increases the ATCase stability and protects the ATCase from activity loss at higher temperatures. Therefore, ATCase activity increased at higher temperatures and more CA was produced. Moreover, it has been shown that metabolic channelling plays an important role in the cell's physiology in microorganisms, and it can protect the labile metabolites from thermal decomposition. Hence, CP could be protected and converted to CA (Legrain et al., 1995; J. Massant & Glansdorff, 2004; Jan Massant & Glansdorff, 2005; Jan Massant et al., 2002; Purcarea et al., 1999; Q. Wang et al., 2008). Finally, hyperthermophile CKase activity increases with an elevation of temperature (Hennessy et al., 2018).

However, in a combination of CKase and ATCase from *E. faecalis,* CA production increased from 10 to 40 °C and then decreased by increasing the temperature from 40 to 60 °C and stopped at 80 °C. This is probably due to the activity loss in the CKase and ATCase mesophilic enzymes. Since both CKase and ATCase were mesophilic enzymes and lost their activity at high temperatures, they could not protect each other from activity loss. Consequently, the CKase and ATCase combination from *T. sibiricus* exhibited better temperature stability and pH stability.

Overall, after characterising the CKase and ATCase combination and confirming the activity of acetate kinase in lysate to recycle ATP as previously stated, it may be advantageous to produce a recombinant lysate from either CKase or ATCase in *E. coli* rather than using exogenous *E. coli* lysate. By using the recombinant lysate, we can minimise the consumption of medium and lysate and reduce the purification step.

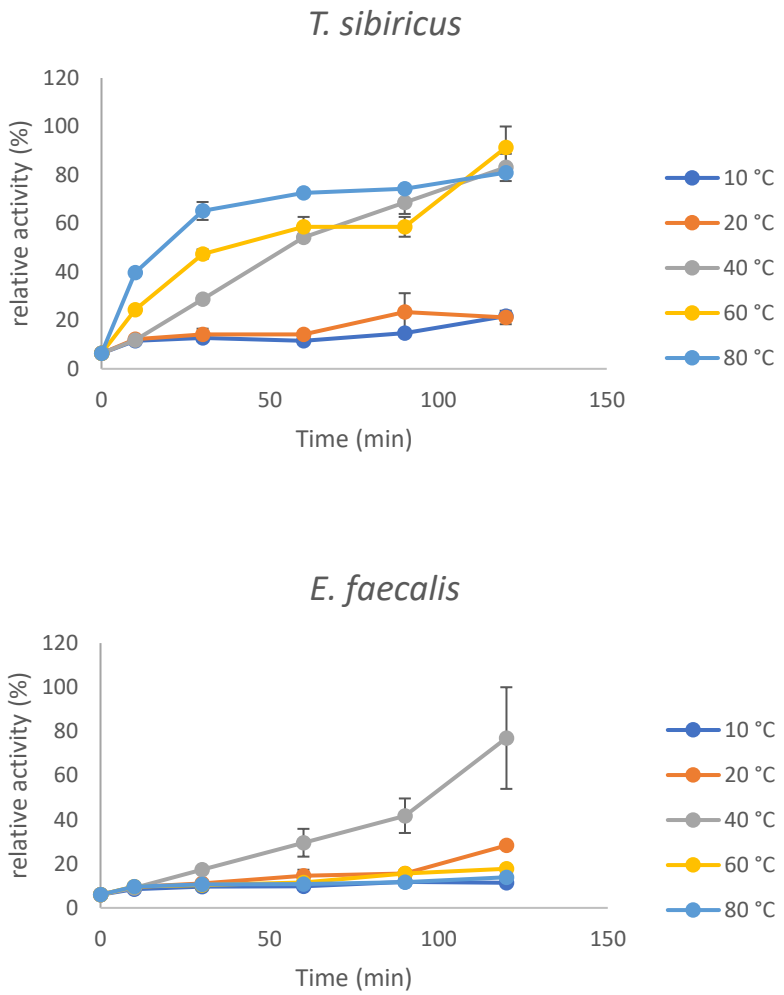


Figure 3.22: The effect of temperature on CA production when using CKase and ATCase combinations. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 10 mM $MgSO_4$, 10 mM aspartate, 10 µL S30 lysate, 20 mM acetyl phosphate (adding portion-wise) and 1 unit CKase and 16 unit ATCase from one of the microorganism *T. sibiricus* and *E. faecalis*. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.14. Combining CKase Lysate, Purified ATCase and ATP Recycling System

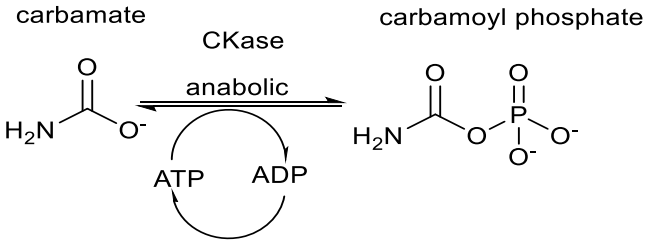
As described fully in the previous sections, it was shown that *E. coli* lysate contains an ATP recycling system comprising acetate kinase and adenylate kinase, which could be coupled with the combination of pure CKase and ATCase assay to recycle ATP. Herein, we aim to develop an alternative process to avoid using separate *E. coli* lysate. In this section, we study the recombinant overexpression of CKase in *E. coli* lysate. This helps to avoid the timely and costly purification step and also minimises the number of reaction mixture

ingredients. In this experiment CKase (and not ATCase) was considered for overexpression in *E*. *coli* as CKase activity was dependent on ATP. Therefore, by producing the recombinant crude lysate, the proof of the concept of CKase activity and ATP recycling system could be examined. Hence, CKase recombinant crude lysates from *T. sibiricus* (as the best CKase and ATCase combination for CA production and stability at a different temperature) and *E. faecalis* (as a mesophilic representative) were prepared, and the activity of these enzymes in the presence of the ATP recycle system in the lysate were studied.

The results illustrated that CKase from *T. sibiricus* and *E. faecalis* in the *E. coli* lysate were present and active in the reaction mixture and produced CP, while ATP was recycling using endogenous enzymes acetate kinase (Figure 3.23).

Further, CKase lysates were combined with pure ATCase (to ensure that ATCase can function with CKase in the crude lysate) from the same organisms, while ATP was recycled using AP. The results indicate that both CKase and ATCase were active in a combination of CKase crude lysate and ATCase pure enzyme, while the ATP recycling system was also active (Figure 3.24).

Moreover, the results showed that CA concentration from the second experiment was greater than ADP concentration from the first experiment from CKase lysate activity, which indicates CP production. Thus, more CP was produced in combination CKase lysate and ATCase compared to CKase lysate alone. The reason might be due to the consumption of CP by ATCase and therefore moves the equation towards CP production.



Scheme 3.11: CP production by CKase.

Consequently, CKase lysate contained all necessary enzymes and was combined with ATCase to produce the final product. Therefore, in the next step, instead of making ATCase crude lysate and adding it to CKase crude lysate, a single lysate containing both CKase, ATCase and the ATP recycling system should develop through the co-expression of the required recombinant enzymes from *T. sibiricus* and *E. faecalis*.

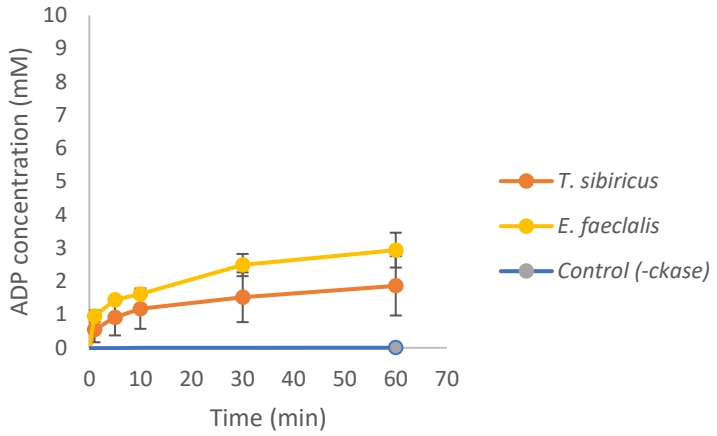


Figure 3.23: ADP production by overexpression of CKase from *E. coli* crude lysate. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ pH 9.4, 10 mM $MgSO_4$, 20 mM AP and 10 µL CKase lysate from one of the enzymes *T. sibiricus* and *E. faecalis* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

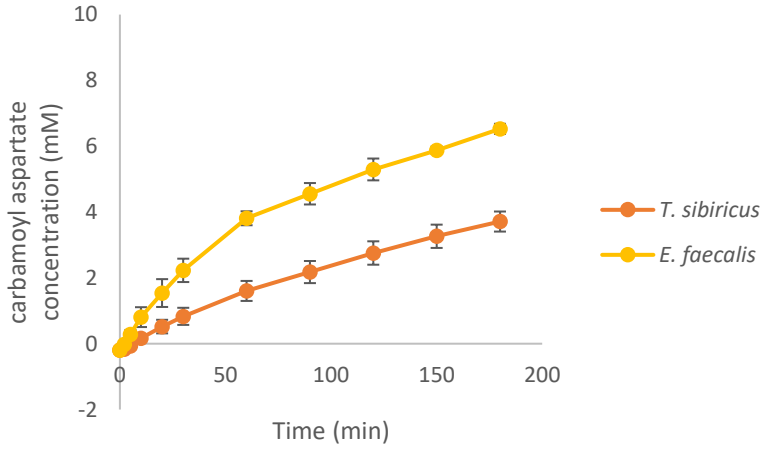


Figure 3.24: CA production while using a combination of CKase crude lysate and pure enzyme ATCase. The CKase and ATCase were used from the same microorganism in each assay. The reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 9.4), 10 mM $MgSO_4$, 10 mM aspartate, 20 mM acetyl phosphate, 10 µL CKase lysate and 16 unit ATCase from one of the enzymes *T. sibiricus* and *E. faecalis* at 40 ºC. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

3.5.15. Cloning, Expression and Purification of Co-Expressed CKase and ATCase

So far, it has been demonstrated that CKase lysate and ATCase could be combined to produce CA by using aspartate, AP, ammonia and bicarbonate. It also has been shown that recombinant *E. coli* lysate contained acetate and adenylate kinase (required for the ATP recycling system).

Therefore, making a single recombinant lysate would be highly efficient to have all the enzymes needed to convert ammonia and bicarbonate to CA. Hence, the focus of this section is the co-expression of CKase and ATCase in one plasmid in *E. coli* and the usage of the bacterial lysate as a source of endogenous and exogenous enzymes.

Before proceeding to the experiment, it is worth mentioning that co-expression of CKase and ATCase in *E. coli* has many advantages. First, it means having a single bacterial culture that has both enzymes and the ATP recycling system. Second, a single bacterial culture reduces the time and cost associated with protein production and purification. And finally, this culture takes advantage of the endogenous enzymes needed to recycle the required cofactor, such as ATP (which is required in costly stoichiometric amounts). Therefore, CKase and ATCase were co-expressed in one plasmid in *E. coli*.

To conduct the expression, co-expression plasmids were constructed using the pRSFDuet-1 vector (Novagen), which has two MCS. It also has compatible replicons, antibiotic resistance markers and T7 promoters for the expression of target genes by IPTG induction. The pRSFDuet-1 vector can easily be sequenced using the pETUPstream primer and the DuetDOWN primer for MCS-1, and the DuetUP2 primer and the T7 Terminator primer for MCS-2. Therefore, the pRSFDuet-1 vector was chosen to co-express the CKase and ATCase. Genes for ATCase were inserted in MCS-1, and genes for CKase were inserted in MCS-2. Four different cross-combinations were made because we were also interested in testing the combination of mesophilic and hyperthermophilic CKase and ATCase in the co-expressed lysate. Here is the list of combinations that were prepared:

- CKase *T. sibiricus*–ATCase *T. sibiricus*
- CKase *T. sibiricus*–ATCase *E. faecalis*
- CKase *E. faecalis*–ATCase *T. sibiricus*

  CKase *E. faecalis*–ATCase *E. faecalis*.

After co-expression, the constructs were sequenced to confirm the correct gene sequence in the vector. Moreover, the SDS-PAGE was run for each lysate to detect the

overexpressed CKase and ATCase (Figure 3.25). A thick band was observed corresponding to CKase and ATCase since their molecular weights are very close and cannot be distinguished in the lysate SDS-PAGE. To ensure the presence of both enzymes in the lysate, the activity of each CKase and ATCase in the lysate should be examined individually.

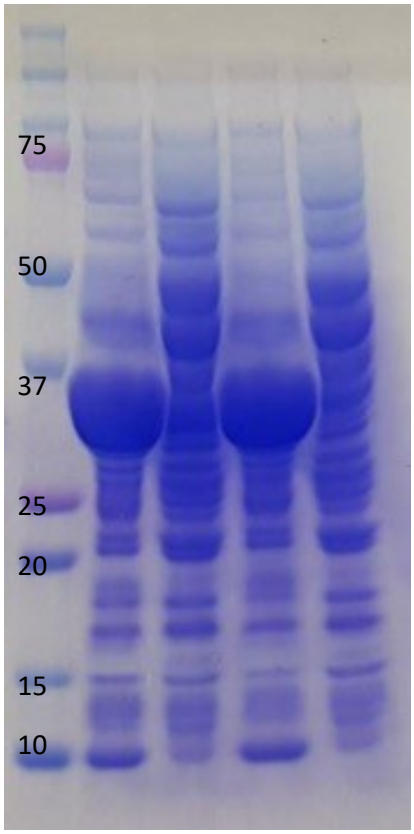


Figure 3.25: SDS-PAGE analysis of the overexpression of co-expressed CKase and ATCase from *T. sibiricus* and *E. faecalis*. From left to right, ladder (NEB broad range 0–250 kDa), CKase *T. sibiricus*–ATCase *T. sibiricus,* CKase *E. faecalis*–ATCase *E. faecalis*. CKase *T. sibiricus*–ATCase *E. faecalis* and CKase *E. faecalis*–ATCase *T. sibiricus*.

3.5.16. Determination of Co-Expressed Lysate Activity

Following the co-expression of the two different sources of CKase and ATCase *T. sibiricus* and *E. faecalis* using pRSFDuet-1 and *E. coli,* a total of four different lysates were produced. Since the band corresponding to each enzyme could not be detected on SDS-PAGE from the previous section, it is important to examine the presence and activity of each enzyme in the lysate. Therefore, each enzyme was examined individually. The ATCase activity was

measured by monitoring the CA production. CKase enzyme activity was measured by monitoring the catalytic conversion of ATP to ADP.

The results confirmed that CKase and ATCase were present and active in the co-expressed lysate (Figure 3.26). The results showed that the CKase activity profile was almost the same in all four different co-expressed lysates, and the difference was not significant. Yet ATCase activity was different in different co-expressed lysates. ATCase *T. sibiricus* from co-expressed lysate CKase *T. sibiricus* and ATCase *T. sibiricus* had the lowest activity, followed by ATCase *T. sibiricus* from co-expressed lysate CKase *E. faecalis* and ATCase *T. sibiricus* and ATCase *E. faecalis* from co-expressed lysate CKase *T. sibiricus* and ATCase *E. faecalis.* The ATCase *E. faecalis* from co-expressed lysate CKase *E. faecalis* and ATCase *E. faecalis* showed the highest activity among the others. It would be difficult to draw any conclusion from ATCase activity from different co-expressed lysates as the amount of enzyme has not been quantified.

Finally, after confirming that all the enzymes were individually present and active in the co-expressed lysate, the combination of CKase and ATCase from each lysate was studied while ATP was recycling. The results showed that all four different combinations of CKase and ATCase in the lysate were able to work together to produce CA from ammonia, aspartate and bicarbonate without adding any cofactor (Figure 3.27). The only requirement was the addition of AP for the ATP recycling system in the lysate.

Moreover, the results from co-expressed combination of CKase and ATCase lysate showed that the highest CA production yield was observed in the co-expressed lysate of CKase *T. sibiricus*–ATCase *E. faecalis* and CKase *E. faecalis*–ATCase *E. faecalis*, followed by CKase *E. faecalis*–ATCase *T. sibiricus* CKase and *T. sibiricus*–ATCase *T. sibiricus* (Figure 3.27). It would be difficult to draw any conclusion from the CA yield from four different co-expressed lysates because the amount of each enzyme could not be measured in the lysate, and we could only measure the total protein.

However, from an analysis of the activity of individual enzymes, it seems that ATCase *E. faecalis* from lysate CKase *E. faecalis*–ATCase *E. faecalis* exhibited more CA production compared to the other ATCase in the lysates. It is probably due to the better expression of ATCase *E. faecalis* in lysate CKase *E. faecalis*–ATCase *E. faecalis* and its possessing higher catalytic efficiency. Since the protein concentration varied in each lysate, it affects the enzyme activity and production of CP and CA, and therefore we could not draw any conclusion comparing the results.

Therefore, a single culture crude lysate has been developed that contains all the recombinant and endogenous enzymes required for the conversion of ammonia and bicarbonate to CA without the addition of any cofactor. This is a significant advantage because it has the potential to sequester undesirable ammonia and carbon dioxide (bicarbonate in our experiment) in a manner that is gentle on the environment since the process is performed in mild conditions in aqueous solution. Moreover, this system is advantageous by representing a cost- and time-effective method that avoids the need for protein production and purification protocols.

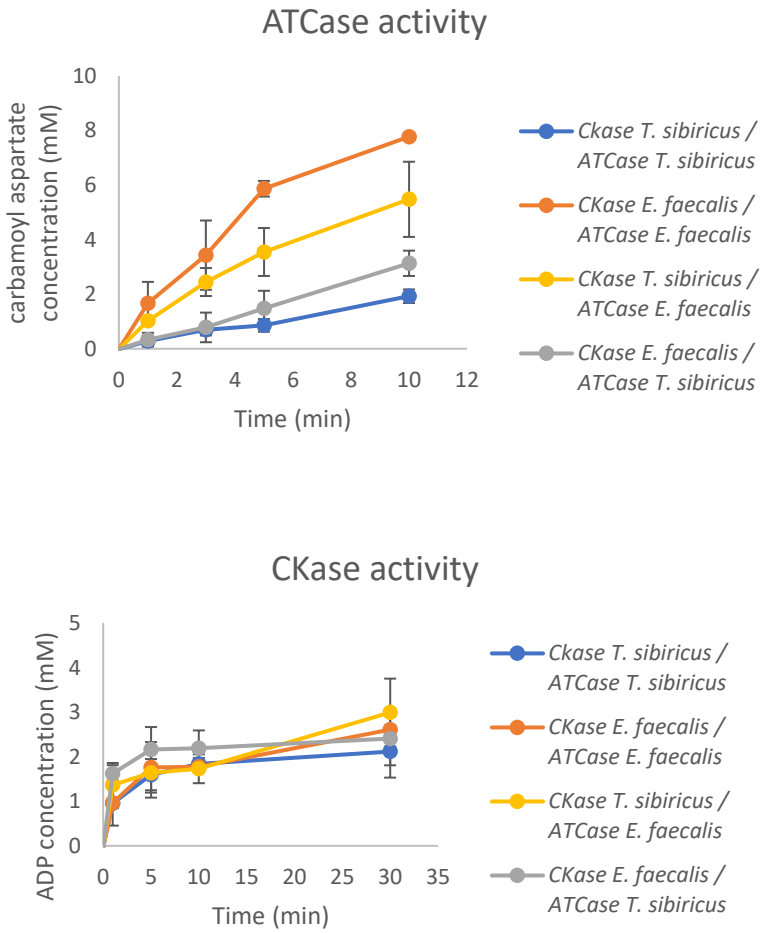


Figure 3.26: CKase (bottom), ATCase (top) assay from co-expressed lysate to examine the presence and activity of each enzyme in the lysate. The reaction mixture contains 200 mM $NH_4OH/NaHCO_3$ pH 9.4, 10 mM CP, 10 mM $MgSO_4$, 10 mM aspartate, 20 mM acetyl phosphate, 10 µL of each co-expressed lysate CKase and ATCase *T. sibiricus* and *E. faecalis*. Values are the average of two replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

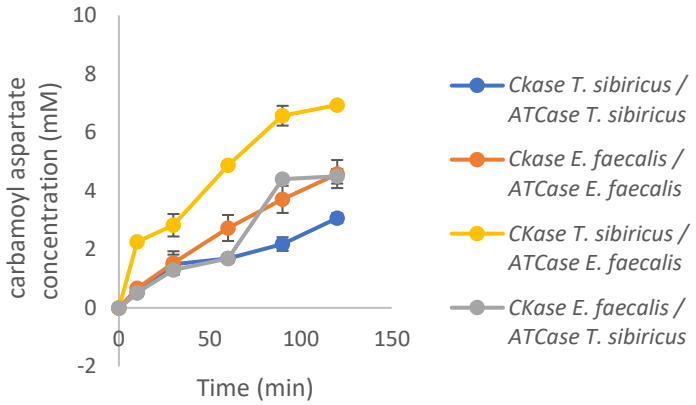


Figure 3.27: Combination of CKase/ATCase assay from the co-expressed lysate to examine the combination enzymes activity together for production of CA from carbamate and aspartate. The reaction mixture contains 200 mM $NH_4OH/NaHCO_3$ pH 9.4, 10 mM $MgSO_4$, 10 mM aspartate, 20 mM acetyl phosphate, 10 µL of each co-expressed lysate CKase and ATCase *T. sibiricus* and *E. faecalis*. Values are the average of two replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

3.5.17. Characterisation of Co-Expressed Lysate

Since it was not clear todraw any conclusions from the previous section regarding a choice of four different co-expressed lysates, we aimed to characterise all four lysates to choose the most stable co-expressed lysate. Therefore, to study the activity under different conditions, co-expressed lysates were assayed at different pH values (8.5, 8.8 and 9.4) and temperatures (10, 20, 40, 60 and 80 ºC).

The results from the pH study demonstrated that there is no significant difference (p>0.05) in CA production at pH 8.5 and 8.8 values in each lysate (Figure 3.28). However, at pH 9.4, all co-expressed lysates except co-expressed lysate CKase *E. faecalis*–ATCase *E. faecalis* (showed the same rate for all pH values*)* showed higher CA production yield. Moreover, at pH 9.4 the CA production was significantly (p<0.05) higher than pH 8.5 in co-expressed lysates CKase *T. sibiricus*–ATCase *E. faecalis* and lysate CKase *T. sibiricus*–ATCase *T. sibiricus*. It might be due to CKase's higher activity at higher pH, which results in CP production rate and, therefore, CA rate.

Finally, CKase *T. sibiricus*–ATCase *T. sibiricus* showed the lowest CA production yield at different pH compared to the other lysates (Figure 3.28).

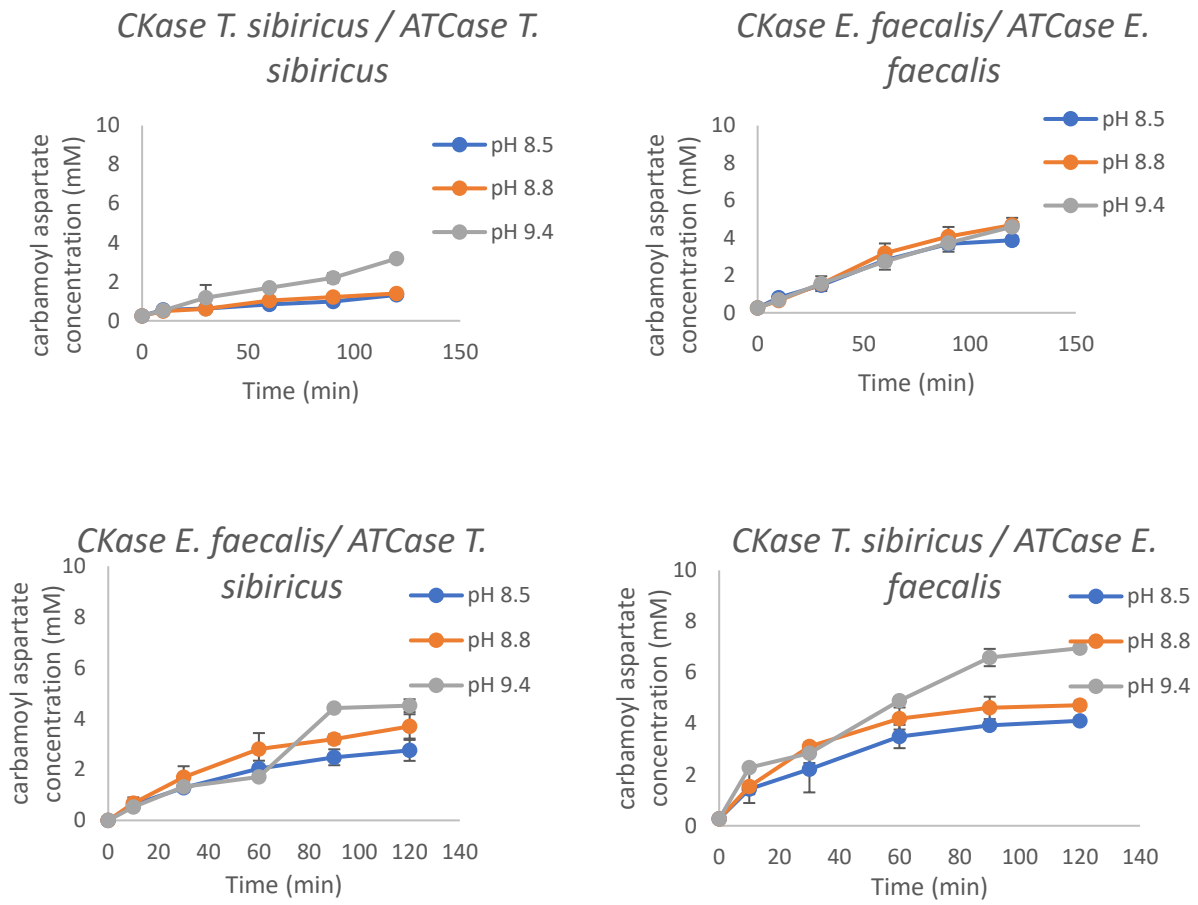


Figure 3.28: CA production when using co-expressed CKase and ATCase at different pH values. The reaction mixture contains 200 mM $NH_4OH/NaHCO_3$, 10 mM $MgSO_4$, 10 mM aspartate, 20 mM acetyl phosphate (adding portion-wise), 10 µL of each co-expressed lysate CKase and ATCase *T. sibiricus* and *E. faecalis*. Values are the average of three sets of two replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

Further, the results from the temperature study manifested that temperature had a large influence on CA production (Figure 3.29). The co-expressed lysate from CKase–ATCase *T. sibiricus* showed a higher CA yield when the temperature was increased. As expected, hyperthermophilic enzyme activity increases by elevating the temperature (Purcarea, 2001; Purcarea et al., 1994; Purcarea et al., 2001) because CKase and ATCase optimal temperature is as high as 60 to 84 ºC for *T. sibiricus* (Miroshnichenko et al., 2001). The lysate from the mesophilic enzyme CKase and ATCase *E. faecalis* demonstrated a decrease in CA production from 40 ºC to 80 ºC. It has been shown in the previous chapter that

ATCase *E. faecalis* can survive at up to 40 °C, but it loses its activity at higher temperatures. The same pattern has been observed for CKase *E. faecalis* (Hennessy et al., 2018). Therefore, the co-expressed lysate behaviour from CKase and ATCase *E. faecalis* follows individual enzyme behaviour and a combination of pure enzymes at different temperatures.

However, the results from the co-expressed lysates from the combinations of mesophilic and hyperthermophilic enzymes were very surprising. The co-expressed lysate from CKase *T. sibiricus*–ATCase *E. faecalis* showed behaviour fluctuations at different temperatures. From 40 to 60 °C, CA production decreased. Then, from 60 °C to 80 °C, the production again increased. It presumably results from protein-protein interaction between CKase and ATCase and the role of hyperthermophile enzyme to protect mesophilic enzyme at a higher temperature. Yet it is not clear why CA production yield decreased at 60 °C. It might be due to the CKase and ATCase structure and their interaction at different temperatures.

Moreover, the co-expressed lysate from CKase *E. faecalis*–ATCase *T. sibiricus* showed different behaviour than the previous combinations. By elevating the temperature to 60 °C, the CA production rate was similar to that observed at 40 °C. However, the CA production rate increased at 80 °C. This could be due to the interaction between CKase and ATCase from hyperthermophilic and mesophilic sources, as explained below.

In the previous studies, biological interaction between CKase and OTCase or ATCase has been confirmed, suggesting that a channelling complex of enzymes protects CP from decomposition at a higher temperature, especially in hyperthermophilic organisms (J. Massant & Glansdorff, 2004; Jan Massant & Glansdorff, 2005; Jan Massant et al., 2002; Purcarea et al., 2003; Purcarea et al., 1996). One of the discussions on the presence of the channelling in the cell, especially in hyperthermophiles, is that if there was no channelling between the two enzymes at high temperatures, CP would diffuse into aqueous solution and degrade to cyanate, which is toxic for the cell. Therefore, the cell should have an enzyme to decompose cyanate. Yet, there is no evidence of the existence of a cyanase to protect the cell from cyanate in hyperthermophilic cells, demonstrating the absence of cyanate in the cell. Hence, the CP would not diffuse to a solution at a high temperature. (Purcarea et al., 2003). Following the mentioned discussion, a transient intermediate channelling CKase–ATCase complex exists between CKase and ATCase by the physical association of the enzymes. Therefore, by elevating the temperature, the hyperthermophile CKase or ATCase transient complex probably protects mesophiles from denaturation.

Consequently, by comparing the effect of temperature on CKase and ATCase from a combination of mesophile and hyperthermophile, it can be concluded that ATCase from hyperthermophiles has a more protecting role compared to CKase from hyperthermophiles probably due to its trimeric structure. Yet, previous studies showed that channelling did not occur between heterologous CPSase (similar to CKase) from hyperthermophiles and ATCase from *E. coli* (Purcarea et al., 2003). Therefore, this is the first time such interactions between heterologous systems have been proposeded.

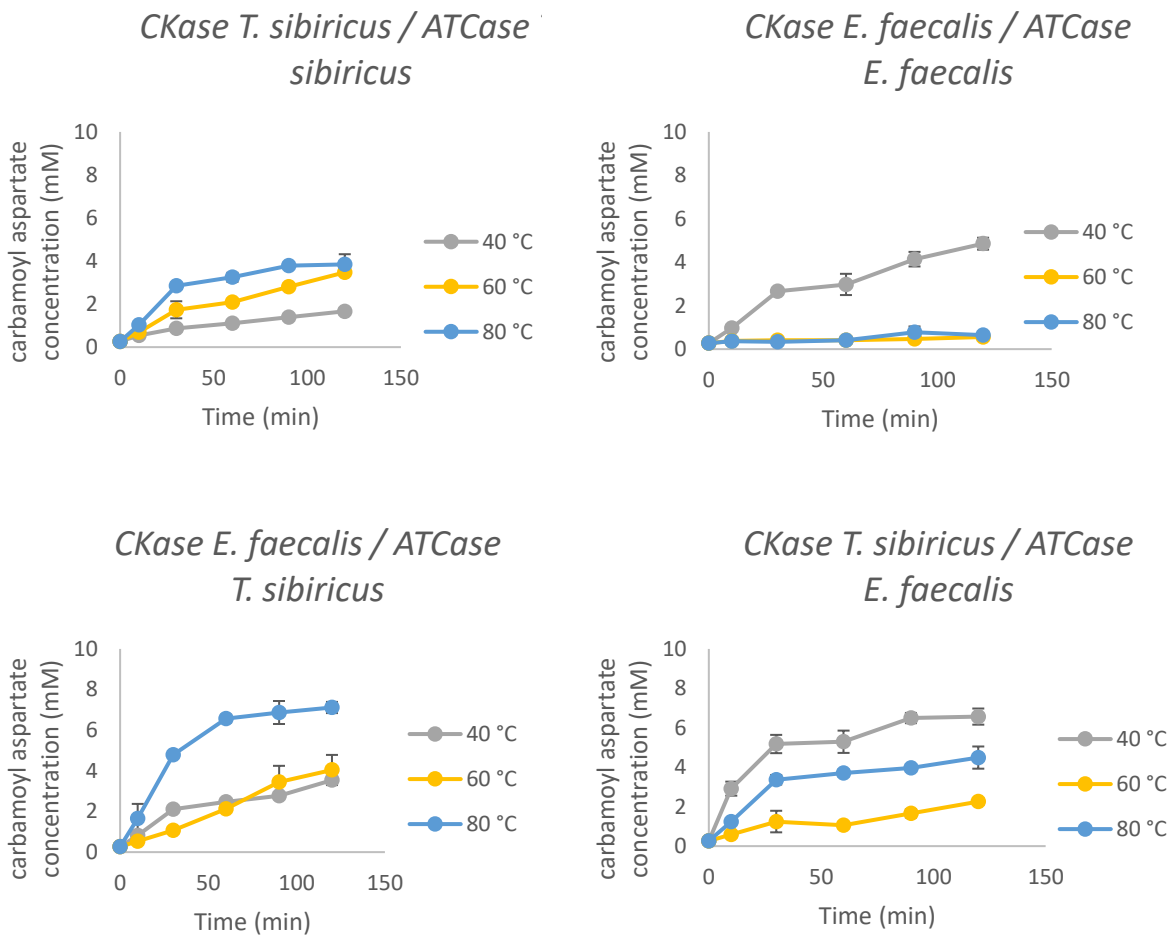


Figure 3.29: CA production using co-expressed CKase and ATCase at different temperatures. The reaction mixture contains 200 mM $NH_4OH/NaHCO_3$ pH 9.4, 10 mM $MgSO_4$, 10 mM aspartate, 20 mM acetyl phosphate (adding portion-wise), 10 µL of each co-expressed lysate CKase and ATCase *T. sibiricus* and *E. faecalis*. Values are the average of three sets of two replicates of each enzyme, and error bars represent the standard deviation of duplicate experiments.

3.6. Conclusion

The ultimate aim of the present research was to examine the combination of CKase and ATCase while ATP was recycled to produce CA.

The first aim of this study was to investigate the possibility of the combination of pure CKase and pure ATCase to produce the final product, CA, while ATP was recycling. Firstly, the study showed that pure CKase and ATCase could successfully be combined to produce the final product. Secondly, the study has identified the best combinations of CKase and ATCase with respect to CA production rate. Thirdly, the experiments confirmed that the ATP recycling system from *E. coli* lysate could be incorporated in the combination of CKase and ATCase using acetyl phosphate and ADP or AMP.

The second aim of this study was to determine the limiting factor(s) for the combination of CKase and ATCase. The finding of this study suggested that ATCase and AP are the rate-limiting enzyme and reagent, respectively. Notwithstanding these limitations, the study suggests that an excess amount of ATCase and AP (added gradually and portion-wise) should have been used in the reactions to achieve the optimal conditions for high CA production rate.

Finally, the main goal of this research was to investigate the possibility of the production of CA from one single recombinant lysate containing all necessary enzymes. The results of this investigation revealed that recombinant enzymes CKase and ATCase could be cloned and expressed in *E. coli.* The findings of this research also indicated that recombinant enzymes and endogenous enzymes from *E. coli* are present and active in the lysate, and there is no need for cost- and time-consuming protein purification steps. The characterisation of co-expressed lysate presented good pH stability and an interesting temperature profile, which sheds new light on the hyperthermophile and mesophile protein interaction. Before this study, there was no evidence of any successful combination of CKase and ATCase from hyperthermophiles and mesophiles. This finding may lay the groundwork for future research into a combination of hyperthermophile and mesophile enzymes.

Taken together, we have developed a one-pot multienzyme pathway to convert ammonia, carbon dioxide and aspartate to CA. This approach constitutes the utility of the cell-free *E. coli* lysate containing homologous and/or heterologous recombinantly expressed CKase and ATCase with the endogenous enzyme's acetate kinase and adenylate kinase to synthesise CA.

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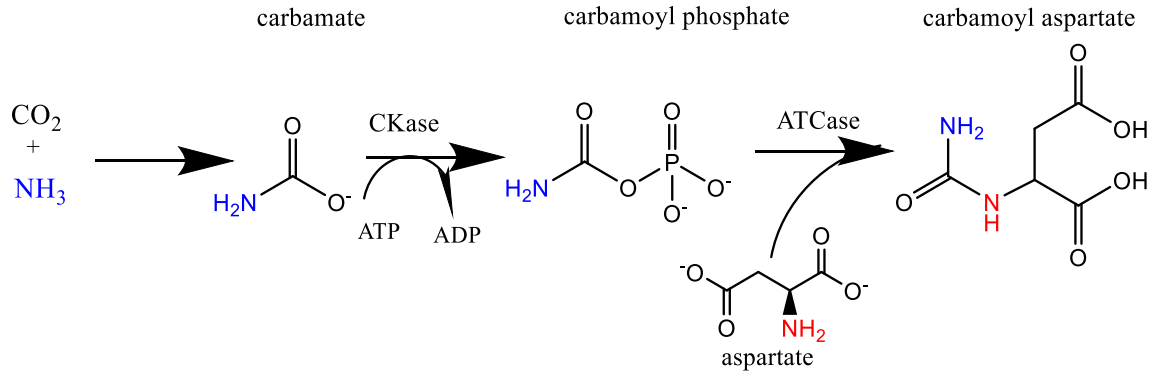
Chapter 4: Study Aspartase activity

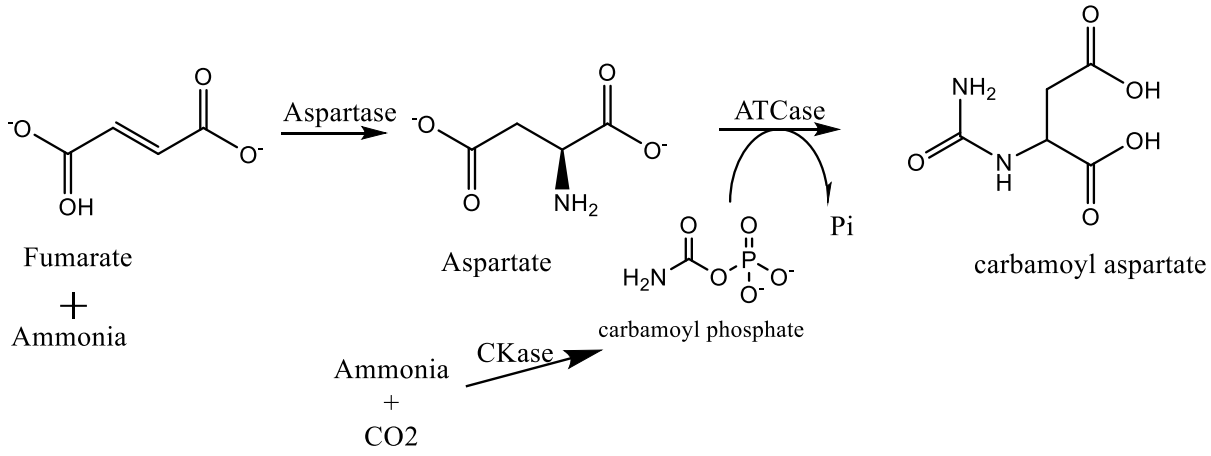
4.1. Introduction

So far, a system has been developed to convert ammonia, aspartate and carbon dioxide (in the form of bicarbonate in our study) to CA by using the combination of two enzymes CKase and ATCase. CA is an intermediate precursor in the pyrimidine pathway to produce pyrimidine. CA has two nitrogen atoms; one nitrogen comes from ammonia in our process (which potentially can come from wastewater) and the other from aspartate (Figure 4.1). Therefore, it will be advantageous if aspartate could be substituted with its nitrogen-free precursor, fumarate. This allows us to receive another nitrogen from ammonia via wastewater. Therefore, both nitrogens in CA would be provided by ammonia from wastewater.



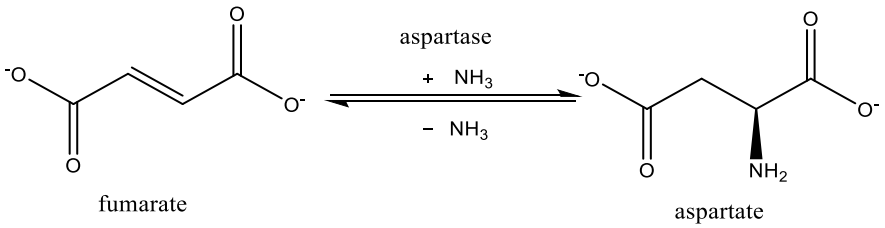
Scheme 4.1: CA production from ammonia, carbon dioxide and aspartate by using CKase and ATCase. The blue colour shows the nitrogen comes from ammonia, and the red shows the nitrogen from aspartate.

Fumaric acid (or fumarate) can be a good alternative to aspartate as it lacks one nitrogen compared to aspartate, and its price is one-tenth of aspartate (from Merck). Therefore, finding an enzyme to convert fumarate and ammonia to aspartate would be promising. A few studies have shown that aspartase can produce aspartate from fumarate and ammonia (Fibriansah, Veetil, Poelarends, & Thunnissen, 2011; Garza, Rodríguez, Hernández, & Rodríguez, 2000; Halpern & Umbarger, 1960; Hubert, Hornsperger, & Wurtz, 1975; Puthan Veetil, Fibriansah, Raj, Thunnissen, & Poelarends, 2012). Therefore, the focus of this study will be investigating the possibility of aspartase usage in a combination of CKase and ATCase experiments to produce CA from ammonia and fumarate. This makes the scheme more efficient due to using two molecules of ammonia from wastewater (Sheme 4.2).



Scheme 4.2: Enzymatic reaction cascade to produce CA from ammonia, fumarate and carbon dioxide.

Having already discussed how aspartase might benefit this project, it is necessary to explain aspartase structure and kinetics. Aspartase (L-aspartate ammonia- lyase, EC 4.3.1.1) is an enzyme that plays an important role in nitrogen metabolism. It catalyses reversible deamination conversion of L-aspartate to fumarate and ammonium ion (Asp A). In the reverse reaction of aspartase, the aspartate is produced from fumarate and ammonia (Asp B). (Weiner, Poelarends, Janssen, & Feringa, 2008) (Sheme 4.3).



Scheme 4.3: Aspartase reaction that converts fumarate to aspartate in an excess of ammonia.

Aspartase is found in plants, some animal tissue and various bacteria, including *E.coli* (Rudolph & Fromm, 1971; Suzuki, Yamaguchi, & Tokushige, 1973a; J. S. Takagi, Ida, Tokushige, Sakamoto, & Shimura, 1985), *B. subtilis* (Sun & Setlow 1991) and *Pseudomonas fluorescens* (Takagi et al.,, 1984; TAKAGI et al.,1986). Aspartase from bacteria have been purified, characterised and vastly studied (William E Karsten, Hunsley, & Viola, 1985; Rudolph & Fromm, 1971) (Y. Kawata et al., 2000; Yasushi Kawata et al., 1999; Sun & Setlow, 1991; Suzuki, Yamaguchi, & Tokushige, 1973b; J. S. Takagi et al., 1985; Jun S TAKAGI et al., 1986; Wilkinson & Williams, 1961; Yoon et al., 1995). In addition, a complete

study has been conducted on aspartase from *E. coli,* and the crystal structure has been acquired (W. Shi, Dunbar, Jayasekera, Viola, & Farber, 1997). Aspartase is a key enzyme in nitrogen metabolism that catalyses the cleavage of C-N bonds without applying oxidation or hydrolysis mechanisms. Aspartase is a tetramer enzyme consisting of four identical subunits of 478 amino acids and a molecular weight of about 52 kDa (Figure 4.1) (J. S. Takagi et al., 1985). Each subunit is composed of three structurally distinct domains: N-terminal domain, having two stranded β-sheets and five α-helices; the C-terminal domain containing seven α-helices and finally, the central helix domains consisting of six α-helices and SS-loop (a flexible loop consists of 10 amino acids with conserved motifs sequence of GSSxxPxKxN) (Figure 4.1). The four subunits form a tetramer structure with a total of four active sites. Further, it has been demonstrated that aspartase is allosterically activated by aspartate and an Mg^{2+} ion (binding to the activator site, different from the enzyme active site) (Falzone, Karsten, Conley, & Viola, 1988; W. E. Karsten, Viola, & Gates, 1986). Finally, several studies have shown the importance of metal ion on aspartase activity at alkaline pH, such as a requirement to Mg^{2+} for activity at higher pH (Weiner et al., 2008). Knowing the *E. coli* aspartase structure, it will be a good choice for this project as an aspartase source, as *E. coli* has been used in the previous studies of this project, and it is beneficial to use it in this study too.

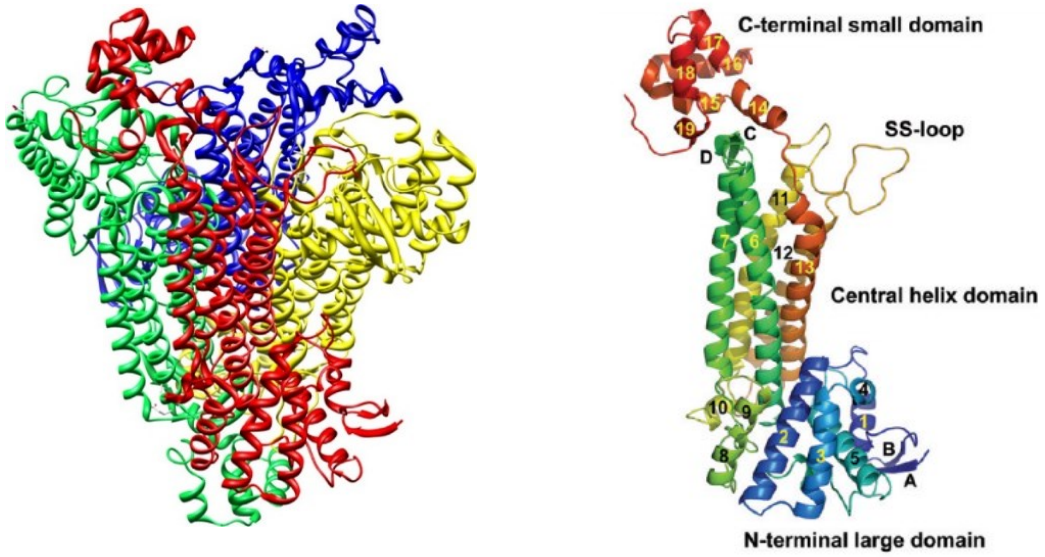


Figure 4.1: Left: Aspartase homotetramer structure (J. Zhang & Liu, 2014), right: Secondary structure of aspartase subunit showing SS-loop and C- and N-terminal domain. The numbers represent α- helices and letters for β-strands (Fibriansah et al., 2011).

4.1.1. Aspartase Mechanism in Amination Direction

It has already been explained that aspartase can catalyse the reversible amination of fumarate and ammonia to aspartate, being the focus of this chapter. However, the amination mechanism has not yet been explored as the crystal structure of aspartase with substrate or product is not available (W. Shi et al., 1997). It is only proposed that in the amination reaction, the aspartase activity is due to two group ionisations on the enzyme. The first one is responsible for changing the metal independent enzyme form to the metal ion-active form. The second group ionisation is responsible for acid-base catalyst on the enzyme (W. E. Karsten & Viola, 1991). Moreover, it is confirmed that the SS-loop has an important catalytic role in aspartase activity as well as amino acid residues: serine 318, lysine 324, his 188, thr 101, thr 141, thr 187, ser 140 and142 (Fibriansah et al., 2011; W. E. Karsten & Viola, 1991; Veetil, Raj, Quax, Janssen, & Poelarends, 2009) Finally, it has been determined that substrate binding at an active site involves the close association of a loop from an adjacent subunit (Figure 4.2)

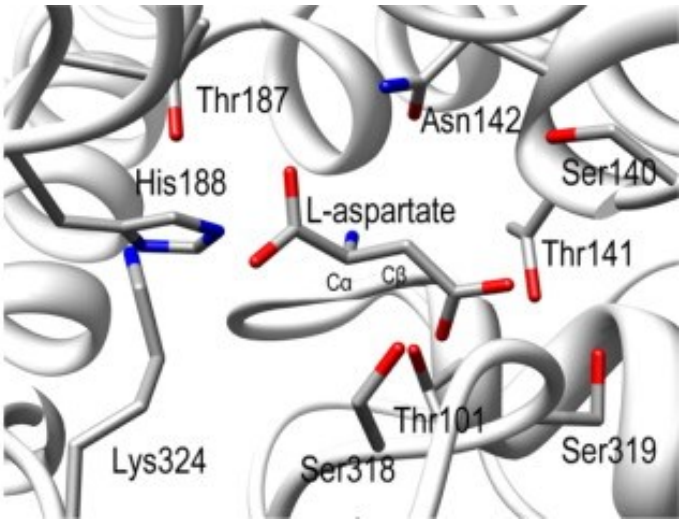


Figure 4.2: Crystal structure of aspartase active site and aspartate coplanar structure (J. Zhang & Liu, 2014).

4.1.2. Kinetic Studies of Aspartase

The study aim is to use aspartase from *E. coli* since the expression of therecombinant enzymes has been conducted in *E. coli*. Several studies have been done on aspartase kinetic properties on *E. coli*; the gene-encoding aspartase has been cloned and expressed, and the pure enzyme has been purified. Moreover, several studies have been conducted on the effect of pH on aspartase from *E. coli*. It has been confirmed that the aspartase pH profile depends on the absence or presence of a metal ion. It has been shown at higher pH

that aspartase has a higher demand for a divalent metal ion, and at lower pH, some activity in the absence of a metal ion has been seen (Rudolph & Fromm, 1971; Suzuki et al., 1973b; V. R. Williams & Lartigue, 1967). The studies have revealed that in the presence of a metal ion in *E. coli*, the optimal pH is 8.5, while in the absence of a metal ion, the optimal pH is 7.7 (Suzuki et al., 1973b). Moreover, the study of aspartase in the amination direction at high pH revealed a lag phase before a linear steady state rate is achieved in time course kinetics (Ida & Tokushige, 1985; W. E. Karsten et al., 1986). The apparent km value for ammonium was 20 mM (Suzuki et al., 1973b). Consequently, aspartase in the deamination direction is very specific, while in the amination direction, it can accept other substrates instead of ammonia. In the amination reaction, other nitrogen source substitutes can be used instead of ammonia as the amine donor to catalyse the amination of fumarate with different nucleophiles, such as hydroxylamine and hydrazine (Weiner et al., 2008). The promiscuity of aspartate in amination reaction makes it a good choice for industrial applications, such as being used to prepare the artificial sweetener aspartame (W. Shi et al., 1997).

4.1.3. Aspartate in Industry

Aspartate is an important precursor for the industrial production of food additives and artificial sweeteners (aspartame) (Liese, Seelbach, & Wandrey, 2006; Wubbolts, 2002). Another application of aspartate is in the fertiliser industry (polyaspartate improves nitrogen intake and water retention in the soil) (Kelling, 2001), biodegradable polymers (such as those used in diapers and feminine hygiene products).

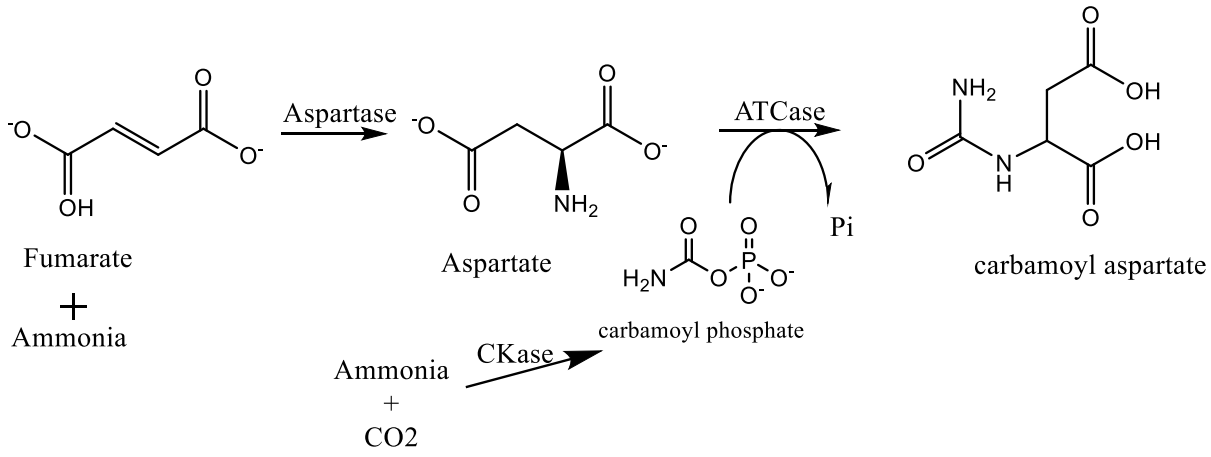
4.2. Aims

The ultimate aim is the production of CA from ammonia, carbon dioxide and fumarate while ATP is recycled from one single recombinant co-expressed lysate using acetyl phosphate.

To achieve the aim, the following steps have to be carried out:

1. investigate the possibility of the enzymatic production of aspartate from fumaric acid and ammonia using aspartase by using *E. coli* lysate as a source of aspartase
2. investigate the combining of aspartase with pure CKases and ATCases while ATP is recycling
3. investigate the aspartase activity from recombinant co-expressed lysate of CKase and ATCase
4. investigate the production of CA from ammonia, carbon dioxide and fumarate while ATP is recycling from one single co-expressed lysate (Figure 4.6)

5. investigate the application of recombinant co-expressed lysate on wastewater for removal of ammonia.



Scheme 4.4: Enzymatic reaction cascade to produce CA from ammonia, fumarate and carbon dioxide.

4.3. Materials and Methods

4.3.1. Materials

Polyacrylamide gels were cast using acrylamide and bis-acrylamide solutions from Bio-Rad and run on a Mini-PROTEAN® Tetra system; Bio-safe® Coomassie Blue was used for staining. Protein markers, restriction enzymes and Quick DNA Ligation™ Kit were purchased from NEB. Alkaline phosphatase was from Roche Diagnostics GmbH. Agarose for electrophoresis was purchased from Invitrogen. Nutrients for the broth were purchased from Bacto Laboratories Pty Ltd. L-Aspartate, ampicillin and N-chloromethylphthalimide were obtained from Sigma-Aldrich®. HPLC analysis was performed on a Hewlett-Packard Series 1100 separation system operated by Chemstation from Agilent Technologies. All materials were purchased from Sigma-Aldrich®.

4.3.2. Strains and Culture

All cell work was carried out with *E. coli* BL21 (DE3). The *E. coli* was cultivated in 100 mL of media with or without fumarate as an inducer (Table 4.1) (Chibata, Tosa, & Sato, 1974; Tosa, Sato, Mori, & Chibata, 1974). The pH was adjusted to 7.4 (as the optimum pH suggested by the literature for the highest yield of aspartase (Garza et al., 2000; Halpern & Umbarger, 1960; W. Shi et al., 1997)) by adding ammonia as it could also be the source of nitrogen for the cells. The cells were then collected from the 100 mL culture by centrifugation

(4000 × g for 10 min), resuspended (1 mL per g wet cell pellet) in Tris-acetate buffer (10 mM, pH 8.3) containing 16 mM KOAc, 14 mM Mg $(OAc)_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The pellets were then lysed over ice using a Thermo Fisher Scientific Omni Sonic Ruptor 400, equipped with an ORT-375 processing tip set at 50% power and 50% pulse for 6 min. The lysate was centrifuged at 20,000 × g for 60 min at 4 °C. The supernatant was aliquoted in an Eppendorf tube located on dry ice for snap-frozen and then stored at –80 °C.

Table 4.1: Different Media for Cultivating Aspartase

Substance	Concentration needed	+ Sodium fumarate	+Sodium fumarate + Ammonium bicarbonate	Control
Salt 5X *	1X	✓	✓	✓
$MgSO_4$	0.01 M	✓	✓	✓
$CaCl_2$	0.01 M	✓	✓	✓
Sodium fumarate	0.187 M	✓	✓	-
Thiamine	0.33 mM	✓	✓	✓
Glucose 20%	20%	✓	✓	✓
Micronutrient	1X	✓	✓	✓
Ammonium bicarbonate	0.1 M	-	✓	-
Water		✓	✓	✓

Note. *Salt 5x = 30 g/L Na_2HPO_4, 15 g/L KH2PO4, 2.5 g/L NaCl, 5 g/L NH_4Cl. Micronutrient 1000X bought from Merck Sigma.

4.3.3. Aspartase Assay

Aspartase enzyme activity was measured through the production of aspartate. The enzyme lysate was pre-incubated at 37 °C for 5 min before adding fumarate. The final reaction mixture (500 μl) contained either 50 mM Tris-HCl or sodium bicarbonate, pH 8.5, 10 mM sodium fumarate, 200 mM NH_4Cl, 25 mM $MgSO_4$ and 10 µL of *E. coli* lysate containing aspartase. The assay was conducted at 37°C.

4.3.4. Determination of Aspartase Activity

An aliquot of 50 µL was withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by the addition of 50 µL of 1 M NaOH to neutralise. The amount of aspartate was measured by a reverse-phase HPLC analysis of a 10 μL aliquot of the resultant solution derivatised with AccQ-Tag™ reagent, following the manufacturer's guidelines as described in materials and methods of Chapter 2.

4.3.5. Combination of Aspartase from *E. coli* Lysate and Pure ATCase

After several hours of incubation of aspartase in the reaction mixture (contained 50 mM bicarbonate (pH 8.5), 10 mM sodium fumarate, 200 mM NH_4Cl, 25 mM $MgSO_4$ and 10 µL of lysate containing aspartase) 0.1 µM ATCase from *T. sibiricus* and 20 mM CP were added to the aspartase reaction mixture and run for another 10 min. Samples were taken at each time point and measured by HPLC or LC/MS as described in Chapter 2.

4.3.6. Combination of Aspartase from *E. coli* lysate with Pure ATCase and Pure CKase

Since the aim of this section is to investigate the aspartase activity in combination with CKase and ATCase activity, only one of the CKase and ATCase (*T. sibiricus*) was examined in this step. Aspartase, CKase and ATCase combination assays were performed at pH 8.5 and 40 °C and in the combination of CKase and ATCase condition assay. The reaction mixture contained 200 mM $NaHCO_3$ and NH_4OH, 10 mM ATP, 10 mM sodium fumarate, 25 mM $MgSO_4$, 10 µL aspartase lysate from *E. coli*, 1 U (mM/min) of CKases and 16 U (mM/min) ATCase from *T. sibiricus*. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by the addition of 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water prior to measurement. The amount of CA production was measured by HPLC/MS.

4.3.7. Preparation of Co-Expressed Lysate of CKase and ATCase Containing Aspartase

The production of co-expressed lysate has been explained in the previous chapter in Section . Since the co-expressed lysate is from *E. coli*, it should have contained endogenous aspartase that can be used for further experiments.

4.3.8. Determination of Aspartase Activity in the Co-Expressed Lysate of CKase and ATCase

The aspartase activity in co-expressed lysate of CKase and ATCase was assayed at pH 8.5 and 40 °C. The 0.5 mL reaction mixture contained 50 mM Tris-HCl pH 8.5, 200 mM NH_4Cl, 10 mM sodium fumarate, 25 mM $MgSO_4$ and 10 µL co-expressed lysate. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by the addition of 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water prior to measurement. The amount of aspartate production was determined by HPLC/MS or AccQ-Tag.

4.3.9. Determination of Carbamoyl Aspartate Production from the Co-Expressed Lysate With or Without ATP Recycling

The co-expressed lysate containing aspartase, CKase and ATCase was assayed at pH 8.5 and 40 °C while ATP was recycled. The 0.5 mL reaction mixture contained 200 mM $NaHCO_3$ and NH_4OH, 10 mM sodium fumarate, 25 mM $MgSO_4$, 10 mM ATP or 20 mM acetyl phosphate and 0.1 mM ADP or AMP and 10 µL co-expressed lysate. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by the addition of 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water prior to measurement. The amount of CA production was measured by HPLC/MS.

4.3.10. Characterisation of Co-Expressed Lysate

The characterisation assays were performed at different pH values (8.5, 8.8 and 9.4). Also, another assay was performed using different quantities of co-expressed lysate (10, 20, 40 and 80 µL). The assay contained 200 mM $NaHCO_3$ and NH_4OH, 10 mM sodium fumarate, 25 mM $MgSO_4$, 20 mM AP, and 10 µL co-expressed lysate. An aliquot of 50 µL was then withdrawn at each time point and mixed with 50 µL of 1 M HCl, followed by the addition of 50 µL of 1 M NaOH to neutralise and then diluted by a factor of 10 with water prior to measurement. The amount of CA was measured by HPLC/MS.

4.4. Results and Discussion

This chapter aimed to investigate aspartate production by using *E. coli* lysate. Since *E. coli* lysate is the source of many endogenous enzymes and the factory for producing our co-expressed enzymes (as explained in Chapter 3), it is beneficial to use it as the source of aspartase enzyme.

4.4.1. Aspartase Production

The aspartase production in *E. coli* was examined in different media due to diversity in aspartase production, as aspartase production can be affected by aspartase induction and the regulation of the biosynthesis of aspartase (Hubert & Wurtz, 1976). Therefore, to achieve the highest aspartase yield, the culture medium was optimised for *E. coli* aspartase with respect to two important components (sodium fumarate and ammonium bicarbonate) for their ability to support growth (Hubert et al., 1975; Hubert & Wurtz, 1975). *E. coli* cells were cultivated in different minimal media containing either sodium fumarate (as an induction agent) or sodium fumarate plus ammonium bicarbonate (as an ammonia source and inducer) or none of the components. The pH was adjusted to 7.4 by adding ammonia, as suggested by the literature (Chibata et al., 1974; Garza et al., 2000; Halpern & Umbarger, 1960; W. Shi et al., 1997; Tosa et al., 1974). The results indicated that the highest *E. coli* cell growth was obtained from the medium containing sodium fumarate and the control (without sodium fumarate and ammonium bicarbonate). The medium having both sodium fumarate and ammonium bicarbonate did not show a high amount of cell growth.

4.4.2. Determination of Aspartase Activity

Aspartase activity in *E. coli* lysate from the culture medium with sodium fumarate was measured in either 50 mM Tris-HCl or bicarbonate. The reason to use two different buffers is because of their application. Tris-HCl is the optimal buffer for aspartase (William E Karsten et al., 1985; La et al., 2002; Suzuki et al., 1973b; J. S. Takagi et al., 1985)). Bicarbonate is the buffer used in the combination of CKase and ATCase assay in the previous chapters) at pH 8.5, which is the optimal pH for aspartase activity from previous studies (J. S. Takagi et al., 1985; V. R. Williams & Lartigue, 1967). The results demonstrated that aspartate production rate was higher in 50 mM bicarbonate buffer compared to Tris-HCl buffer (Figure 4.3). There is no clear explanation for this behaviour as there is not enough study on aspartase catalytic mechanism. However, it is obvious that the type and ionic strength of buffer affects the aspartase activity in aqueous solution, as explained by the (Kunz, Henle, & Ninham, 2004). Moreover, the results showed no Tris-HCl inhibition on aspartase activity,

although Tris-HCl has the primary amine group, and it assumes to react with aspartase. Therefore, aspartase is ideal for converting sodium fumarate and ammonia to aspartate.

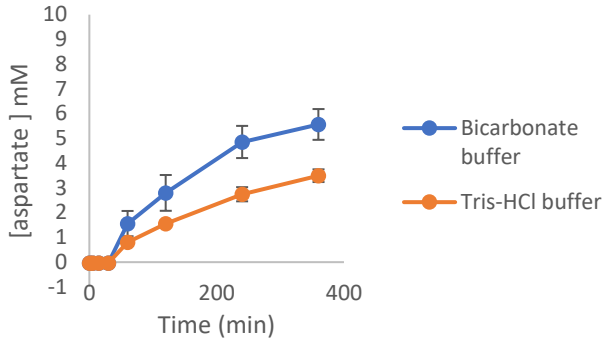


Figure 4.3: Aspartase activity (from induced culture medium with sodium fumarate) in the presence of either Tris-HCl or Bicarbonate buffer. The 0.5 mL reaction mixture contained either 50 mM Tris-HCl or sodium bicarbonate (pH 8.5), 10 mM sodium fumarate, 200 mM NH_4Cl, 25 mM $MgSO_4$ and 10 µL of lysate containing aspartase. They were incubated for 6 hours at 37 °C. The samples were measured by reverse-phase HPLC using AccQ-Tag derivatisation reagents. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

Next, the effect of an induction agent (sodium fumarate) on the production of aspartase activity was measured. The results indicated that the aspartate production rate from the control medium (without sodium fumarate) is surprisingly higher than in the medium with sodium fumarate (Figure 4.4). It might be due to higher aspartase concentration due to either better *E. coli* growth or a better expression rate in the medium without sodium fumarate as an induction component. Overall, it appeared that sodium fumarate did not significantly affect the aspartase expression rate in *E. coli* and might even have suppressed the aspartase expression rate. Taken together, the aspartate production rate was very high in both assays and indicated having sufficient endogenous aspartase in *E. coli* lysate. Therefore, *E. coli* lysate from the culture medium without sodium fumarate (control medium) will be used as an aspartase source for this project hereafter.

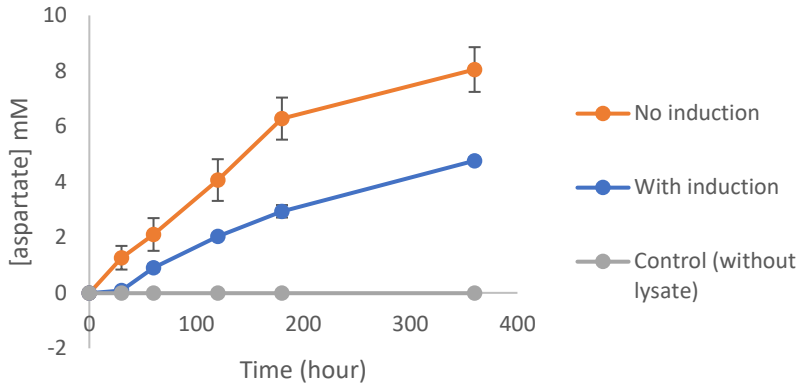


Figure 4.4: Aspartase activity from either induced (with sodium fumarate) or uninduced culture medium. The 0.5 mL reaction mixture contained 50 mM bicarbonate, pH 8.5, 10 mM sodium fumarate, 200 mM NH4Cl, 25 mM MgSO4 and 10 µL of lysate containing aspartase. They were incubated for 6 hours at 37 °C. The samples were measured by reverse-phase HPLC using AccQ-Tag derivatisation reagents. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

4.4.3. Determining the Activity of Aspartase and Pure ATCase Combination

The previous section confirmed that aspartase is present and active in *E. coli* lysate as an endogenous enzyme from induced or uninduced media. It was also demonstrated that the *E. coli* control medium contained enough aspartase to convert fumarate to aspartate. This finding is very beneficial in this project. A single *E. coli* culture is enough to express all necessary enzymes for the whole project. Therefore, the focus of this section is the investigation of CA production from the combination of pure ATCase and aspartase from *E. coli* lysate (Scheme 4.2). For this purpose, after incubation of *E. coli* lysate containing aspartase at 37 °C for a few hours, ATCase from *T. sibiricus* (since it showed the best activity in Chapter 2 and also in the combination of CKase and ATCase from the Chapter 3 among hyperthermophiles) and CP were added to the reaction mixture.

To establish if aspartase and pure ATCase can work cooperatively, the aspartate production was studied over time. The results demonstrated that the aspartate production rate was around 92% with respect to sodium fumarate after 6 hours. Also, the results from addition of ATCase and CP to the aspartase reaction mixture revealed that aspartate produced by aspartase was consumed by pure ATCase (Figure 4.5). Therefore, ATCase was able to combine with *E. coli* lysate containing aspartase to produce CA from sodium fumarate,

ammonia and CP, as shown in Scheme 4.2. Consequently, CKase can be added to the reaction mixture to produce CP from ammonia and bicarbonate.

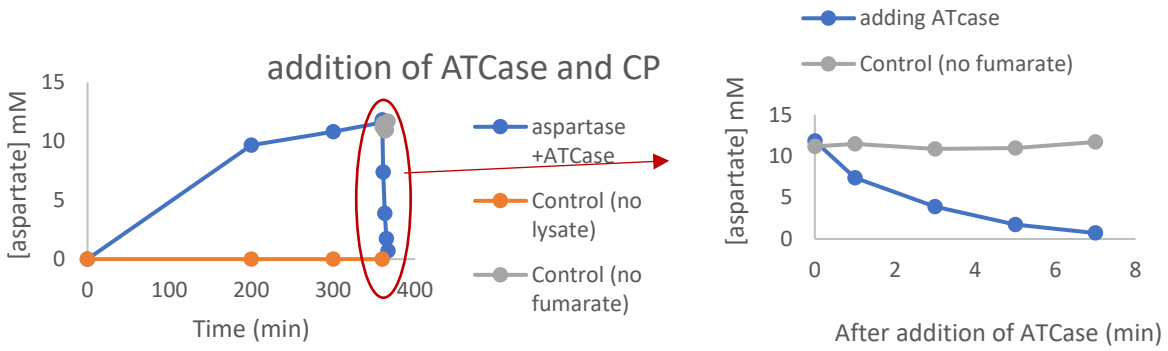


Figure 4.5: The combination of aspartase in *E. coli* lysate and ATCase from *T. sibiricus*. After 6 hours of incubating aspartase at 37 °C, the ATCase and CP were added and ran for another 7 min. The 0.5 mL aspartase reaction mixture contained 50 mM bicarbonate pH 8.5, 12 mM sodium fumarate, 200 mM NH_4Cl, 25 mM $MgSO_4$ and 10 µL of lysate containing aspartase at 37 °C. The sample was analysed by reverse-phase HPLC using AccQ-Tag derivatisation reagents.

4.4.4. Determination of Activity of Combining Aspartase with CKase and ATCase

It was shown in the previous section that aspartase from *E. coli* lysate was able to work with pure ATCase to produce CA from fumarate, ammonia and CP. As explained in the introduction, the ultimate purpose of using aspartase was to use ammonia from wastewater as a nitrogen source for aspartate production in the CA production pathway. Therefore, the combination of all three enzymes, aspartase, CKase and ATCase, should be studied (Scheme 4.7). For this purpose, pure CKase and pure ATCase were sourced from *T. sibiricus*. The reasons to choose CKase and ATCase from *T. sibiricus* were their high pH and temperature stability and robustness compared to mesophile and other hyperthermophiles. Then aspartase (in *E. coli* lysate), pure CKase and ATCase were assayed at 40 °C (the condition for CKase and ATCase combination assay). It was previously shown (Chapter 3, Section 4.3.13) that a pH range of 8.5 to 9.4 slightly and not significantly affects the CA production yield in the pure CKase and ATCase combination

assay. Therefore, pH 8.5 was chosen to perform the assays for this chapter as it is the optimal pH for the aspartase.

The production of CA from combination of three enzymes, aspartase, ATCase and CKase, was investigated (Scheme 4.7). The results from the combination of aspartase, CKase and ATCase demonstrated that these three enzymes are able to work with each other. The product from one enzyme can be consumed as a substrate for the next enzyme to produce the final product, CA (Figure 4.6). However, the CA production rate was lower than the combination of CKase and ATCase. It might result from combining the three enzymes with different velocities and the fact that aspartase is used in this assay in lysate. Lysate cannot be fully investigated in detail as a multifactor exists in it and restricts our study on the combination of three enzymes. However, the outcome of the three enzyme combinations meets our expectation, being CA production.

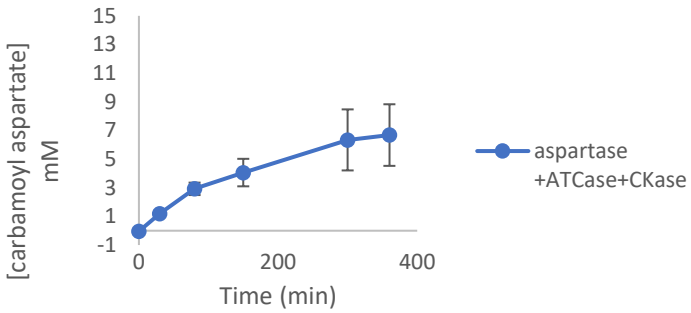


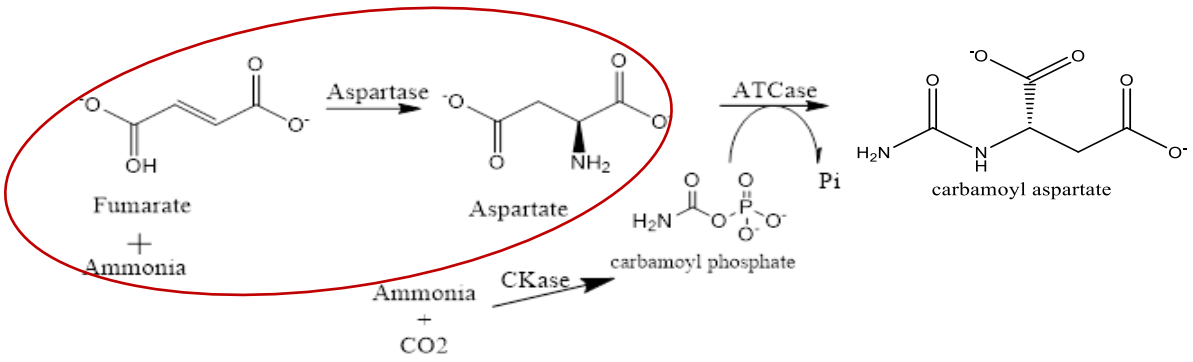
Figure 4.6: CA production from the combination of aspartase, CKase and ATCase from *T. sibiricus*. The 0.5 mL reaction mixture contained 200 mM NH_4OH and $NaHCO_3$ (pH 8.5), 10 mM sodium fumarate, 10 mM ATP, 25 mM $MgSO_4$, 10 µL of lysate containing aspartase, 1 U CKase and 16 U ATCase *T. sibiricus* at 40 °C. The samples were measured by LC/MS. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

4.4.5. Determination of Aspartase Activity in the Co-Expressed Lysate of CKase and ATCase

In the previous chapter, the CKases and ATCases from *E. faecalis* and *T. sibiricus* were co-expressed in *E. coli. F*our different lysates were made with respect to different CKases and ATCases. Also, it was confirmed both CKase and ATCase were active in the lysates and produce CA in high yield by using ammonia, bicarbonate and aspartate (Chapter 3, Section 3.4.17). In this part, the activity of aspartase in the co-expressed lysate of CKase and ATCase was examined (Scheme 4.5). From previous sections of this chapter, it was

confirmed that sufficient endogenous active aspartase exists in *E. coli* lysate to be able to produce aspartate from fumarate and ammonia. Therefore, the presence of aspartase in recombinant co-expressed lysate from *E. coli* was examined by measuring aspartase activity. The aspartase activity was measured using Tris-HCl buffer (and not bicarbonate as it is one of the substrates for CKase) to avoid the production of CP by CKase. Therefore, in the absence of CP, ATCase will not be able to convert aspartate to CA and aspartase activity could be measured by aspartate production.

The aspartase activity was investigated in co-expressed lysate from *E. coli*. The results showed aspartase is present and active in the recombinant co-expressed lysate from *E. coli* and produces aspartate in high yield (Figure 4.7). The results demonstrated that aspartate production was higher in the co-expressed lysate of CKase *T. sibiricus*–ATCase *T. sibircus* compared to another co-expressed lysate. It is difficult to draw any conclusion on why the aspartate production is higher in the co-expressed lysate of the combination of CKase *T. sibiricus*–ATCase *T. sibircus* as there are many factors in the lysate that cannot be investigated in detail in this study. Moreover, there is no significant difference in aspartate production from other recombinant co-expressed lysates (expect CKase *T. sibiricus*–ATCase *T. sibircus*) and non-recombinant lysate.



Scheme 4.5: Recombinant co-expressed lysate from *E. coli* contains all three enzymes that can produce CA from ammonia, fumarate and carbon dioxide. The red circle shows the aspartase enzyme that converts ammonia and fumarate to aspartate.

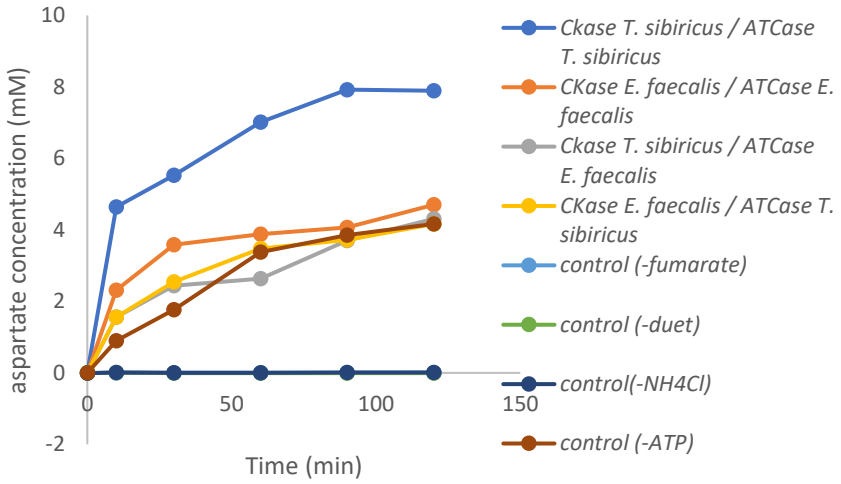


Figure 4.7: Aspartate production by aspartase in lysate from co-expressed CKase and ATCase. The 0.5 mL reaction mixture contained 50 mM Tris-HCl pH 8.5, 200 mM NH_4Cl, 10 mM sodium fumarate, 25 mM $MgSO_4$ and 10 µL of recombinant co-expressed lysate containing aspartase at 40 °C.

4.4.6. Determination of Carbamoyl Aspartate Production from Lysate Using Multienzyme CKase, ATCase, Aspartase and Acetate Kinase

It was confirmed in the previous section that aspartase exists in recombinant co-expressed lysate and produces aspartate. In this section, we investigated the activity of the three enzymes aspartase, CKase and ATCase, in co-expressed lysate to confirm that they can work together to produce the final product, CA. The CA production was investigated in THE co-expressed lysate and results showed all three enzymes aspartase, CKase and ATCase, are active in co-expressed lysate and can produce the final product, CA (Figure 4.8).

Next, the activity of endogenous acetate kinase (by using ADP or AMP) and adenylate kinase (by using AMP) was investigated at pH 9.4 (which is the optimal pH for a combination of CKase and ATCase). It has been shown (Chapter 3, Section 3.5.8) that there is sufficient ATP/AMP or ADP in *E. coli* lysate to initiate the ATP recycling system. Therefore, this will also be investigated without using any exogenous cofactor. The results from the investigation of the addition of AMP, ADP and ATP indicated no significant difference (p>0.05) in the CA production rate while using ATP, ADP or AMP. This means ATP was recycled by acetate kinase and adenylate kinase. In addition, the absence of cofactors does not affect the CA production yield, which demonstrates that there are sufficient endogenous (in trace amount) cofactors (Loan et al., 2019) in the co-expressed lysate to initiate the ATP recycling system. After two hours of incubation of lysate at 40 °C, the yield of CA production

without using any cofactor for recycling ATP was as follows: CKase and ATCase *E. faecalis* around 60% (Figure 4.8b); for CKase and ATCase *T. sibiricus* around 81% (Figure 4.8a); CKase *T. sibiricus* and ATCase *E. faecalis* around 59% (Figure 4.8c) and CKase *E. faecalis* and ATCase *T. sibiricus* around 78% (Figure 4.8d) with respect to sodium fumarate concentration. The CA production rate was higher in co-expressed lysate CKase *T. sibiricus*–ATCase *E. faecalis* (~4.7 mM in 2 h) (Figure 4.8c). The same behaviour with respect to CA production was also seen in the previous chapter from co-expressed lysate CKase *T. sibiricus*–ATCase *E. faecalis*. Therefore, it assumes that either CKase *T. sibiricus* and ATCase *E. faecalis* concentration is higher in this lysate or the fact that ATCase *E. faecalis* catalytic activity is higher, as shown in Chapter 2.

Overall, the results demonstrated that co-expressed lysate containing recombinant enzymes CKase and ATCase and endogenous enzymes including aspartase, acetate kinase adenylate kinase can work together to produce the final product, CA. Therefore, by using one lysate, we can take advantage of using five different enzymes from different sources without the addition of any exogenous cofactor to produce the ultimate product.

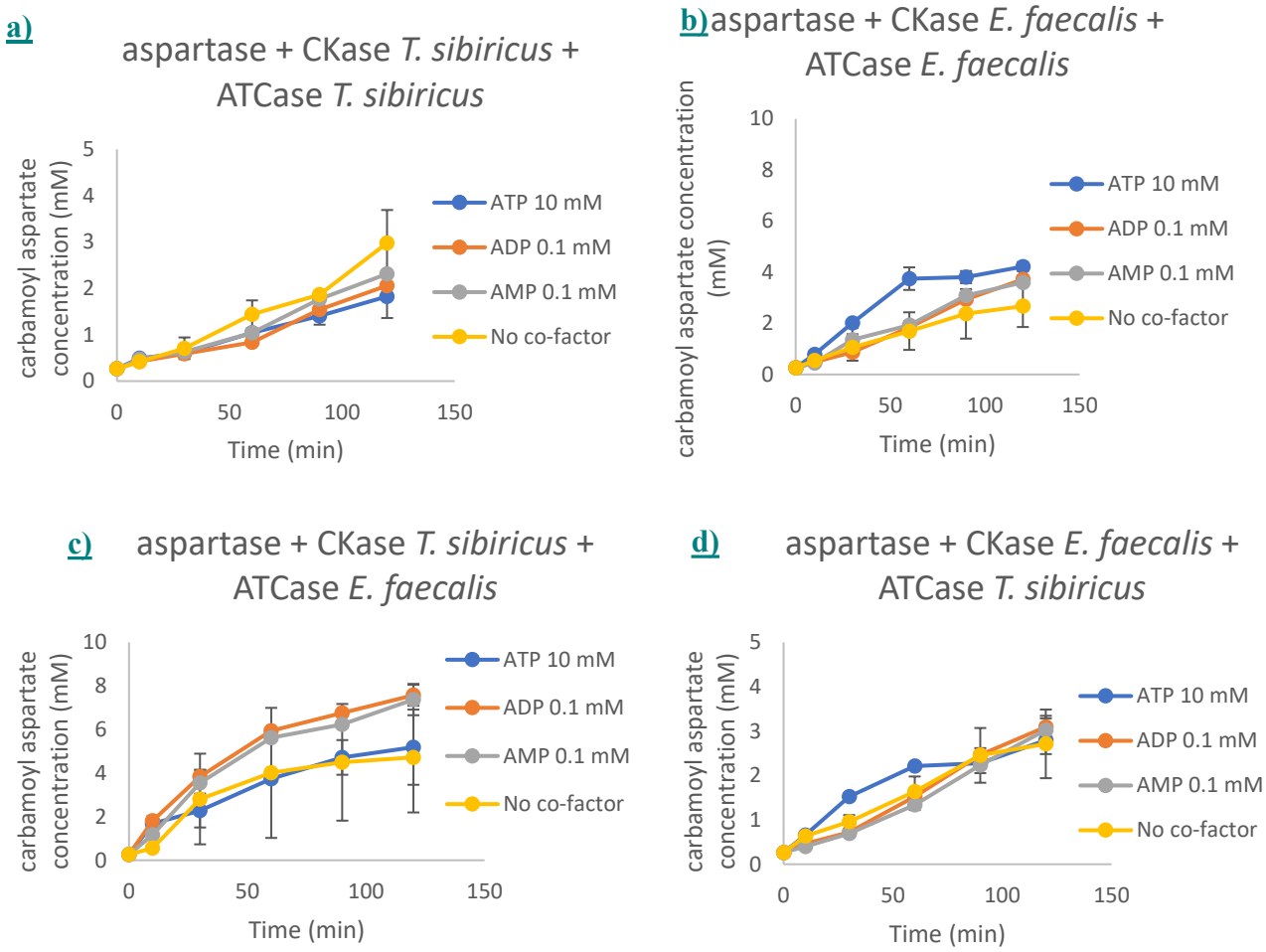


Figure 4.8: Carbamoyl aspartate production from recombinant co-expressed lysate of CKase and ATCase containing endogenous aspartase, acetate kinase and adenylated kinase. The 0.5 mL reaction mixture contains 200 mM NH_3 and $NaHCO_3$ pH 9.4, 25 mM MgSO4, 10 mM sodium fumarate, 20 mM acetyl phosphate, either 0.1 mM ADP or AMP and 10 µL of each co-expressed lysate of CKases and ATCases *T. sibiricus* and *E. faecalis* at 40 °C. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

4.4.7. Characterisation of Co-Expressed Lysate Containing Multienzyme CKase, ATCase, Aspartase and Acetate Kinase

In the previous section, the activity of all recombinant and endogenous enzymes in the co-expressed lysate was confirmed. In this section, the recombinant co-expressed lysate from *E. coli* was assayed at different pH values (8.5, 8.8 and 9.4, similar to the CKase and ATCase pH value range) while ATP was recycling. The pH study is an important factor in wastewater treatment. The effect of different pH on CA production from co-expressed lysate was investigated, and the results indicated no significant difference (p>0.05) in CA

production yield at different pH values in a varied combination of CKase and ATCase (Figure 4.9 a-d). Therefore, aspartase activity in this pH range is not significantly affected. This is similar to the results obtained from the co-expressed combination of CKase and ATCase discussed in the previous chapter (Section 3.4.18). Therefore, the crude lysate could be used in a pH range of 8.5 to 9.4 without any concern about losing any enzyme activity. Moreover, the ATP recycling system (enzymes and substrates) is active at different pHs and is not affected by varying pH levels.

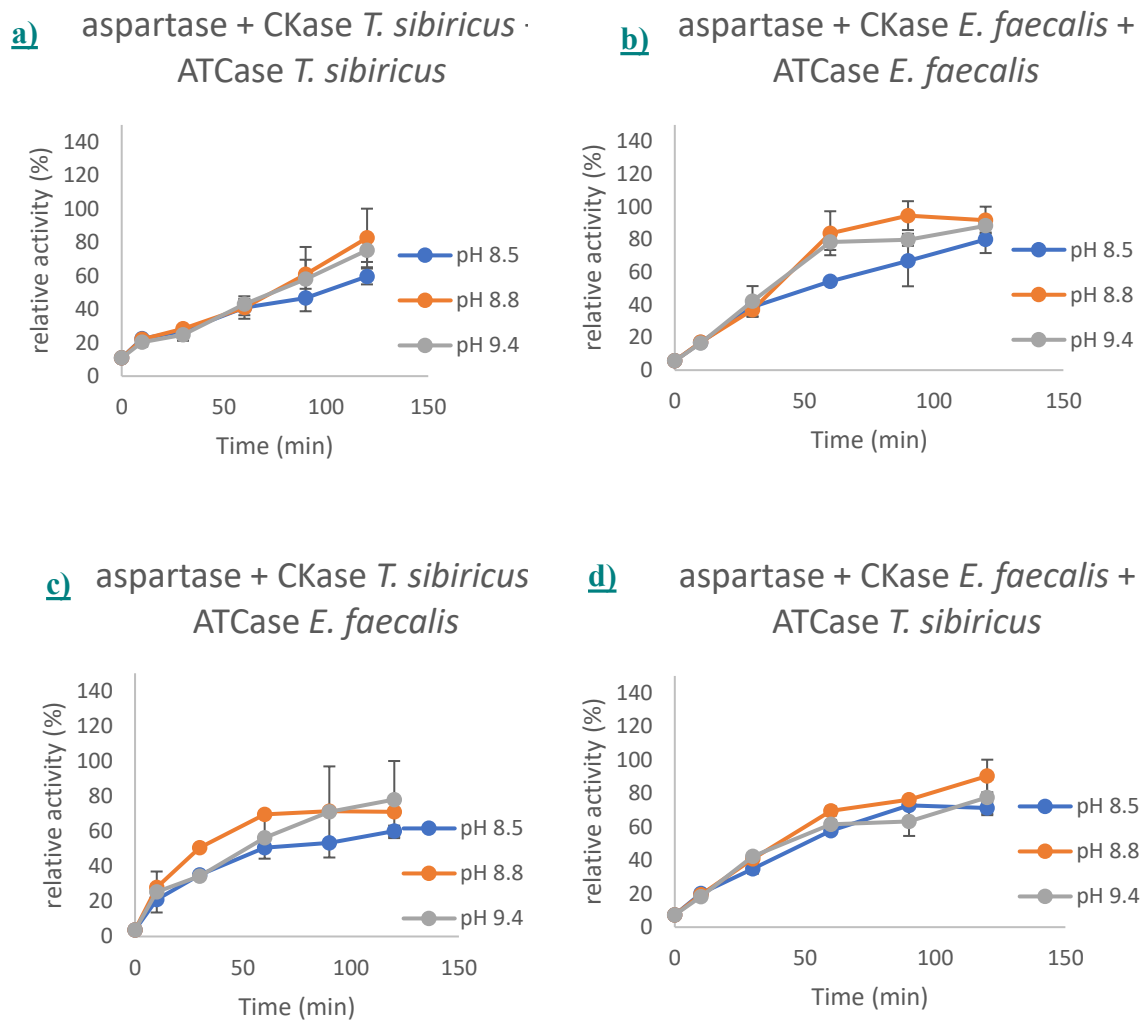


Figure 4.9: Co-expressed CKase and ATCase with the ATP recycling system at different pH values. The 0.5 mL reaction mixture contains 200 mM NH_4OH and $NaHCO_3$, 25 mM $MgSO_4$, 10 mM sodium fumarate, 20 mM acetyl phosphate and 10 µL of each co-expressed lysate of CKases and ATCases *T. sibiricus* and *E. faecalis* at 40 °C. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

4.4.8. Determination of Carbamoyl Aspartate Production After 24 Hours Incubation of Lysate Containing Multienzyme CKase, ATCase, Aspartase and Acetate Kinase

In the previous sections, the experiments were run for only two hours, and therefore, 100 per cent production yield with respect to sodium fumarate was not achieved. Therefore, in this section, the experiment runs for a longer time to achieve the highest production yield. Thus, the production of CA from recombinant co-expressed lysate containing aspartase, CKase, ATCase and acetate kinase was investigated in 24 hours incubation time at 40 °C. The results from incubation of co-expressed lysate at 40° C for 24 hours showed that the CA production accumulation increases with time and after 24 hours although the rate slows down after 10 hours assaying. The CA yield for different lysates was as follows: CKase–ATCase *E. faecalis* around 80%, CKase–ATCase *T. sibiricus* around 85%, CKase *T. sibiricus*–ATCase *E. faecalis* around 100% and CKase *E. faecalis*–ATCase *T. sibiricus* around 84% with respect to sodium fumarate (Figure 4.10). There was no significant difference *(p*>0.05) among CKase–ATCase *T. sibiricus,* CKase–ATCase *E. faecalis* and CKase *E. faecalis*–ATCase *T. sibiricus*.

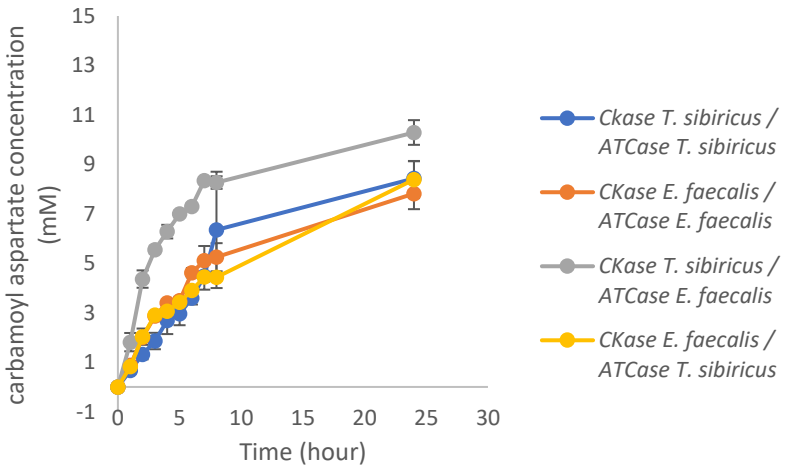


Figure 4.10: Co-expressed CKase and ATCase with ATP recycling system assaying for 24 hours. The reaction mixture contains 200 mM NH_3 and $NaHCO_3$ pH 8.5, 25 mM $MgSO_4$, 10 mM sodium fumarate, 20 mM acetyl phosphate and 10 µL of each co-expressed lysate of CKases and ATCases *T. sibiricus* and *E. faecalis.* Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

4.4.9. Saturation Amount of Co-Expressed Lysate Containing Multienzyme

The previous section confirmed that a high production yield for CA with respect to sodium fumarate could be achieved. Therefore, in this section, the focus is on investigating the effect of lysate concentration on CA production yield in a shorter time to achieve 100 per cent CA yield. Therefore, different amounts of the recombinant co-expressed lysate (from 2% to 16% v/v lysate/reaction mixture) were assayed to measure the saturation concentration of lysate for the production of CA. The results demonstrate that the CA production rate from lysate CKase–ATCase *T. sibiricus* increased substantially by increasing the lysate (Figure 4.11a). At 16% (v/v) lysate to the reaction mixture, more than 83% production yield was achieved.

The CKase–ATCase *E. faecalis* production yield was similar, between 10 to 20μL (2% to 4% of total volume). There was also no significant difference (p>0.05) in CA production between 10 and 20μL lysate. However, there was a sharp increase when using 60 to 80μL of lysate in the assay (8% to 16% of total volume), reaching 73% CA yield after 2 hours (Figure 4.11b).

Further, in lysate from CKase *T. sibiricus*–ATCase *E. faecalis*, the CA sharply increased from 10 to 20μL (2% to 4% of total volume), reaching 70% CA production yield. After that, the production rate gradually increased to 84% using 80μL lysate (16% of total volume). There was no significant difference (p>0.05) in CA production among 20, 40 and 80μL lysate (Figure 4.11c).

Finally, CKase *E. faecalis*–ATCase *T. sibiricus* CA production yield gradually increased to 90% by elevating the lysate amount to 40μL in the assay (8% of total volume) and decreased when using 80μL (it might be due to the existence of an inhibition factor in the lysate preventing the enzymes to fully function (Bisswanger, 2014)). There was no significant difference (p>0.05) in CA production among 20, 40 and 80μL lysate (Figure 4.11d).

Overall, all co-expressed lysates demonstrated more than 75% and 45% CA production rates with respect to sodium fumarate and acetyl phosphate respectively, in two hours when more than 8% (v/v) lysate was used in the assay.

Finally, it would be difficult to draw any other conclusion from these results, as the accurate concentration of each enzyme is not measurable in the crude lysate. CKase and ATCase have similar molecular weight, and after running SDS-PAGE, a thick band was observed corresponding to both CKase and ATCase. It is also difficult to identify the endogenous enzymes on the SDS-PAGE.

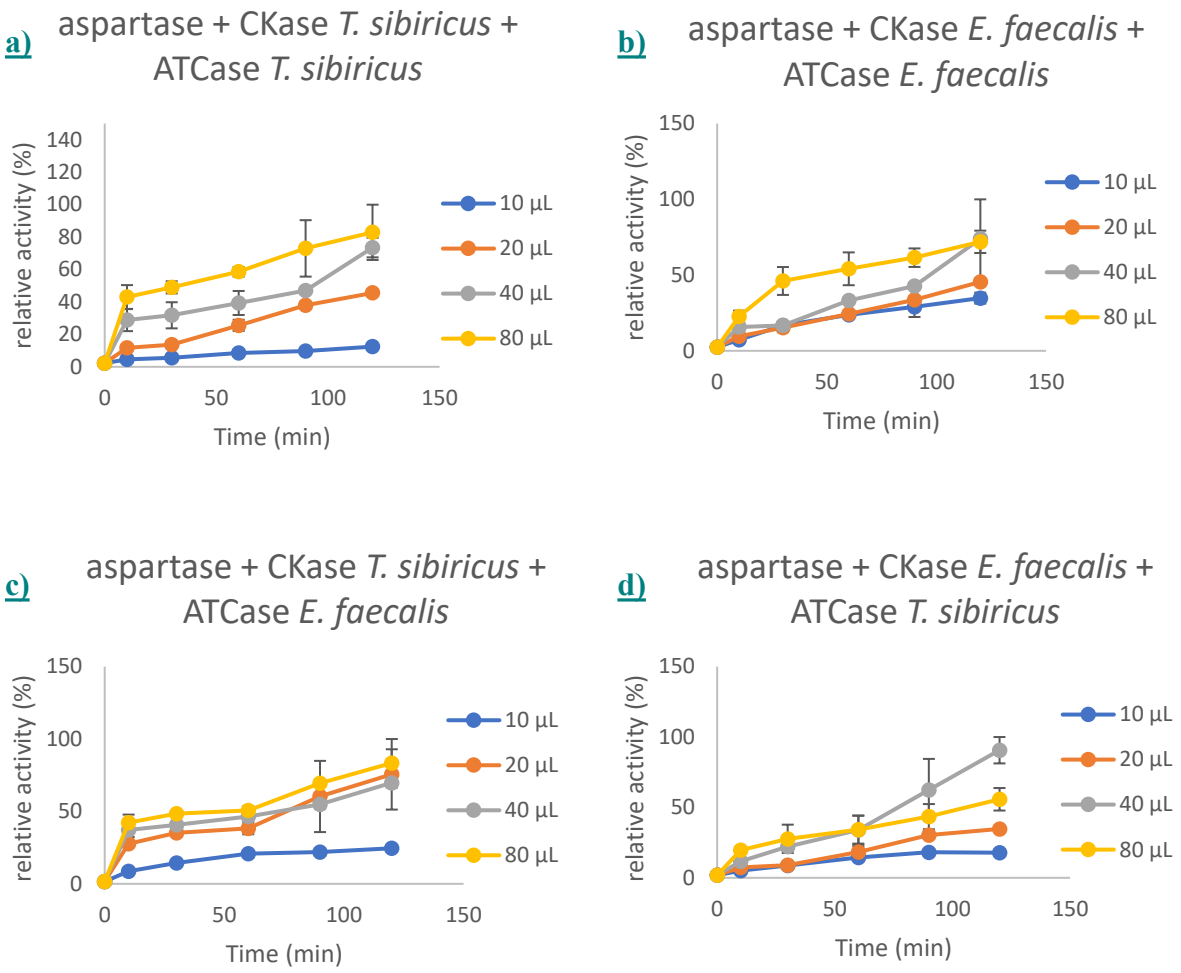


Figure 4.11: Study the effect of different amounts of co-expressed CKases and ATCases on CA production yield. The 0.5 mL reaction mixture contains 200 mM NH_4OH and $NaHCO_3$ (pH 8.5), 25 mM MgSO4, 10 mM sodium fumarate, 20 mM acetyl phosphate and different amount of each co-expressed lysate of CKases and ATCases *T. sibiricus* and *E. faecalis.* 10 µL is 2% v/v lysate to reaction mixture, 20 µL is 4% v/v and 40 µL is 8% v/v and 80 µL is 16% v/v. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

4.5. Conclusions

We have developed a system that mitigates two important environmental factors, carbon dioxide and ammonia, through a biocatalytic transformation process. We demonstrated that *E. coli* lysate can be used as a source of aspartase and contains enough aspartase to convert ammonia and fumarate to aspartate. Moreover, it was demonstrated that co-expressed lysate contains enough aspartase to produce aspartate for ATCase usage.

Further, it was confirmed that all endogenous and exogenous enzymes in co-expressed lysate are active and can work together to produce the ultimate product, CA.

Taken together, a multiple enzyme system has been established to produce CA by carbamoylation of aspartate in aqueous solution from ammonia and carbon dioxide. Importantly, the enzyme cascade is performed by *E. coli* lysate that was prepared from one single bacterial culture with the recombinant co-expressed and endogenous enzymes required for this cascade.

Overall, the research showed that the combination of CKase and ATCase was able to not only sequester the ammonia and carbon dioxide but also convert them to a value-added product, CA, a valuable precursor in the pyrimidine pathway and a good potential fertiliser.

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Chapter 5: Immobilisation

5.1. Introduction

In the previous chapter, CKase and ATCase showed promising results with respect to transforming ammonia into CA. However, the CKase and ATCase enzymes would not be recycled by themselves; therefore, the process would not be economical for industrial applications.

As it has already been explained in the main introduction, the use of enzymes as a catalyst has many advantages over chemical catalysts, such as high specific activity, mild reaction conditions, high catalytic activity and improved economic viability. However, the use of enzymes would not be economical if the enzyme is not recovered and cannot be reused. Immobilisation of the enzymes is a suggested method for recycling the enzyme and fully utilising their capacity (Knežević-Jugović et al., 2017). Therefore, this chapter focuses on CKase and ATCase enzyme immobilisation to recover and reuse the enzymes.

Immobilisation is a technique to improve enzyme performance as a biocatalyst (R. A. Sheldon, 2007). Immobilisation allows the enzyme to be easily separated from the reaction mixture, simplifying the enzyme application and providing an efficient and reliable process. Immobilisation also helps recover and reuse the enzymes, which dramatically decreases the cost, an essential prerequisite for enzyme catalyst systems (Tischer & Wedekind, 1999). Moreover, immobilisation helps the enzymes be more stable in the soluble form. It induces enzyme stability and activity under extreme conditions, such as pH, temperature and organic solvents (Cantone et al., 2013).

There are many methods for immobilising enzymes, each with pros and cons. They vary from binding on prefabricated carrier material to in situ prepared carriers. The binding force can differ from weak to strong or in having one binding point or multiple binding points. The use of different immobilisation methods depends on the desired application.

The choice of an appropriate immobilisation method is critical as it determines the enzyme activity and characteristics of the reaction. Many factors should be considered in selecting an immobilisation method, such as the cost of the immobilisation procedure, the overall enzymatic activity, the desired final properties of the immobilised enzymes and the toxicity of the immobilisation reagents and the regeneration characteristics (Chiou & Wu, 2004).

In this chapter, the synthetic organic carrier called Eupergit® C 250 L was used for immobilisation because it is simple, reliable and uses covalent binding. It has already been explained in the introduction of Chapter 2 that the ATCase catalytic subunit is a trimer (composed of three monomers). Therefore, the ATCase structure makes immobilisation very complicated since the position of the active site is located at the interface of the trimeric structure. However, with our immobilisation techniques and choice of carrier, ATCase and CKase were successfully immobilised. Moreover, ATCase and CKase enzymes maintain more than 90 per cent of their activities. Also, the findings from stability studies exhibit higher enzyme stability when immobilised.

5.1.1. Enzyme Immobilisation Techniques

Immobilisation can be performed in two classical ways: the physical and chemical methods (Figure 5.1).

1. Physical methods are characterised by the weaker and monovalent interaction such Van der Waals forces, hydrophobic interaction, hydrogen bonds, affinity binding and ionic binding of the enzyme with a carrier or by entrapment (Herrmann, Kragl, & Wandrey, 1993) using media such as gel, and fibres and by microencapsulation (Reetz, Zonta, Simpelkamp, Rufinska, & Tesche, 1996), and;
2. Chemical methods use interactions that form weak or strong covalent bonds between enzymes and carriers, such as cross-linking (Fernandez-Lafuente et al., 1995) and carrier-bound enzymes (Tischer & Wedekind, 1999).

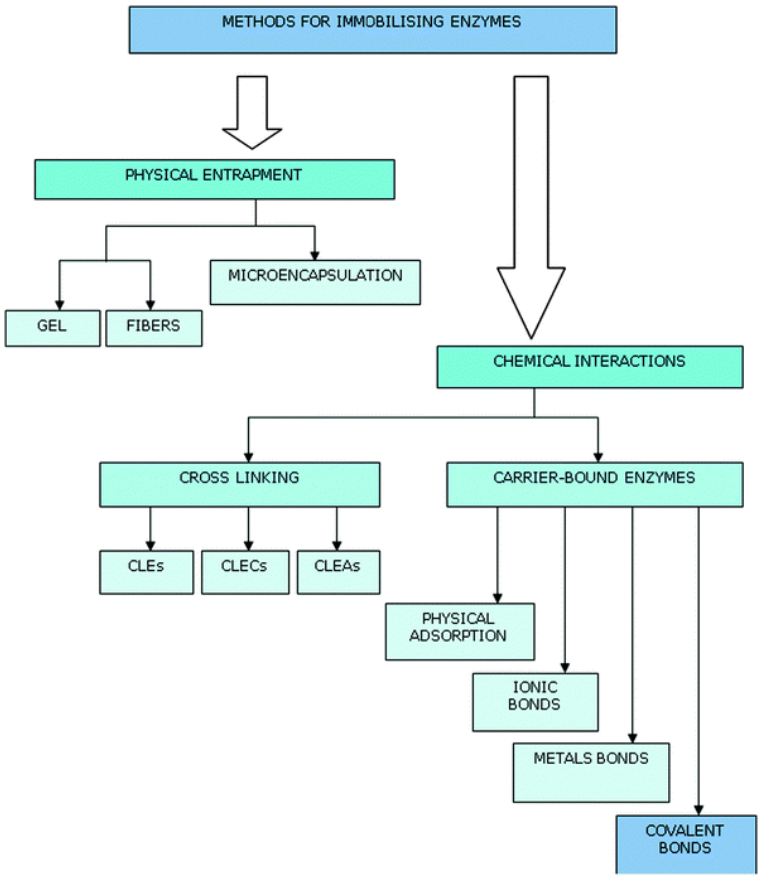

Figure 5.1: Carrier bond immobilisation methods versus other different immobilisation techniques. CLEs = Cross-Linked Enzymes; CLECs = Cross-Linked Enzyme Crystals; CLEAs = Cross-Linked Enzyme Aggregates (Cantone et al., 2013).

In another classification, immobilisation can be categorised into three different techniques: adsorption, entrapment and covalent/cross-linking. This section explains the advantages and disadvantages of different techniques, focusing on covalent binding being used for this project.

Depending on the nature of enzyme structure, one of these three methods can be used (Figure 5.2). Adsorption technique is one of the easiest methods that is reversible. The enzyme is physically adsorbed or attached to the carrier. The bonding strength varies from weak bonds, such as Van der Waals and hydrophobic interactions, to stronger bonds such as ionic bonding. The advantages of this method are simplicity, low-cost, chemical-free enzyme and the maintenance of high enzyme activity (Flickinger & Drew, 1999). However, the drawbacks of this method are overtaken by the advantages, such as being pH-dependent and subject to temperature and buffer ionic strength changes. Therefore, it is not the best method for my project as the enzymes' ultimate usage will be in wastewater that could have different a pH and temperature. Also, the bonds in this technique are generally

weak. Therefore, the enzymes can be detached from the carrier and prone to leaching, resulting in potential substrate contamination during industrial usage. (Mohamad, Marzuki, Buang, Huyop, & Wahab, 2015; Pryakhin, Chukhrai, & Poltorak, 1977; R. Sheldon, 2007).

Another method is entrapment, which is an irreversible method. In this technique, the enzyme is entrapped inside a carrier, such as a lattice fibre or a polymer membrane. The entrapment restricts the enzyme from leaching and improves the mechanical stability of the enzyme. It also creates an optimal microenvironment for the enzyme that prevents enzyme denaturation (Subramanian et al., 1999). The disadvantages of this method include limitations on the mass transfer of substrate or analytes to the enzyme's active site, a low loading capacity and the erosion of the carrier material during utilisation (Górecka & Jastrzębska, 2011; R. Sheldon, 2007; Subramanian et al., 1999). Hence, it would not be a suitable choice for this project as it limits the mass transfer of substrate to enzymes.

Cross-linking is the other method for immobilisation that is irreversible. In this method, no carrier is used, and the enzyme acts as its own carrier (R. Sheldon, 2007). Therefore, the advantages and disadvantages of using a carrier are avoided. (Hanefeld, Gardossi, & Magner, 2009). This method occurs by cross-linking the enzyme molecules using bi or multifunctional reagents. The drawback of this method is that it is costly, has low space-time yields and has pH dependency on the cross-linking method (Cao, Langen, & Sheldon, 2003; Migneault, Dartiguenave, Bertrand, & Waldron, 2004). This method does not fit this project's purpose as ATCase is a trimeric enzyme and cross-linking the subunits will substantially minimise the enzyme's activity.

Finally, the covalent bonding method can be used for irreversible immobilisation and is one of the most commonly used techniques for immobilisation. In this method, a functional group on the enzyme side chains of lysine, cysteine, aspartic acid, and glutamic acids is usually involved in binding the enzyme to an activated group on the carrier (Guisan, 2006; Singh, 2009). The shape, size, and composition of the carrier materials, the specific conditions used during the immobilisation and the nature of the immobilisation methods are the factors that influence the activity of covalently bonded enzymes (Obzturk, 2001). In covalent bonding between the enzyme and carrier, the stability is determined by the direction of the enzyme binding. If the active centre amino acids of the enzyme do not take part in the binding, the highest level of enzyme activity would be achieved. This method could benefit ATCase immobilisation as most of the active site amino acids (including His 134, Gln 137, Ser 52, Thr 55, Lys 84, Arg 105, Thr 53, Arg 54, Arg 229, Ser 80, Glu 231 and Arg 167) are different from the amino acids that are usually involved in binding the enzyme (lysine, cysteine,

aspartic acid, and glutamic acids) to a carrier. The advantages of this method are a powerful bond between the enzyme and the carrier is created (that allows the easy recovery and reuse of the enzymes and prevents the enzyme from leaching into the reaction), increased thermostability and unlimited covalent binding between the enzyme and substrate. (Ispas et al., 2009; Ovsejevi et al., 2013; R. Sheldon, 2007). A good example of this method is the immobilisation of lipase on silica nanoparticles and glutaraldehyde-activated aminopropyl glass beads for ester synthesis in industry (Damnjanović et al., 2012; Dandavate, Keharia, & Madamwar, 2009; Yilmaz, Can, Sezgin, & Yilmaz, 2011). Therefore, the covalent bonding method is the most promising method used in this project for CKase and ATCase enzyme immobilisation.

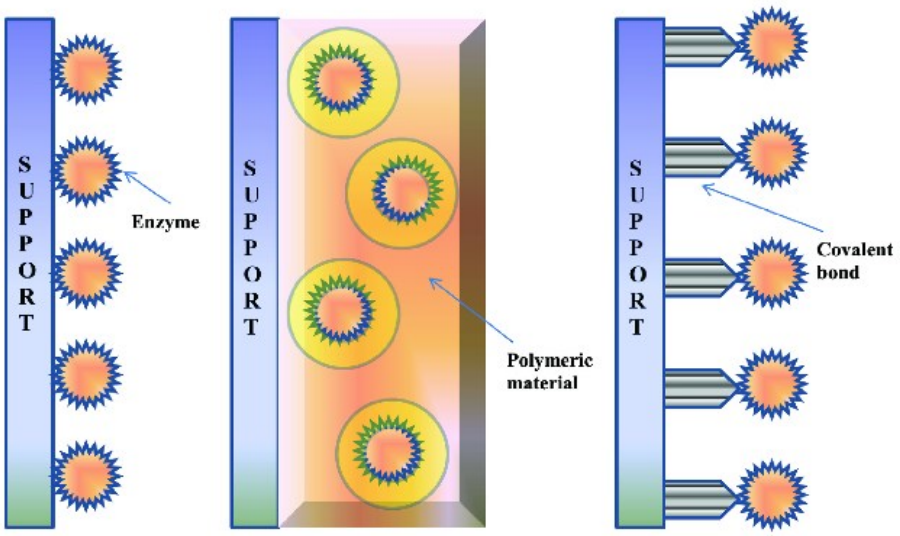

Figure 5.2: Schematics of three common enzyme immobilisation techniques. From left to right: physical adsorption, entrapment and covalent attachment/cross-linking (Spahn & Minteer, 2008).

5.1.2. Immobilisation onto a Surface

Classically, there are four different methods to covalently immobilise an enzyme onto a surface. Firstly, activate the enzyme by suitable reaction prior to binding. The disadvantage of this method is the enzyme's catalytic and structure, essential residue modifies and affects the intra- and intermolecular cross-linking. Therefore, the enzyme activities significantly decreased by this method (Kurzer & Douraghi-Zadeh, 1967; Solomon & Levin, 1974; Tischer & Wedekind, 1999), and it would not be suitable for this project.

Secondly, using an intermediate between the carrier and enzyme functional groups. The intermediate can be a bi or multifunctional coupling agent. This method can result in intra- and intermolecular enzyme cross-linking, which is a drawback and makes this method unsuitable for our project (Dandavate, Keharia, & Madamwar, 2009; Yilmaz, Can, Sezgin & Yilmaz 2011; Damnjanović, et al., 2012).

Thirdly, adsorption of a specific enzyme to a specific carrier. In this method the enzyme is engineered by recombinant DNA techniques to produce an enzyme with 'biospecific' group that can adsorb to a specific carrier by affinity binding. The drawback of this technique is that it is very costly (X. L. Huang, Walsh, & Swaisgood, 1996; Ong, Gilkes, Warren, Miller, & Kilburn, 1989; Stempfer, Höll-Neugebauer, Kopetzki, & Rudolph, 1996) and therefore is avoided in this project.

Finally, binding the native enzyme to a modified and activated support. This method is based on providing a covalent bond between the enzyme and the carrier surface and is the most promising technique and is used for this project.

5.1.3. Carriers

To immobilise the enzyme, one of the most important components to consider is the choice of enzyme carrier, which contributes to enzyme performance (Knežević-Jugović et al., 2017). The carriers can be categorised into three different groups.

5.1.3.1. Natural Organic Carrier

The early research studies used natural organic carriers. They are cheap starting materials, available in large quantities, highly hydrophilic and can bind covalently to enzymes. Yet, they are poor in mechanical characteristics and compressibility and need to be activated before use. Therefore, it is not considered for usage in this project. Examples of natural organic carriers are keratin, collagen, globular proteins or carbohydrates (Stempfer, Höll-Neugebauer, Kopetzki & Rudolph, 1996).

5.1.3.2. Inorganic Carrier

They show good mechanical properties, resistance against microbial attack and organic solvents and have good thermal stability. However, it depends on the type of the inorganic carrier as they can show some drawbacks such as having a small binding surface, high production costs, limited stability under alkaline conditions. They also may need activation and modification. Hence, due to many drawbacks of this kind of carrier, the usage for this

project is avoided. A few examples of inorganic carriers are glass, silica gel, alumina, bentonite, hydroxyapatite, nickel/nickel oxide and titania. (Buchholz, Duggal, & Borchert, 1980; Buchholz & Klein, 1987) (Gemeiner et al., 1994).

5.1.3.3. Synthetic Organic Carrier

Finally, recent studies present synthetic organic carriers. They show great physical and chemical characteristic variation, such as adaptation to a wide range of different enzymatic processes. They are resistant to microbial attacks. They are found in many forms, such as purely adsorptive resins, ion-exchange resins with different basic and acidic groups or preactive support carriers like those with epoxide and azlactone groups. Examples of synthetic organic carriers are Eupergit, Emphaze, polystyrene, polyacrylate and polyvinyl. (Gemeiner et al., 1994). Therefore, synthetic organic carriers seem to be the best option for enzyme immobilisation for our project. Eupergit is the most often used ‘ready to go’ enzyme carrier in this group. Eupergit is an epoxy-activated organic carrier and is one of the best-known supports for enzyme immobilisation. It has excellent mechanical and chemical properties (Katchalski-Katzir & Kraemer, 2000). It also exhibited other good properties, such as a low swelling tendency in solvents and good performance in stirred batch reactors.

Eupergit® C 250 L demonstrates having the most criteria for carrier selection. The following criteria for selecting an effective carrier need to be considered (Foresti & Ferreira, 2007; Tischer & Wedekind, 1999).

Table 5.1: Selection Criteria for Choosing an Effective Carrier

Selection criteria	Explanation	Eupergit® C 250 L
Functional group	determines the activity yield of an immobilisation reaction, enzyme stability and operational stability	√
Permeability and surface area	mostly using a large size carrier with high porosity is desirable that allows the enzyme and substrate to penetrate	√
Hydrophilicity/hydrophobicity of the carrier matrix	can affect the adsorption, availability of the substrate and product, and the type and strength of the non-covalent enzyme-carrier interaction	√
Insolubility	very important factor in immobilisation as it avoids enzyme loss and contamination of the product by dissolved enzyme and carrier	√
Mechanical stability/rigidity	this depends on the application, for example, the carrier using a bioreactor should be stable against sheer forces	√
Form and size of support	an important factor when used in a bioreactor and filtration as it should not pass filtration—the ideal spherical size is from 150 to 300 μm	√
Resistance to microbial attack	the carrier should be stable against microbial attack during long-term usage of the carrier	√
Regenerability	important for expensive carrier materials to be able to recover and reuse them	√

5.1.3.4. Eupergit®

Eupergit® is microporous, epoxy-activated acrylic beads with a diameter of 100–250 μm (pore size r = 100 nm and oxirane density 300 μmol/g dry beads) and is made by copolymerisation of N,N′-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Eupergit epoxy groups make binding sites for enzymes that make it very suitable as a matrix for enzyme immobilisation (Figure 5.3). The enzyme directly binds to the polymer via an epoxy group by covalent bonds on beads, which results in an enzyme orientation shift. This keeps the active site of the enzyme the maximum distance from the support, providing better access to the substrate by using good geometrical congruence between enzyme and support, proper immobilisation protocol and

enhancement the reactivity of nucleophilic groups of the enzyme surfaces(Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007).

B-galactosidase (AOG) from *Aspergillus oryzae* is a good example of enzyme immobilisation on Eupergit®. AOG could not only be fully immobilised on the Eupergit® but also achieved a 130% activity yield. Immobilisation also improved enzyme usage and storage (Aslan, Taher, & Cavİdoğlu, 2018). Another example is serum paraoxonase (PON1), a high-density protein associated enzyme that protects low- and high-density proteins from oxidation. This enzyme was immobilised on Eupergit®, and the immobilisation helped the enzyme maintain its catalytic activity and increase its thermostability (Sayin & Guler, 2015). Since Eupergit® showed promising immobilisation outcomes for many enzymes, Eupergit® was chosen in this project for CKase and ATCase immobilisation.

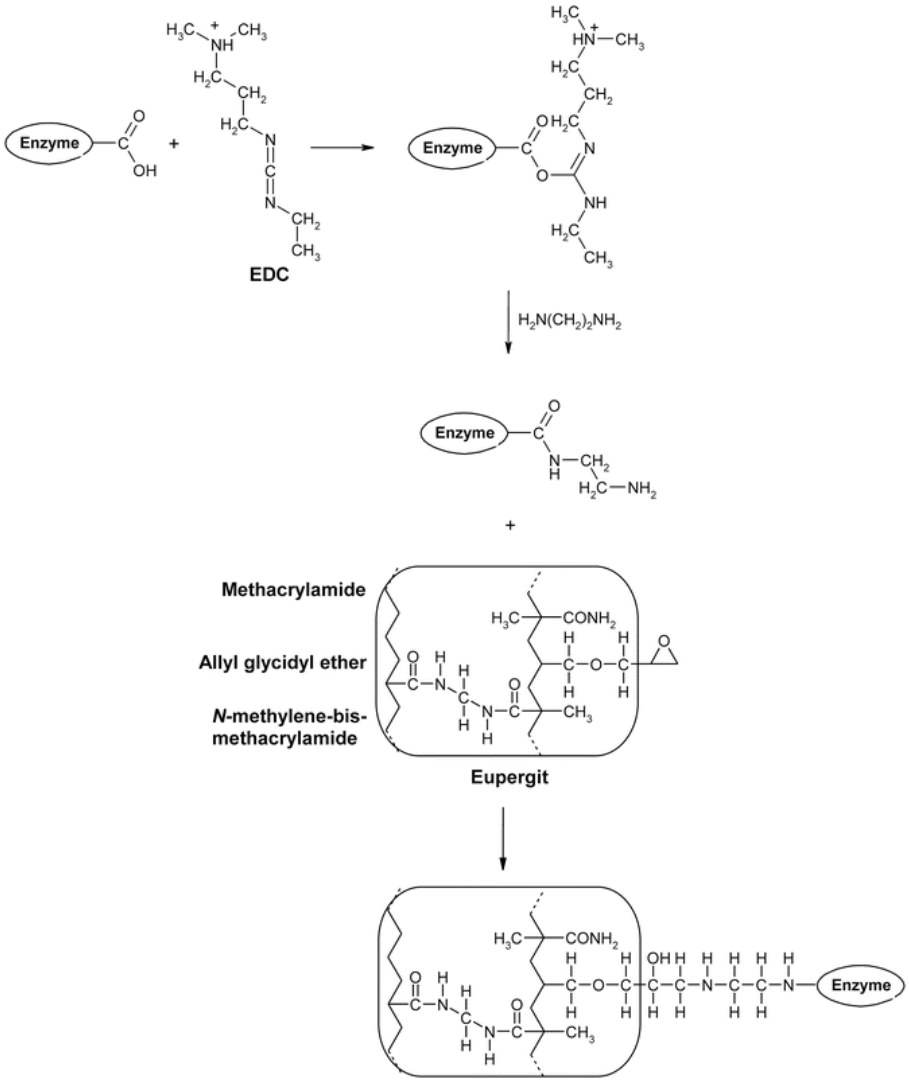

Figure 5.3: Schematic illustration of the modified conventional method for enzyme immobilisation on epoxy carriers based on the immobilised enzyme post-treatment consisting of the chemical amination of the biocatalyst with ethylenediamine (after activation of the carboxylic function with carbodiimide) (Knežević-Jugović et al., 2017).

5.2. Aim

This chapter aims to use immobilised enzymes for further usage in wastewater treatment to remove and use ammonia. To achieve this aim, the first objective is to immobilise ATCase *T. sibiricus* and ATCase *E. faecalis* and CKase *T. sibiricus* on Eupergit® C 250 L as the carrier. The second objective is to immobilise a combination of CKase and ATCase (CKase *T. sibiricus* and ATCase *T. sibiricus*; CKase *T. sibiricus* and ATCase *E. faecalis*) on Eupergit® C 250 L. The third objective is to characterise the immobilised enzymes at different temperatures and pH values, and the final objective is to use the immobilised enzymes in wastewater to produce CA from ammonia.

5.3. Materials and Methods

5.3.1. Materials

Eupergit® C 250 L was bought from Sigma-Aldrich®

5.3.2. Immobilisation

Purified ATCase *E. faecalis* (5 mg), ATCase *T. sibiricus* (5 mg) and CKase *T. sibiricus* (5 mg) were individually dissolved in 5 mL of 1 M potassium phosphate buffer pH 8.5.

Eupergit® C 250 L (500 mg) was dry weighted and added to the enzyme solution of ATCase *T. sibiricus*, ATCase *E. faecalis* and CKase *T. sibiricus* in a 20 mL glass jar. The reaction mixture was incubated at room temperature with orbital shaking for 72 hours.

After 72 hours, the beads were collected using a sintered glass filter, and the solution was drained by vacuum. The beads were washed five times with 10 mL 100 mM potassium phosphate buffer pH 8.5 and then treated with 5 mL glycine 2 M for two hours to block the remaining active groups. The reaction mixture was washed thoroughly (five times) with 10 mL 100 mM potassium phosphate buffer pH 8.5, air dried and then kept at 4 ºC for further usage.

In another experiment, a combination of CKase *T. sibiricus* and either ATCase *E*. *faecalis* or *T. sibiricus* was immobilised on Eupergit® C 250 L. Two different processes have been conducted for a combination of CKase and ATCase immobilisation. In the first method, the CKase and ATCase were added simultaneously to Eupergit® C 250 L and left at room temperature for 72 hours before washing and drying. In the second method, the CKase was firstly added to Eupergit for 24 hours at room temperature. Then the ATCase was added to the reaction mixture and kept at room temperature for another 48 hours until washed and air dried for further usage.

5.3.3. Determination of Immobilised CKase *T. sibiricus* and ATCase *T. sibiricus* or *E. faecalis* Activity

The activity of CKase was measured by HPLC by measuring the ATP and ADP concentration, as described in Chapter 3 in the Material and Methods section. The ATCase activity was measured by aspartate consumption and CA production, as described in Chapter 2. The combination of CKase and ATCase activity was measured by aspartate consumption and CA production, as described in Chapter 3.

5.3.4. Effects of Temperature and pH on Immobilised ATCase *T. sibiricus* and *E. faecalis*

The thermal stability of (5 mg), the immobilised catalytic subunit of enzymes ATCase *T. sibiricus* and ATCase *E. faecalis* were measured. To determine the optimal temperature for immobilised enzyme activity, the reaction solutions of ATCase containing 50 mM Tris-HCl, pH 8.5, 10 mM CP, 10 mM aspartate and immobilised catalytic subunit of enzymes (5 mg *T. sibiricus* and *E. faecalis*) were pre-incubated at 20, 40, 60 and 80 ºC for 5 min and assayed after at 20, 40, 60 and 80 ºC for 10 min. The assays were initiated by adding CP.

In the second assay to measure thermostability, the immobilised enzymes were pre-incubated at 40 ºC in 50 mM Tris-HCl pH 8.5 without substrates for up to 30 days. The samples were assayed at 40 ºC as a function of time.

Two buffers were used over a broad range of pH values to determine the optimal pH for immobilised enzyme activity. We used 50 mM Tris-HCl for pH values 6.0 to 8.8, and 50 mM bicarbonate was used for pH values 8.8 to 11. The reaction solution was contained immobilised catalytic subunits of the enzymes (5 mg *T. sibiricus* and *E. faecalis*), 50 mM of either buffer, 10 mM CP and 10 mM aspartate. Reagents were pre-incubated at 40 ºC for 5 min, and the reaction was initiated by adding CP and then assayed for 10 min.

5.3.5. Effects of Temperature and pH on Immobilised CKase *T. sibiricus*

The thermal stability of the immobilised catalytic subunit of enzyme CKase *T. sibiricus* (5 mg) was measured. To determine the optimal temperature for immobilised enzyme activity, the reaction solutions for CKase containing 200 mM NH_3/$NaHCO_3$ pH 9.4, 10 mM $MgSO_4$, 10 mM ATP and immobilised catalytic subunit of enzymes (5 mg *T. sibiricus*) were pre-incubated at 20, 40, 60 and 80 ºC for 5 min and then assayed after at 20, 40, 60 and 80 ºC for 60 min. The assays were initiated by adding ATP.

In the second assay to measure thermostability, the immobilised enzymes were pre-incubated at 40 ºC in 200 mM NH_3/$NaHCO_3$ pH 9.4 without substrates for up to 30 days, and the samples were assayed at 40 ºC as a function of time.

To determine the optimal pH for immobilised enzyme activity, NH_3/$NaHCO_3$ was used over a pH range of 8.5 to 9.4. The reaction solution contained immobilised catalytic subunits of enzymes (5 mg *T. sibiricus*) 200 mM NH_3/$NaHCO_3$, 10 mM $MgSO_4$ and 10 mM ATP. Reagents were pre-incubated at 40 ºC for 5 min, and the reaction was initiated by adding ATP and then assayed for 60 min.

5.3.6. Effects of Temperature and pH on Immobilised Combination of CKase *T. sibiricus* and ATCase *T. sibiricus* OR *E. faecalis*

The thermal stability of (5 mg) immobilised catalytic subunit of enzymes CKase *T. sibiricus* and ATCase from either *E. faecalis*, or *T. sibiricus* was measured. To determine the optimal temperature for immobilised enzymes activity, the reaction solutions containing 200 mM NH_3/$NaHCO_3$, pH 8.5, 10 mM $MgSO_4$, 10 mM aspartate and 10 mM ATP and immobilised enzymes were pre-incubated at 20, 40, 60 and 80 ºC for 5 min and assayed after at 20, 40, 60 and 80 ºC for 60 min. The assays were initiated by adding ATP.

In the second assay to measure thermostability, the immobilised enzymes were pre-incubated at 40 ºC 200 mM NH_3/$NaHCO_3$ pH 8.5 without substrates for up to 30 days, and the samples were assayed at 40 ºC as a function of time.

To determine the optimal pH for immobilised enzyme activity, NH_3/$NaHCO_3$ was used over the pH range of 8.5 to 9.4. The reaction solution contained immobilised enzymes (5 mg) 200 mM NH_3/$NaHCO_3$, 10 mM $MgSO_4$, 10 mM aspartate and 10 mM ATP. The reagents were pre-incubated at 40 ºC for 5 min, and the reaction was initiated by adding ATP and then assayed for 60 min.

5.3.7. Application of Immobilised Combination of CKase *T. sibiricus* and ATCase *T. sibiricus* or *E. faecalis* in Wastewater

A wastewater sample from Perth, Australia, was filtered by using 0.2 µm filter. Then 5 mg of immobilised CKase *T. sibiricus* and ATCase *E. faecalis* or CKase *T. sibiricus* and ATCase *T. sibiricus* were weighted and added to the wastewater in a 1.5 mL Eppendorf tube. The reaction mixture contained 2 mM $MgSO_4$, 1 mM acetyl phosphate, 10 µL *E. coli* lysate and 0.5 mM sodium fumarate to 1 mL wastewater. The reaction mixture was incubated at 40 ºC for 6 hours. The CA production was measured as an index of ammonia conversion, firstly to CP and later to CA.

5.4. Results and Discussion

This chapter aims to immobilise the ATCase and CKase enzymes for usage in wastewater treatment to remove ammonia and convert it to CA. This provides the opportunity to reuse, isolate, and recover the enzymes and, therefore, substantially decrease the cost and time of enzyme production. This system is very efficient, versatile, cheap, easy to prepare and cost-effective.

5.4.1. ATCase, CKase and CKase/ATCase Immobilisation Yield

In this section, the immobilisation of ATCase, CKase and the combination of CKase and ATCase was investigated using Eupergit (Figure 5.4). The purified ATCase and CKase were added to Eupergit® C 250 L to immobilise and increase the enzyme capacity for recovering and reusing. Three individual enzymes were immobilised from CKase and ATCase from different sources. Moreover, the combinations of CKase and ATCase were immobilised to study the possibility of simultaneous immobilisation of two enzymes together from either the same source or different sources. Therefore, ATCase *E. faecalis*, ATCase *T. sibiricus* and CKase *T. sibiricus* were chosen for immobilisation as they showed higher residual activity compared to other enzymes investigated in Chapters 2 and 3. Further, the combination of CKase and ATCase from *T. sibiricus* was chosen as both are hyperthermophiles. It will be the first time two hyperthermophillic enzymes will be immobilised together. Additionally, CKase *T. sibiricus* and ATCase *E. faecalis* were chosen for immobilisation because they were a combination of a hyperthermophile and a mesophile in addition to their higher residual activity to produce CA compared to CKase *E. faecalis* and ATCase *T. sibiricus*.

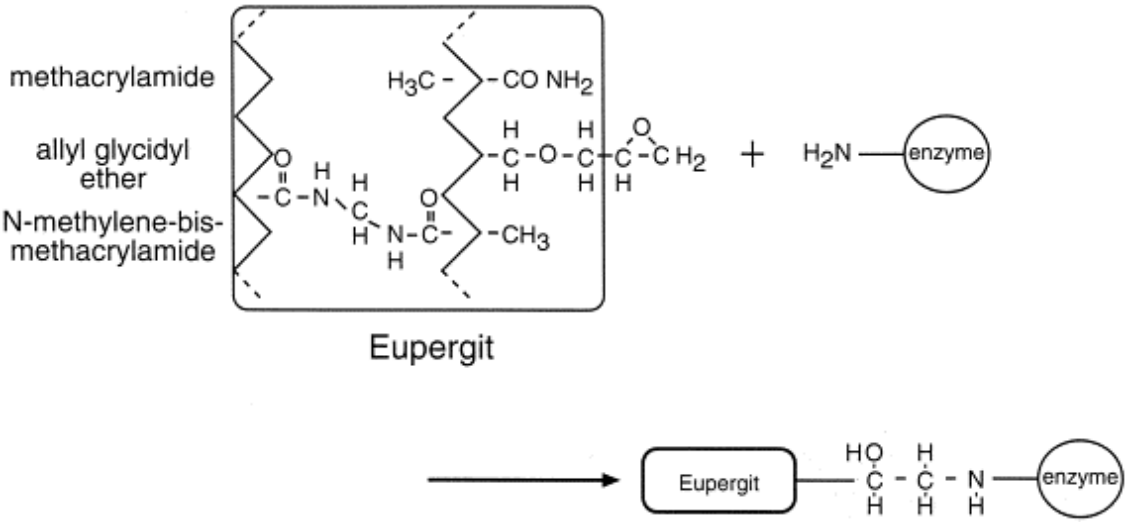

Figure 5.4: The immobilisation of enzyme on Eupergit® C 250 L.

The ATCase and CKase are attached to the oxirane groups of Eupergit® C 250 L by covalent bonds through their lysine side chain. The amount of the soluble enzyme and immobilisation method determines the immobilisation enzyme yield (Tischer & Wedekind, 1999).

The immobilisation yield and enzyme activity yield were defined as follows:

$$\text{Immobilisation yield (\%)} = [(U_a\text{-}U_r) / U_a] \times 100$$

$$\text{Activity yield (\%)} = [U_x/(U_a\text{-}U_r)] \times 100$$

Where U_a is total activity of the enzyme added to the solution

U_r activity of residual enzyme recovered in the washings.

U_x activity of the immobilised enzyme.

The activity calculated by measuring substrate consumption or product formation unit (mM) per time (min) under linear reaction conditions.

The results from immobilisation of individual ATCase *E. faecalis*, ATCase *T. sibiricus* and CKase *T. sibiricus* on Eupergit® C 250 L demonstrated that the enzymes were successfully immobilised on Eupergit® C 250 L. The results showed that ATCase from *T. sibiricus* and *E. faecalis* were immobilised between 97% to 91%, respectively, and CKase was immobilised at 100% on Eupergit (Table 5.2).

Moreover, the immobilisation of a combination of CKase and ATCase on Eupergit® C 250 L investigated. The results demonstrated that the combination of CKase and ATCase was successfully immobilised on Eupergit® C 250 L. The immobilisation yields from the combination of CKase and ATCase were between 74% to 79%, as shown in Table 5.2. The immobilisation yield of the combination of CKase *T. sibiricus* and ATCase *T. sibiricus* in which ATCase was added at the beginning or after 24 hours was very similar. Therefore, both CKase and ATCase could be added at the beginning of the process to Eupergit.

Further, the effect of immobilisation on enzyme activity was investigated. The results showed the activity yield of immobilised enzymes varied with the different enzymes and their combination (Table 5.2). ATCase *T. sibiricus* has the lowest immobilisation activity yield, followed by ATCase *E. faecalis* and then the combination of CKase and ATCase *T. sibiricus*. The low immobilisation activity yield from ATCase might be due to their trimeric subunit catalytic structure and the position of the active site at the interface of the trimeric structure of ATCase (Figure 5.5 top). Since Eupergit binds to a lysine from ATCase, it probably prevents some of the ATCase from forming trimeric subunits structure by binding to the monomer of ATCase (Figure 5.5 bottom). Therefore, the attachment of Eupergit to the ATCase monomer subunit affects the trimer formation and, as a result, decreases the

activity yield. Moreover, there is one lysine in the active site of ATCase that can bind to Eupergit, resulting in a loss of ATCase activity. Further, immobilisation also reduces the activity of the immobilised enzyme due to the mass transfer effect. The mass transfer effect reduces the enzyme molecule availability within the pores or by slowing the circulating substrate molecules (Tischer & Wedekind, 1999). Yet, this disadvantage of immobilisation may be compensated by increasing the enzyme stability. The immobilisation effect on CKase activity yield revealed that CKase immobilised activity yield was more than 90%. This demonstrates that immobilisation on Eupergit does not inhibit enzyme activity and that less than 10% of activity was missing.

Finally, the effect of immobilisation on the combination enzyme activity yield was investigated. The results from immobilisation of the combination of CKase and ATCase demonstrated that they all have more than 80% activity yield, which is higher than individual ATCase activity yield (Table 5.2). The higher activity yield of the combination of CKase and ATCase compared to individual ATCase might be due to an interaction between CKase and ATCase. It might be that the trimeric ATCase has been attached and interacted with the immobilised CKase rather than Eupergit and therefore retained its trimeric structure and activity. Another explanation might be that the CKase and ATCase interact in the solution before being added to the Eupergit. As a result, ATCase was able to keep its trimeric structure while attaching to Eupergit through its lysine side chain. Also, the results showed that the immobilised CKase *T. sibiricus*/ATCase *E. faecalis* has higher activity yield than CKase *T. sibiricus*/ATCase *T. sibiricus.* This could be due to the higher activity of ATCase *E. faecalis*, as shown in Chapters 2 and 3.

Table 5.2: The Immobilisation of Enzymes Yields on Eupergit

Microorganism	**Enzyme immobilisation yield (%)**	**Immobilised activity yield (%)**
ATCase *T. sibiricus*	91.4 ±0.06	23.6 ± 5.4
ATCase *E. faecalis*	97.7 ± 0.2	59.2 ± 6.3
CKase *T. sibiricus*	100 ± 0.2	91.2 ± 4.7
CKase *T. sibiricus*/ATCase *T. sibiricus**	78.8 ± 0.2	82.4 ± 6.5
CKase *T. sibiricus*/ATCase *T. sibiricus***	77.9 ± 0.4	88.3 ± 17.2
CKase *T. sibiricus*/ATCase *E. faecalis*	73.8 ± 3.4	91.2 ± 17.0

* ATCase is added at the beginning of immobilisation along with CKase. ** ATCase was added 24 hours after CKase was added to Eupergit.

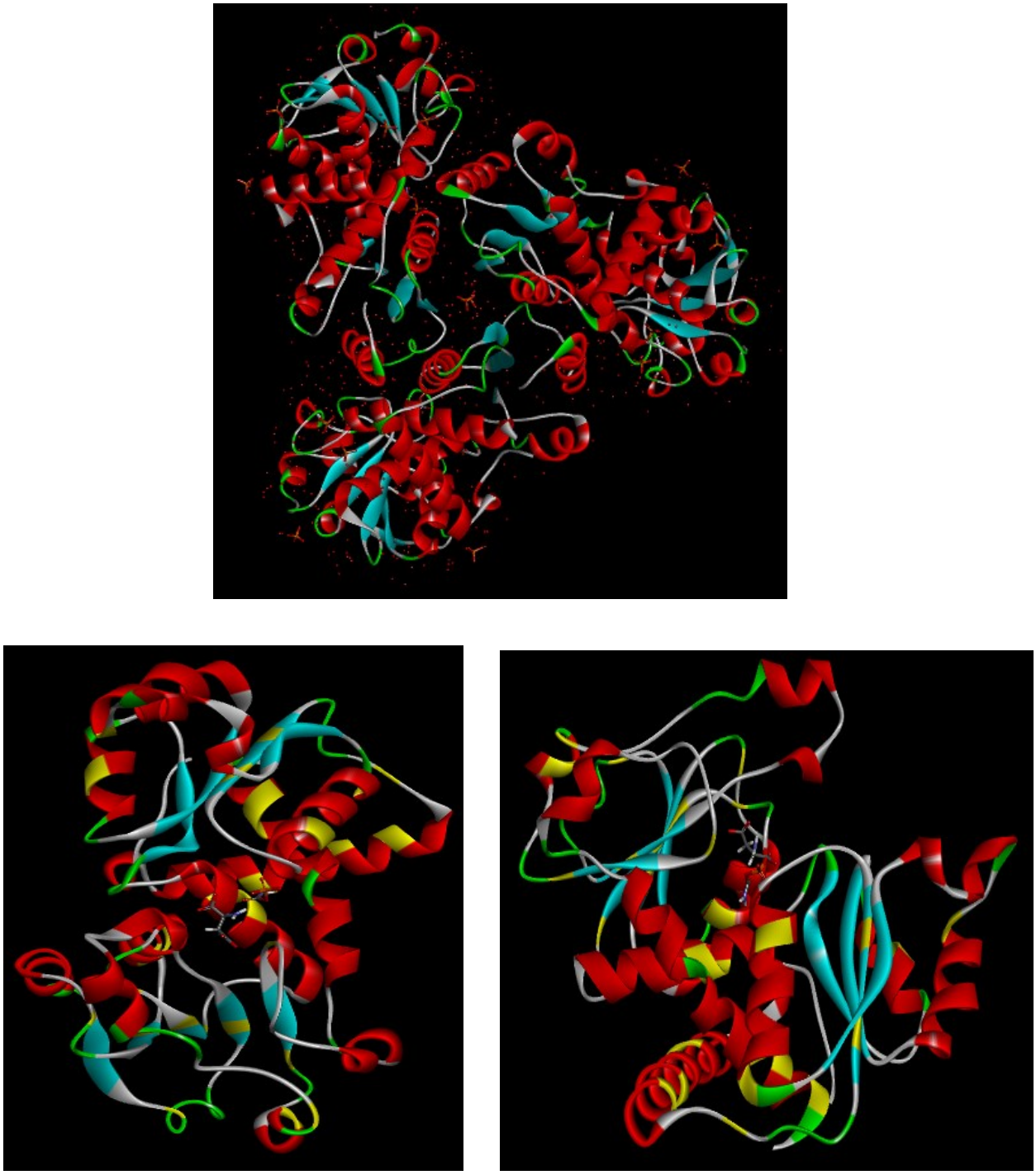

Figure 5.5: Top: ATCase *T. sibiricus* trimer subunit's structure. The active catalytic site is located in the interface among three subunits. The figure has been drawn by Chimera 1.8.1 and SWISS-MODEL.Bottom: ATCase *T. sibiricus* monomer subunit structure. Left: shows the ATCase monomer subunit from above. Right: shows the monomer from the side. The yellow colour shows the lysine position in the monomer. Carbamoyl phosphate and aspartate are the molecules in the middle of the structure.

5.4.2. Effect of pH On Immobilised Enzyme Activity

The previous section showed that individual CKase and ATCase and the combination of CKase and ATCase were successfully immobilised. Moreover, the immobilised enzymes were all active at different rates. In this section, the effect of pH on immobilised enzymes was investigated as the pH in wastewater can vary.

Therefore, the enzyme activity was measured at different pH values, and their effects on the activity of the immobilised enzymes were measured in comparison to the non-immobilised enzymes. The results show that immobilisation changes the optimal pH for ATCase (Figure 5.7). Non-immobilised ATCase *E. faecalis* and *T. sibiricus* exhibited optimum activity at a pH of around 9 and 8, respectively (Figures 5.6a and 5.6b). While immobilised ATCase showed optimal activity for immobilised ATCase *E. faecalis* and *T. sibiricus* at pH 8 to 8.8 and 8.8, respectively. This might be due to the new microenvironment of the enzyme's properties. Further, immobilised ATCase *E. faecalis* and *T. sibiricus* mostly showed higher residual activity at different pH values, especially at higher pH compared to non-immobilised ATCase *E. faecalis* and *T. sibiricus*. From pH 6 to 11, both immobilised ATCase *E. faecalis* and *T. sibiricus* maintained more than 44% and 49% of relative residual activity, respectively. At pH 11, immobilised ATCase *E. faecalis* and ATCase *T. sibiricus* showed 2.5 and 1.3 times more residual activity, respectively, compared to non-immobilised enzyme (Figures 5.6a and 5.6b).

Overall, the immobilised enzymes exhibited higher activity at different pH values compared to non-immobilised enzymes. This might be because many properties of enzymes improve by immobilisation, such as functional stability and pH tolerance (Guzik et al., 2014). Also, the effect of the new microenvironment in immobilisation increases the enzyme activity, as confirmed by previous research studies. For example, immobilisation of trehalose synthase on Eupergit shows no change in different pH ranges. Therefore, the studies have confirmed the effect of immobilisation on enzyme stability and activity at different pH values (Cho, Park, & Shin, 2006).

The effect of pH on immobilised enzyme activity was investigated on CKase *T. sibiricus.* The pH study was done in the pH range of 8.5–9.4 because it showed the lowest specific activity in the non-immobilised CKase study (Hennessy et al., 2018), and it is the pH range in the majority of wastewater. Previous studies assume that immobilisation might increase the specific activity in that pH range (Guzik et al., 2014). The results from the pH study on CKase showed that the relative residual activity profile of non-immobilised and immobilised enzymes was similar (p>0.05) and that it increased by elevating the pH (Figure 5.6c). At

pH 8.5 and 8.8, the immobilised CKase demonstrated higher relative residual activity compared to non-immobilised CKase enzymes. In comparison, at pH 9.4, the non-immobilised relative activity was higher than the non-immobilised CKase (Figure 5.6c). However, the difference between CKase from immobilised and non-immobilised at varied pH levels was not significant (p>0.05). The relative low residual activity at a lower pH might be due to the restricted availability of carbamate at lower pH (more carbamate is produced at higher pH due to a higher ratio of ammonia to ammonium ions).

Moreover, the immobilised combination of CKase *T. sibiricus* and ATCase *T. sibiricus* (from two different immobilisation methods) relative residual activity increased from pH 8.5 to 8.8. It slightly decreased from pH 8.8 to 9.4 (p>0.05) (Figures 5.6e, f). While non-immobilised combinations relative residual activity increased by increasing the pH (p>0.05). The immobilised combination of CKase *T. sibiricus* and ATCase *T. sibiricus* relative residual activity was higher than non-immobilised at pH 8.5 and 8.8 but was lower at pH 9.4 (Figures 5.6e, f). However, there was no significant difference (p>0.05) in varied pH.

Further, CKase *T. sibiricus* and ATCase *E. faecalis* showed a similar pH profile, and there was no difference in residual activity in the pH range of 8.8 to 9.4 (Figure 5.6d). However, immobilised combination relative residual activity was higher than non-immobilised combination activity (Figure 5.6d).

It seems that the combination of CKase and ATCase has its own pH properties and is more stable compared to individual ATCase and CKase properties. The interaction between the two enzymes might make them more stable, as we saw in Chapter 3.

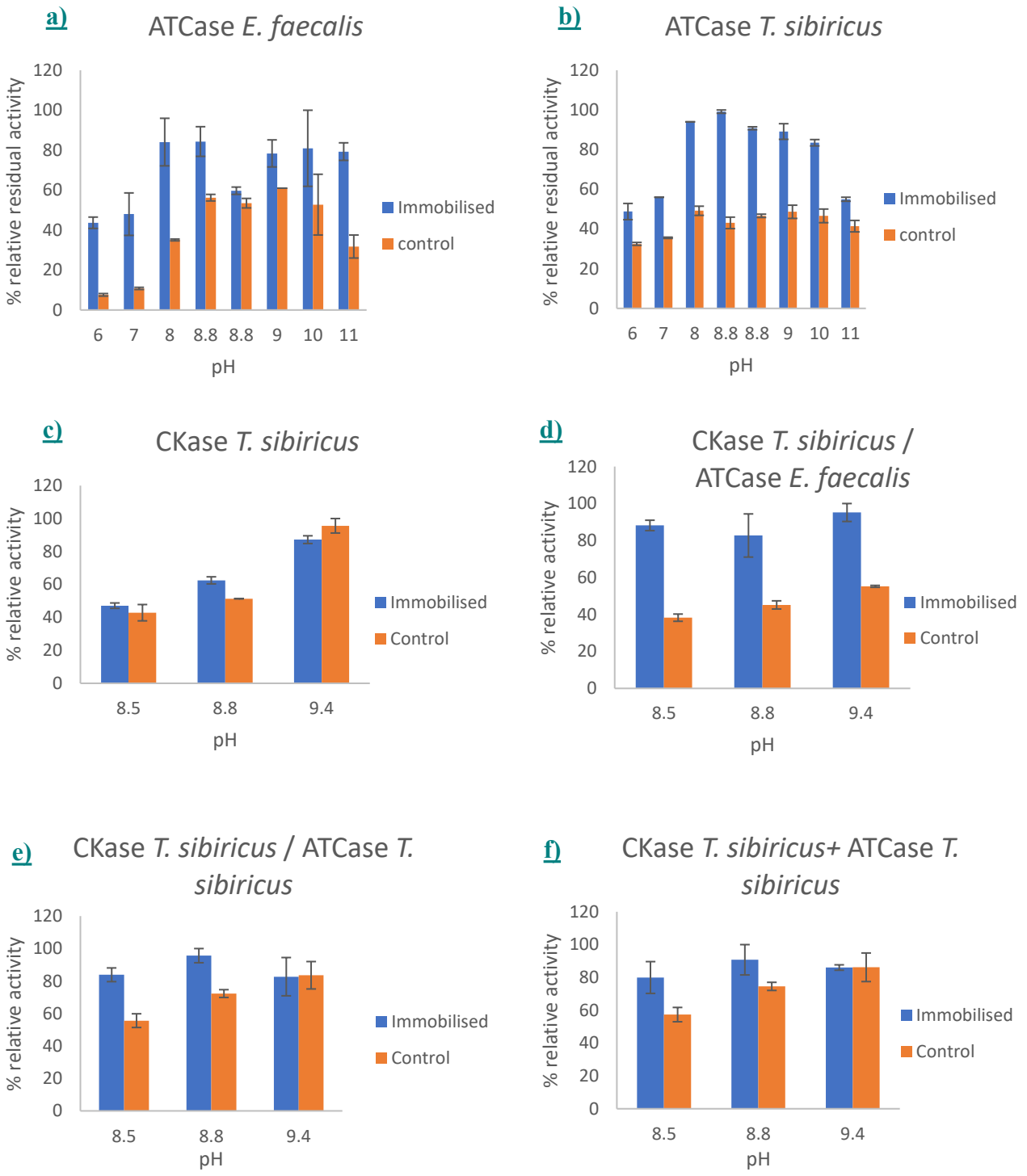

Figure 5.6: The effect of pH on the activity of non-immobilised and immobilised CKase and ATCase and combination of CKase/ ATCase. The reaction mixture for ATCase contains either 50 mM Tris-HCl or 50 mM $NaHCO_3$, 10 mM CP and 10 mM aspartate at 40 °C. The reaction mixture for CKase and combination of CKase/ATCase contains 200 mM $NH_3/NaHCO_3$, 10 mM $MgSO_4$, 10 mM aspartate at 40 °C. Values are the average of duplicate experiments, and error bars represent the standard deviation of experiments.

5.4.3. Effect of Temperature on Immobilised Enzyme Activity

In this study, the effect of temperature on the activity of the immobilised enzymes was studied in comparison to the non-immobilised enzymes.

The results demonstrated that the non-immobilised and immobilised ATCase *E. faecalis* optimum temperature was around 40 ºC (Figure 5.9a). However, immobilised enzymes maintained more than 87% activity at 60 °C, 14% at 80 °C and 75% at 20 °C (Figure 5.9a). Overall, immobilisation has improved the stability of mesophilic ATCase *E. faecalis* at different temperatures. It might be due to different mechanisms that could happen during immobilisation, such as rigidification, multisubunit immobilisation, hydrophilisation of the microenvironment or multipoint covalent immobilisation (Figures 5.7–5.8), which makes the enzyme more stable at a higher or lower temperature without affecting the enzyme function (Cho et al., 2006; D. A. Cowan & Fernandez-Lafuente, 2011; C. Mateo et al., 2002; Mateo, Grazú, et al., 2007; Mateo, Palomo, et al., 2007) (Figure 5.9). Yet it was observed that the immobilised enzyme lost most of its activity at 80 °C, similar to that of the non-immobilised enzyme.

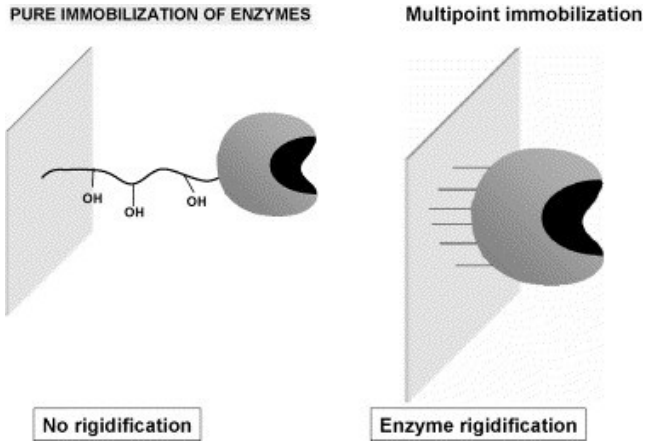

Figure 5.7: The effect of enzyme immobilisation on stability sourced (Mateo et al., 2007).

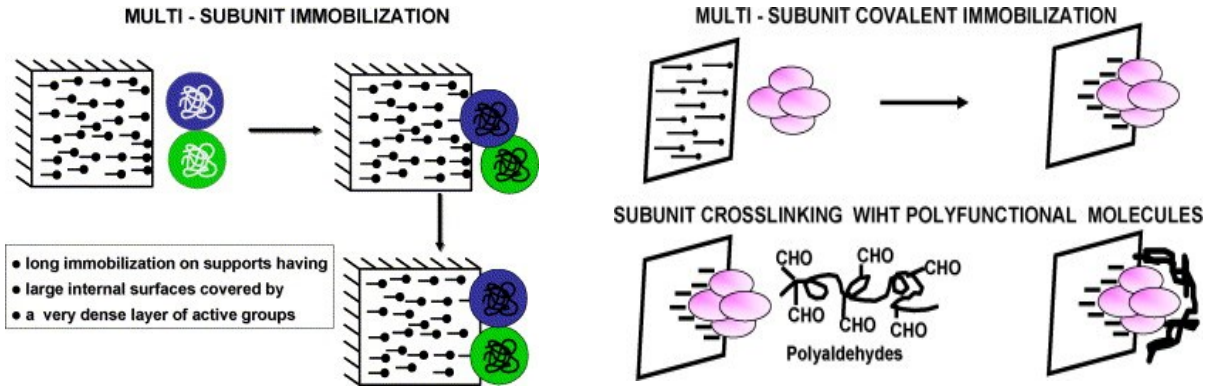

Figure 5.8: The effect of enzyme immobilisation on stability sourced (Mateo et al., 2007).

Immobilised and non-immobilised ATCase *T. sibiricus* showed a similar temperature profile from 20 to 80 ºC except that the highest relative activity for non-immobilised was 40 ºC; for immobilised, it was 60 ºC (Figure 5.9b). Non-immobilised ATCase *T. sibiricus* showed more than 76% relative activity at a temperature of 40 ºC (55% at 60 ºC), while immobilised ATCase *T. sibiricus* showed more than 96% relative activity at a temperature of 60 ºC (88% at 40 ºC) (Figure 5.9b). At 20 ºC, immobilised ATCase maintained 64% of its activity compared to 61% activity for non-immobilised ATCase (Figure 5.9b). At 80 ºC, immobilised ATCase T. *sibiricus* maintained around 63% of its activity, while non-immobilised maintained 40%. Therefore, the immobilisation improved the ATCase activity at a higher temperature (Figure 5.10b).

Further, CKase *T. sibiricus* showed different behaviour between immobilised and non-immobilised enzymes. The relative residual activity of non-immobilised CKase increased sharply from 20 to 60 ºC and decreased slightly from 60 to 80 ºC (Figure 5.9c). In contrast, the immobilised CKase *T. sibiricus* relative residual activity slight increased by elevating the temperature from 20 to 40 ºC and then had a sharp increase from 40 to 80 ºC due to immobilisation (Figure 5.9c). Immobilised CKase *T. sibiricus* had lower relative residual activity at temperatures 20 to 60 ºC compared to the non-immobilised enzyme, which was higher at 80 ºC (Figure 5.9c).

Finally, the non-immobilised and immobilised combination of CKase *T. sibiricus* and ATCase *T. sibiricus* (from two different additional timings of CKase to immobilisation solution) showed similar temperature profiles (Figure 5.9 e&f). The enzyme residual activities increased by elevating the temperature (similar to the CKase *T. sibiricus* temperature profile). The difference between relative residual activity of the immobilised and non-immobilised enzymes combination of CKase *T. sibiricus* and ATCase *T. sibiricus* was not significant (p>0.05) except for 20 ºC (Figures 5.9e, 5.9f).

The immobilised combination of CKase *T. sibiricus* and ATCase *E. faecalis* residual activity was higher compared to non-immobilised at temperatures 20, 40 and 60 °C (Figure 5.9d). In comparison, the relative residual activity was the same in immobilised and non-immobilised at 80 °C *(p*>0.05). In both immobilised and non-immobilised combinations of CKase *T. sibiricus* and ATCase *E. faecalis*, the residual activity increased by elevating the temperature (Figure 5.9d).

Overall, the effect of immobilisation is more obvious in mesophile enzymes at high temperatures, as the results from this section showed. Therefore, it is very important to apply immobilisation to use mesophile enzymes in industries such as wastewater.

On the other hand, the hyperthermophile enzymes showed high stability with and without immobilisation at high temperatures. However, the immobilisation still improves the hyperthermophile enzyme stability. Therefore, the application of immobilisation does not appear necessary regarding enzyme stability. However, the importance of immobilisation in recovering and reusing enzymes, including hyperthermophiles, should not be dismissed.

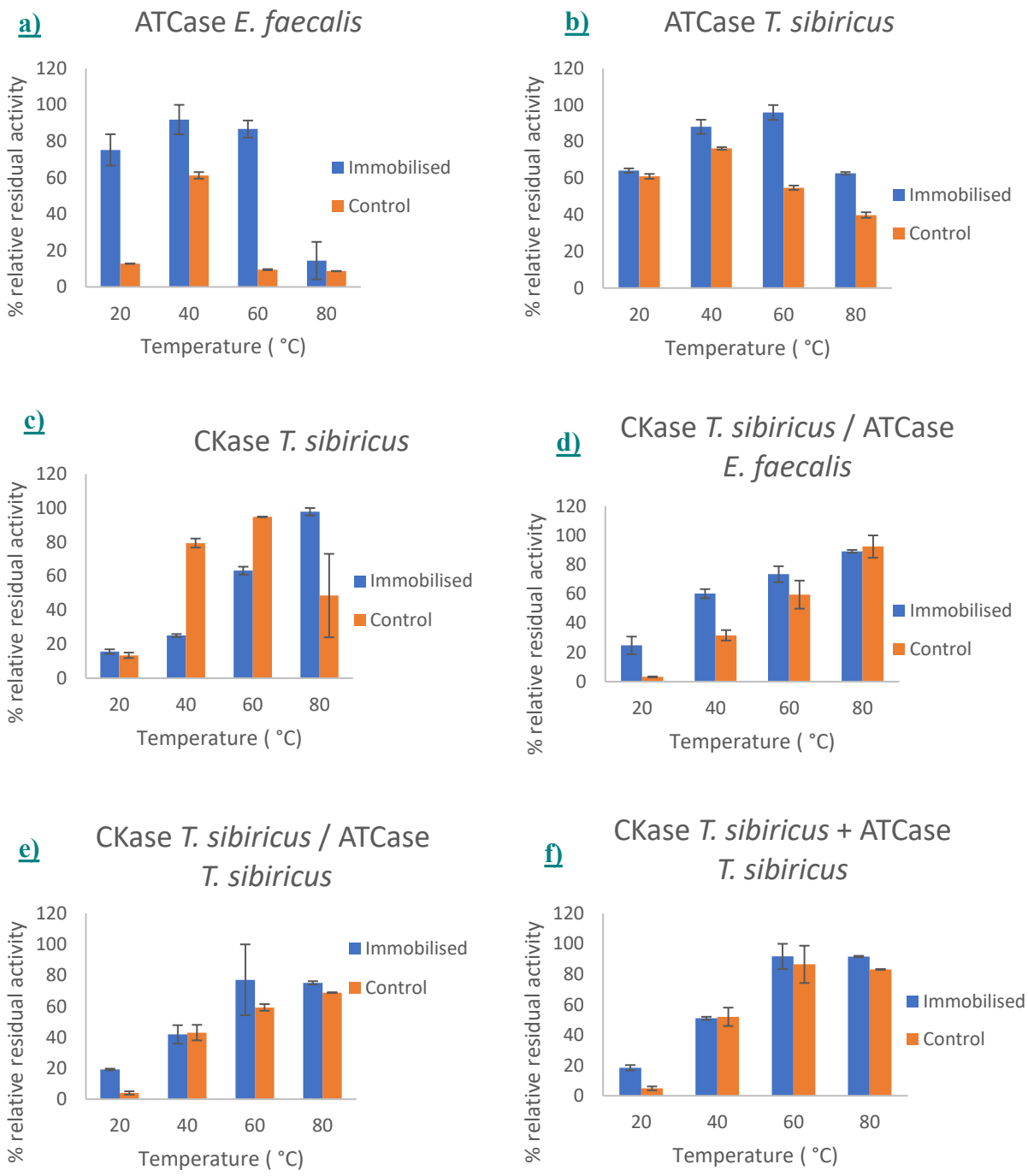

Figure 5.9: The effect of temperature on the activity of non-immobilised and immobilised CKase and ATCase and a combination of CKase/ ATCase. The reaction mixture for ATCase contains 50 mM Tris-HCl pH 8.5, 10 mM CP and 10 mM aspartate. The reaction mixture for CKase and CKase/ATCase contains 200 mM NH_3 and $NaHCO_3$ pH 9.4 (CKase) and pH 8.5 (CKase/ATCase), 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate at 40 °C. Values are the average of duplicate experiments, and error bars represent the standard deviation of experiments.

5.4.4. Immobilised Enzymes Thermostability

The previous section shows that the immobilisation maintained the mesophile enzyme residual activity at a high temperature. However, there was a slight improvement in enzyme activity at different temperatures in hyperthermophile enzymes. In this section, the thermostability of enzymes with or without immobilisation was studied. Therefore, thermostability of the immobilised enzymes was measured at 40 °C for 30 days and compared to non-immobilised enzymes.

The results from stability showed that immobilised ATCase *E. faecalis* presented significant thermostability over 30 days of incubation at 40 ºC compared to the non-immobilised enzyme (Figure 5.10a). Non-immobilised ATCase *E. faecalis* lost around 98% of its activity up to 4 days and lost almost all residual activity after that. Meanwhile, immobilised ATCase *E. faecalis* maintained more than 99% of its residual activity for four days, and around 37% at Day 30 (Figure 5.10a). Therefore, immobilisation could stabilise the mesophilic enzyme ATCase *E. faecalis* at 40 °C for 30 days by maintaining more than a third of its residual activity. Moreover, immobilised and non-immobilised hyperthermophile ATCase *T. sibiricus* relative residual activity decreased gradually from Day 1 to Day 30 (Figure 5.10b). However, they maintained more than 30% of their activity after 30 days. Moreover, immobilised ATCase *T. sibiricus* showed higher residual activity compared to the non-immobilised enzyme. Non-immobilised ATCase *T. sibiricus* maintained 35% of its activity after 30 days, while the immobilised enzymes maintained 49% of their activity after 30 days (Figure 5.10b). In terms of CKase *T. sibiricus,* immobilised and non-immobilised enzymes showed high activity after several days' incubation at 40 ºC (Figure 5.10c). There was no significant difference (p>0.05) between immobilised or non-immobilised CKase. It might be due to the high thermostability of hyperthermophilic CKase *T. sibiricus*.

Moreover, the immobilisation of the combination of CKase *T. sibiricus*–ATCase *E. faecalis* showed higher relative residual activity compared to non-immobilised enzyme after several days (Figure 5.10d). Non-immobilised CKase *T. sibiricus* and ATCase *E. faecalis* lost around 94% of their activity up to five days, while the immobilised enzymes maintained 82% of their activity. After 5 days, the activity of the immobilised enzymes gradually decreased to around 20% at Day 30, while it was around 4% for the non-immobilised combination of CKase *T. sibiricus*–ATCase *E. faecalis* (Figure 5.10d). Finally, the immobilisation of CKase *T. sibiricus*–ATCase *T. sibiricus* demonstrated higher relative residual activity compared to non-immobilised enzyme after a few days of incubation (Figure 5.10e). The non-immobilised CKase *T. sibiricus*–ATCase *T. sibiricus* lost around 44% of its activity up to Day five and

more than 93% after 30 days, while the immobilised enzymes maintained 43% of their activity after five days and gradually lost around 85% of its activity by Day 30 (Figure 5.10e).

Overall, immobilisation increased the thermostability of both mesophilic and hyperthermophilic enzymes individually or in the combination of CKase and ATCase. Moreover, the effect of immobilisation on stability was mostly revealed in mesophilic enzymes as they are less stable than hyperthermophile enzymes and, therefore, benefit more from immobilisation. The enzyme activity improvement by immobilisation might be due to the fact that immobilisation rotates the enzyme in a way that exposes the active site and is, therefore, more accessible to the substrate. Also, immobilisation protects the enzyme from denaturation (Cesar Mateo et al., 2002; Mateo, Palomo, et al., 2007). Taken together, immobilisation is an important technique for this project as it increases the enzyme stability and, more importantly, the enzymes can recycle and reuse. This approach enables us to use the enzymes in the wastewater treatment industry to remove ammonia and convert it to a value-added product, which is not hazardous to the environment.

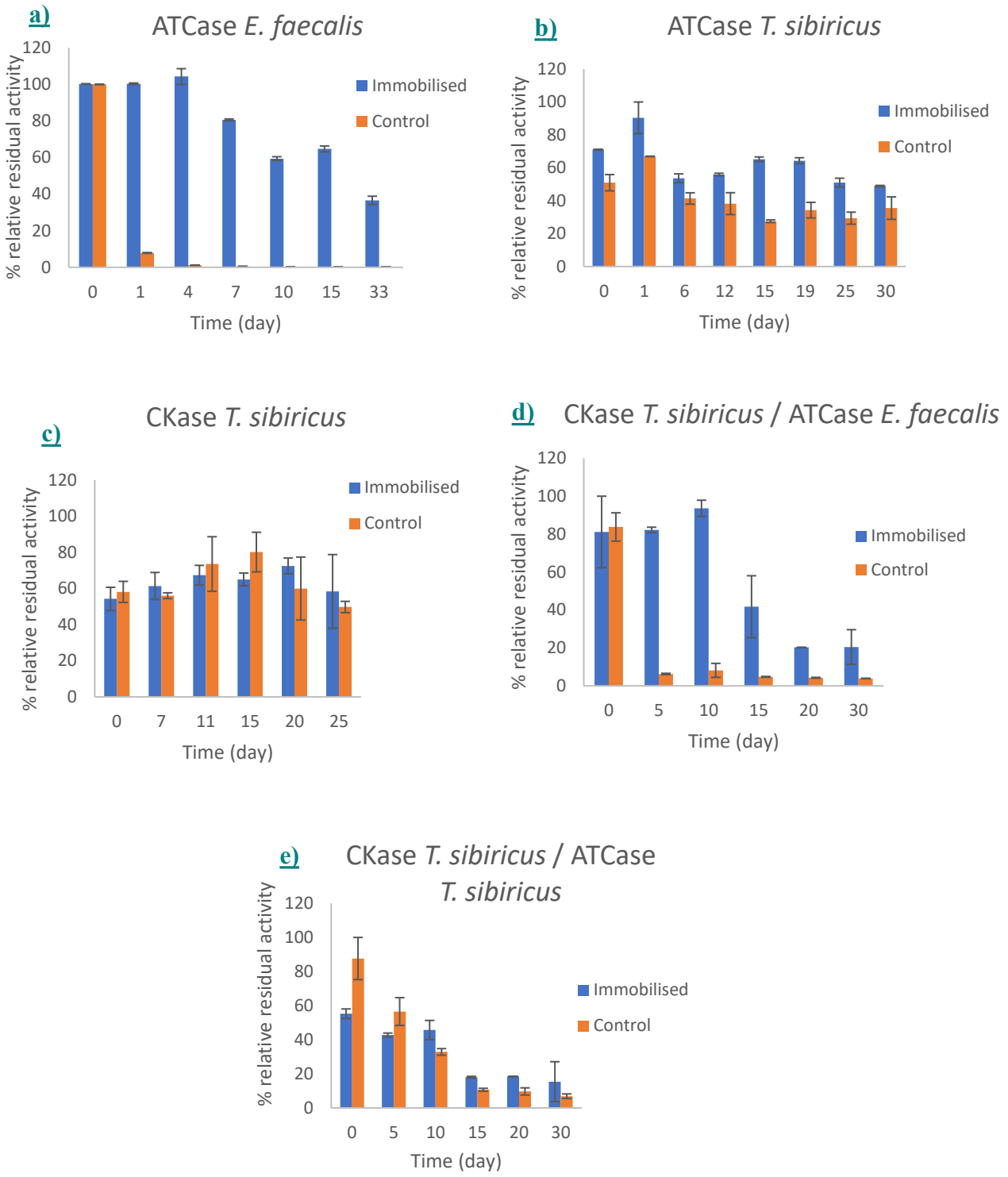

Figure 5.10: The effect of temperature on the activity of non-immobilised and immobilised CKase and ATCase and a combination of CKase/ ATCase. The reaction mixture for ATCase contains 50 mM Tris-HCl pH 8.5, 10 mM CP and 10 mM aspartate. The reaction mixture for CKase and CKase/ATCase contains 200 mM NH_3 and $NaHCO_3$ pH 9.4 (CKase) and pH 8.5 (CKase/ATCase), 10 mM ATP, 10 mM $MgSO_4$, 10 mM aspartate at 40 °C. Values are the average of duplicate experiments, and error bars represent the standard deviation of experiments.

5.4.5. Sequestration of Ammonia from Wastewaters Using Immobilised/Non-Immobilised Recombinant Co-Expressed Lysate from *E. coli* Containing Multienzymes

Waste ammonia is found in different amounts and types in wastewater. The ammonia concentration in wastewater varies from less than 100 mg/L from municipal or animal waste to more than 1 g/L from industrial wastewaters (Ip & Chew, 2010; Malovanyy et al., 2013). Therefore, ammonia must be removed from wastewater to prevent eutrophication and the evolution of nitrogenous greenhouse gases. Since our recombinant co-expressed lysate from *E. coli* has the required enzymes for the mitigation of ammonia and its conversion to value-added products. This section is aimed to test immobilised and non-immobilised enzymes on wastewaters containing ammonia. For this purpose, two different immobilised and non-immobilised combinations of CKase and ATCase were assayed in a wastewater sample using 10µL *E. coli* S30 lysate. From the result from Chapter 4, all lysates showed high CA yield after 24 hours of incubation, and there was no significant difference among them regarding CA production yield. Therefore, firstly, the combination of CKase *T. sibiricus*–ATCase *T. sibiricus* was chosen. This combination has hyperthermophile recombinant enzymes and is, therefore, more stable, and probably more tolerant to wastewater. Secondly, the combination of CKase *T. sibiricus–ATCase E. faecalis* was chosen as it showed highest CA production rate in a shorter time and more thermostability. The wastewater sample was provided from municipal wastewater treatment from Perth, Australia, containing about 1.2 mM ammonia concentration.

The results demonstrated high-yield CA production with respect to ammonia (Figure 5.11). There is no significant difference between immobilised and non-immobilised enzymes regarding CA production concentration. The results showed a sharp increase in CA production in the first 30 min (immobilised) and 60 min (non-immobilised) from both CKase *T. sibiricus*–ATCase *T. sibiricus* and CKase *T. sibirius*–ATCase *E. faecalis*. After 30 min (for immobilised) and 60 min (for non-immobilised), the CA production rate only slightly increased up to 180 min (Figure 5.11). The reason that curves were slightly flattened after 30 min (for immobilised) and 60 min (for non-immobilised), might be due to inhibitor substances in the wastewater. Moreover, the result demonstrated that CA production rate was similar in immobilised and non-immobilised in both combinations up to 180 minFurther, as a control, the combination of CKase *T. sibiricus–ATCase E. faecalis was* added to wastewater without adding any chemicals (acetyl phosphate, sodium fumarate and $MgSO_4$). Surprisingly, the control also showed CA production, although in lower yields, indicating the existence of sufficient chemicals such as bicarbonate ions, magnesium, acetate and phosphate in the wastewater that would be enough for the enzyme activity and assay.

Overall, the finding indicated that all immobilised non-immobilised enzymes and all exogenous and endogenous enzymes in recombinant lysates were active in wastewater and not substantially inhibited by wastewater components. Moreover, all enzymes were able to work with each other to produce the final product, CA. The majority of ammonia from wastewater was converted to CA through CP and aspartate. The concentration of ammonia in the wastewater sample was around 1.2 mM. The concentration of CA production for immobilised enzymes was around 0.45, 0.46 and 0.35 mM for lysate CKase *T. sibiricus*–ATCase *T. sibiricus* and lysate CKase *T. sibiricus*–ATCase *E. faecalis* and finally controls, respectively. The concentration of CA production for non-immobilised enzymes was around 0.49, 0.51 and 0.39 mM for lysate CKase *T. sibiricus*–ATCase *T. sibiricus* and lysate CKase *T. sibiricus*–ATCase *E. faecalis* and finally controls, respectively. Since one molecule of CA requires one molecule of aspartate and one molecule of CP; therefore, it is assumed ammonia has been consumed equally by CKase and aspartase. Therefore, the CA production yields based on ammonia concentration are as follow: CKase *T. sibiricus*–ATCase *T. sibiricus* immobilised 75% and non-immobilised 80%, lysate CKase *T. sibiricus*–ATCase *E. faecalis* immobilised 82% and non-immobilised 85%, and control immobilised 58% and non-immobilised 65%.. From previous study, it is shown that higher ammonia concentration yields higher enzyme activity rate (2.5 mM- 2 M) however, the enzyme is still able to function at ammonia concentration as low as 0.0001 M (Alissandratos et al., 2019). Therefore, in the previous chapters of this thesis the higher ammonia concentration was used to first examine the enzymes in the extreme concentration of ammonia and second increase enzymes rates for reasonable assay timing. However, in the wastewater sample the concentration of ammonia was around 1.2 mM which is lower that suitable range for CKase and probably for aspartase. Yet, as in this project a system of multienzymes cascade is being used in which the product of one enzyme is consumed by other enzymes. Therefore, the system always shifts toward product formation and ammonia consumption and prevent any reverse reaction.

Next, aspartase and CKase were able to use and convert ammonia from wastewater to aspartate and CP, respectively. Aspartase could only consume 0.6 mM ammonia equal to sodium fumarate concentration added to the solution. Therefore, it won't be any ammonia shortage for CKase usages. Furthermore, ATCase could consume the produced CP and aspartate to produce the final product, CA. The only drawback of this system is the production of acetate, which is a by-product of acetate kinase activity for recycled ATP. Fortunately, the acetate could be potentially used in wastewater treatment by anaerobic and aerobic bacteria involving phosphorus removal from wastewaters as a carbon substrate (Cech & Hartman, 1993; Schuler & Jenkins, 2003, 2004). Therefore, by using our system,

the ammonia is removed from wastewater without adding any pollutant chemicals to wastewater.

Finally, we have developed a 'one-pot' multienzymatic cascade process to convert ammonia from wastewater to CA using immobilised enzymes and a single *E. coli* culture without releasing any hazardous elements into the wastewater. This system is very efficient, versatile, easy to prepare, cost-effective and environmentally friendly.

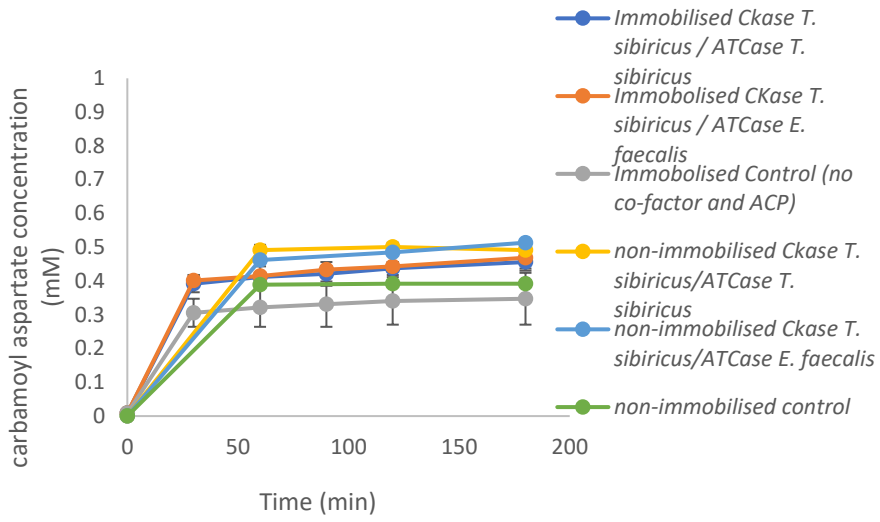

Figure 5.11: Applying a recombinant combination of immobilised and non-immobilised CKase and ATCase lysate to wastewater containing 1.2 mM ammonia (consumes by CKase and aspartase). The reaction mixture contains 2 mM $MgSO_4$, 0.6 mM sodium fumarate (only consumes by aspartase), 1 mM acetyl phosphate, 10 µL of *E. coli* S30 and an immobilised combination of CKase *T. sibiricus*–ATCase *T. sibiricus* and combination CKase *T. sibiricus*–ATCase *E. faecalis*. Control includes a combination of CKase *T. sibiricus*–ATCase *E. faecalis* and 10 µL of *E. coli* S30 in wastewater sample without the addition of any chemicals. Values are the average of duplicate experiments, and error bars represent the standard deviation of duplicate experiments.

5.5. Conclusions

The ultimate goal of the thesis was the application of ATCase and CKase to wastewater to remove ammonia and convert it into a value-added product. The results from this and other chapters confirmed that ammonia could successfully mitigate from wastewater, and CKase and ATCase can be immobilised, individually or in combination.

Moreover, it was shown in this chapter that CKase and ATCase can be immobilised by using covalent binding on a Eupergit carrier. It also confirmed an improvement in the stability and activity of these enzymes by immobilisation, especially for mesophilic enzymes. The immobilisation increased enzymes' thermostability as well.

Therefore, we developed a process that mitigates two important environmental factors, carbon dioxide and ammonia, through a biocatalytic transformation.

Consequently, our immobilised enzymes were successfully applied in the treatment of wastewater. Ammonia from wastewater was sequestrated and reused in the enzymatic cascade to produce valuable CA.

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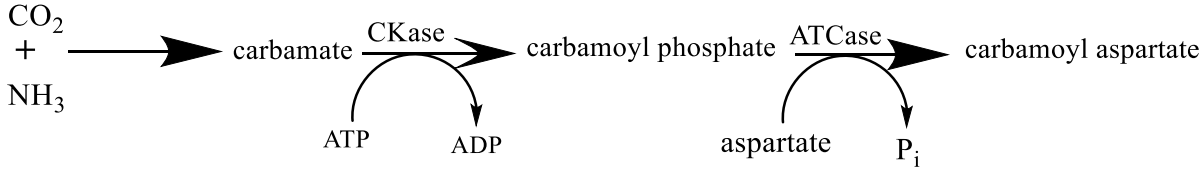
Chapter 6: Conclusions

This thesis presents work undertaken towards the biotransformation of ammonia and carbon dioxide to carbamoyl aspartate (CA) using *in vitro* multienzyme cascade using cell-free process. This work focus on Aspartate transcarbamoylase (ATCase) enzymes from hyperthermophile combining with *E. coli* endogenous enzymes in cell lysate.

In the teams' previous work (Hennessy et al., 2018), a system was developed in which carbamate kinase (CKase) from hyperthermophilic microorganisms could produce carbamoyl phosphate (CP) from ammonia, ATP and carbon dioxide. By screening various sources of CKase, the hyperthermophilic enzymes were identified as the best option for producing CP. However, CP is labile at physiological pH and temperature with a half-life (t ½) of 5 minutes. It can easily decompose at 100°C and convert to cyanate which is toxic to cells. To prevent CP from decomposition, ATCase could be utilised to convert the CP to value added product CA (Scheme 6.1).



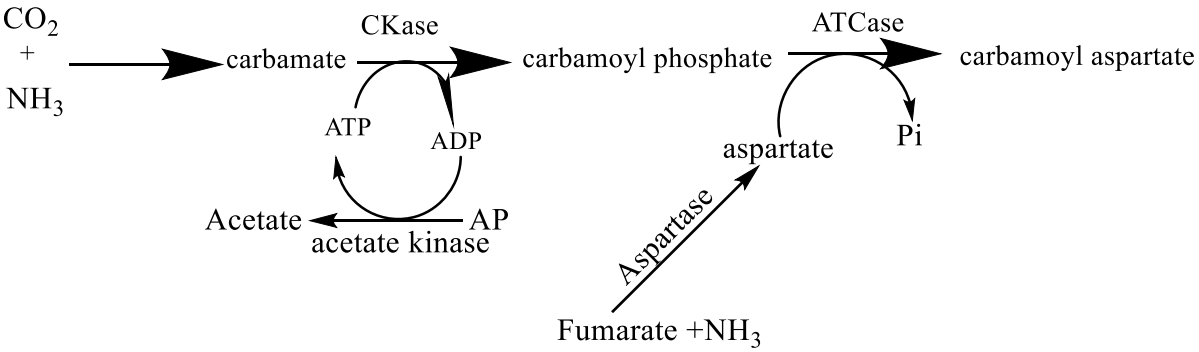
Scheme 6.1: The combination of CKase and ATCase to produce CA from ammonia and carbon dioxide.

ATCases from hyperthermophile were initially explored in chapter two as a potential enzyme for converting CP to CA. The focus of chapter two was cloning, expressing and study the kinetic properties of the catalytic subunit of ATCase from three different hyperthermophilic microorganisms *T. sibiricus*, *T. barophilus* and *P. furiosus and* one mesophilic microorganism *E. faecalis*. The reasons to choose ATCase hyperthermophilic enzymes were the promising properties of hyperthermophillic Ckase from our previous work (Hennessy et al., 2018) such as robustness, pH and temperature stability. This work demonstrated the successful cloning and expression in high yield for all hyperthermophilic and mesophilic enzymes in *E. coli*. Also, aspartate and CA could successfully have identified by HPLC and HPLC/MS and ATCase activity could be measured by HPLC and HPLC/MS. Moreover, it was shown that ATCases hyperthermophile have high pH and temperature stability which were similar to CKase properties. Therefore, Hyperthermophile ATCases are the best options for using in cell-free multienzyme cascade and wastewater treatment.

In chapter three the possibility of combination of ATCase and CKases to produce CA from one single recombinant lysate containing all necessary enzymes (endogenous, exogenous, homogenous& heterogenous) were explored. At first step, Ckases and ATCases pure enzymes were successfully combined to produce CA. Next, CKases crude lysate and pure ATCases were successfully coupled without adding any external ATP. CKase crude lysate contains sufficient endogenous acetate kinase that regenerate ATP from cheap and easy to prepare acetyl phosphate. Then, Ckases and ATCases were successfully co-expressed in one vector in *E. coli*. Last, co-expressed crude lysate of recombinant CKases and ATCases from a single bacterial culture was employed to convert ammonia, carbon dioxide and aspartate to CA without using external ATP. The usage of cell-free lysate substantially decreases limitations related to protein production and purification. Furthermore, this work demonstrated the improvement of the limiting factors in combining CKases and ATCases.

Additionally, the characterisation of co-expressed lysate revealed new findings on the hyperthermophile and mesophile protein interaction. Prior to this finding, there was no evidence of any successful combination of CKase and ATCase from hyperthermophiles and mesophiles. The exploitation of combination of hyperthermophile and mesophile enzymes could be potential future work which might benefit other enzymes cascade.

The works presented in chapter four focuses on the production of aspartate from fumarate and ammonia by using endogenous aspartase from *E. coli* lysate. The main focus was to increase the usage of ammonia in the enzyme cascade. First, the production of aspartase by *E. coli* lysate was successfully demonstrated. Then, it successfully revealed that co-expressed crude lysate of recombinant CKase and ATCase from *E. coli* contains aspartase. Additionally, it was shown that aspartase from crude lysate of recombinant CKase and ATCase coverts ammonia and fumarate to aspartate. Moreover, it was demonstrated that aspartate production from aspartase can covert to CA by ATCase and the production yield meets ATCase requirement. It confirmed all endogenous and exogenous enzymes in co-expressed lysate are active and work together to produce the ultimate product, CA (Scheme 6.2).



Scheme 6.2: The combination of CKase, acetate kinase, ATCase and aspartase to produce CA from ammonia, carbon dioxide and fumarate.

Accordingly, a cell-free multiple enzyme system was successfully established to produce CA by carbamoylation of aspartate in aqueous solution from ammonia and carbon dioxide. Importantly, the enzyme cascade is performed by *E. coli* lysate that was prepared from one single bacterial culture with the recombinant co-expressed and endogenous enzymes required for this cascade.

In chapter five, the work presented the ultimate goal of this thesis which was the application of ATCase and CKase in wastewater to remove ammonia and convert it into CA. The focus of this chapter was immobilisation of CKase and ATCase to be able to recover the enzymes. CKase and ATCase individually or in combination were successfully immobilised on the Eupergit carrier. Also, all immobilised enzymes were active and work together to produce CA. By using this technique, the cost substantially decreased and made the project practical by recovering the enzymes from wastewater. Additionally, immobilisation improved mesophile enzyme stability. This is very extraordinary result because ATCas has three catalytic subunits and immobilisation of an enzyme with three catalytic subunits is very challenging. This work is one of the pioneer works which was successfully conducted. This work may lay a ground for future work for immobilisation of enzymes with more than one subunit.

Finally, the immobilised CKase and ATCase enzymes were applied into wastewater and the removal of ammonia from the wastewater was successfully demonstrated. No additional cost-effective material was added to the solution. Ammonia from wastewater was sequestrated and reused in the enzymatic cascade to produce valuable CA.

Therefore, a process was developed to mitigate two important environmental factors, carbon dioxide and ammonia and convert them to a value-added product, CA. CA is a valuable

precursor in the pyrimidine pathway and a good potential fertiliser. This process occurred through a biocatalytic transformation using a greenprocess.

The findings from this study make several contributions to the current literature. First, the whole process was performed using a single bacterial culture and in a mild aqueous solution, which is an attractive process for wastewater treatment. Second, it is a simple, low-cost and energy-efficient technology that is readily scalable for wastewater treatment or biotechnology purposes. Third, using a cell-free lysate allows the direct monitoring of the reaction progress, control over reaction, substrate concentration and efficiency of ammonia and carbon dioxide transformation to CA. Forth, the development of endogenous ATP recycling mechanism allows to overcome the need to add costly cofactor ATP. Fifth, using immobilisation technique make the process effective as it recovers the enzyme.

Appendix

The native genes of the catalytic subunits of ATCase (PyrB) from Uniprot:

>tr|F0LI09|F0LI09_THEBM Aspartate carbamoyltransferase OS=Thermococcus barophilus (strain DSM 11836 / MP) OX=391623 GN=pyrB PE=3 SV=1
MQGWKGRDVVSIRDFSKEDIEYVLNTAERLERELKEKGSLEYAKGKILATLFFEPSTRTR
LSFESAMHRLGGSVIGFAEASTSSVKKGESLRDTIKTVEQYSDVIVIRHPKEGAARLAAE
VAEIPVINAGDGSNQHPTQTLLDLYTIKREFGKIDGLKIALLGDLKYGRTVHSLSEALSH
YDVELYLISPGLLRMPRHIVEELKEKGVKVYETSDLESVIGELDVLYVTRIQKERFPDEQ
EYLKVKGSYVIDLEILKKAKETLKIMHPLPRVDEIHPSVDSTKHAIYFRQVFSGIPVRMA
LLALTLGVIE

>tr|A0A124FFJ7|A0A124FFJ7_9EURY Aspartate carbamoyltransferase OS=Thermococcus sibiricus OX=172049 GN=pyrB PE=3 SV=1
MKFQDVISINDFGKEEIEYVLKTAKRLENELNEQKILQYAKGKVLATLFFEPSTRTRLSF
ESAMHRLGGSVIGFAEASSTSVKKGESLMDTIRTVEKYADVIVIRHPREGAARLAAEVAE
IPVINAGDGANQHPTQTLLDLYTIKKEFGRINGLKIALLGDLKYGRTVHSLAKALSYYNV
KLYFIAPQGLEMPRHIIEEISDKVEITETNDLESVIPEVDVLYATRIQKERFPDPAEYNK
IKGSYRIDLKILNGAKEGLKIMHPLPRVDEISYEVDKTPYSAYFRQVWSGVPVRMALLSI
VLGVIE

>sp|Q8U373|PYRB_PYRFU Aspartate carbamoyltransferase OS=Pyrococcus furiosus (strain ATCC 43587 / DSM 3638 / JCM 8422 / Vc1) OX=186497 GN=pyrB PE=3 SV=1
MDWKGRDVISIRDFSKEDIEIVLSTAERLEKELKEKGQLEYARGKILATLFFEPSTRTRL
SFESAMHRLGGAVIGFAEASTSSVKKGESLADTIKTVEQYSDVIVIRHPKEGAARLAAEV
AEIPVINAGDGSNQHPTQTLLDLYTIRKEFGKIDGLKIGLLGDLKYGRTVHSLAEALAYY
DVELYLISPELLRMPRHIVEELREKGVTVYETSDLMSVIGELDVLYVTRIQKERFPDEQE
YLKVRGSYQVNLQVLSKAKETLKVMHPLPRVDEIHPEVDKTKHAIYFKQVFNGIPVRMAL
LGLVLGVI

>sp|Q9L4T8|PYRB_ENTFA Aspartate carbamoyltransferase OS=Enterococcus faecalis (strain ATCC 700802 / V583) OX=226185 GN=pyrB PE=3 SV=1
MIITSERISLKHLLTAEALTDREVMGLIRRAGEFKQGAKWHPEERQYFATNLFFENSTRT
HKSFEVAEKKLGLEVIEFEASRSSVQKGETLYDTVLTMSAIGVDVAVIRHGKENYYDELI
QSKTIQCSIINGGDGSGQHPTQCLLDLMTIYEEFGGFEGLKVAIVGDITHSRVAKSNMQL
LNRLGAEIYFSGPEEWYDHQFDVYGQYVPLDEIVEKVDVMMLLRVQHERHDGKESFSKEG
YHLEYGLTNERATRLQKHAIIMHPAPVNRDVELADELVESLQSRIVAQMSNGVFMRMAIL
EAILHGKA

Carbamate Kinase gene details:

Carbamate kinase (CKase)	Mass (Da)	Ext. Coefficient (reduced)	PDB code	Length	Uniprot Code
T.barophilus	34,678	35410	N/A	316	F0LK57
T. sibiricus	34,561	32430	N/A	315	C6A5J0
P.furiosus	34,429	40910	1E19	314	P59625
E. faecalis	32,927	18450	2WE4/ 2WE5	310	P0A2X8

CKase from Uniprot:

>sp|P95474|CPKA_PYRFU Carbamate kinase OS=Pyrococcus furiosus (strain ATCC 43587 / DSM 3638 / JCM 8422 / Vc1) OX=186497 GN=cpkA PE=1 SV=1
MGKRVVIALGGNALQQRGQKGSYEEMMDNVRKTARQIAEIIARGYEVVITHGNGPQVGSL
LLHMDAGQATYGIPAQPMDVAGAMSQGWIGYMIQQALKNELRKRGMEKKVVTIITQTIVD
KNDPAFQNPTKPVGPFYDEETAKRLAREKGWIVKEDSGRGWRRVVPSPDPKGHVEAETIK
KLVERGVIVIASGGGGVPVILEDGEIKGVEAVIDKDLAGEKLAEEVNADIFMILTDVNGA
ALYYGTEKEQWLREVKVEELRKYYEEGHFKAGSMGPKVLAAIRFIEWGGERAIIAHLEKA
VEALEGKTGTQVLP

>sp|P0A2X8|ARCC1_ENTFC Carbamate kinase 1 OS=Enterococcus faecium OX=1352 GN=arcC1 PE=1 SV=2
MGKKMVVALGGNAILSNDASAHAQQQALVQTSAYLVHLIKQGHRLIVSHGNGPQVGNLLL
QQQAADSEKNPAMPLDTCVAMTQGSIGYWLSNALNQELNKAGIKKQVATVLTQVVVDPAD
EAFKNPTKPIGPFLTEAEAKEAMQAGAIFKEDAGRGWRKVVPSPKPIDIHEAETINTLIK
NDIITISCGGGGIPVVGQELKGVEAVIDKDFASEKLAELVDADALVILTGVDYVCINYGK
PDEKQLTNVTVAELEEYKQAGHFAPGSMLPKIEAAIQFVESQPNKQAIITSLENLGSMSG
DEIVGTVVTK

>tr|F0LK57|F0LK57_THEBM Carbamate kinase OS=Thermococcus barophilus (strain DSM 11836 / MP) OX=391623 GN=TERMP_01794 PE=3 SV=1
MRKRVVIALGGNAILQRGQKGTYDEQMENVKKTAKQIVDIILNNDYEVVITHGNGPQVGA
LLLHMDAGQQLYGIPAQPMDVAGAMTQGQIGYMIQQAITNELKRRGIYKPVATIVTQVLV
DKNDPAFQNPSKPVGPFYDEETAKRLAKEKEWVVVEDAGRGWRRVVPSPDPKDIIEKDII
RDLVEKGFIVIASGGGGIPVIEENGQLKGVEAVIDKDLAGEKLAEVVNADIFMILTDVNG
AAINYGKPNERWLHKVAVDELRKYYEEGHFKKGSMGPKVLAAIRFVEWGGERAVIAALDK
AVDALEGRTGTQVIKM

>tr|C6A5J0|C6A5J0_THESM Carbamate kinase OS=Thermococcus sibiricus (strain DSM 12597 / MM 739) OX=604354 GN=TSIB_1834 PE=3 SV=1
MRKRVVIALGGNAILQRGQKGTYEEQMENVRKTARQIVDIILDNEYEVVITHGNGPQVGA
LLLQQDAGEHVHGIPAQPMDVCGAMSQGQIGYMIQQAIMNELRRRGVERPVATIVTQTIV
DKNDPAFQHPSKPVGPFYSEETAKKLAKEKGWVVIEDAGRGWRRVVPSPDPKGHVEAPII
QDLVEKEFIVISSGGGGIPVVEENGELKGVEAVIDKDLAGERLAEEVNADIFMILTDVNG
AAINYGRPNEKWLEKVTLGEIKRYYEEGHFKKGSMGPKVLAAIRFIEWGGERAIIAALDK
AVEALEGKTGTQITR

pETMCSIII expression vector

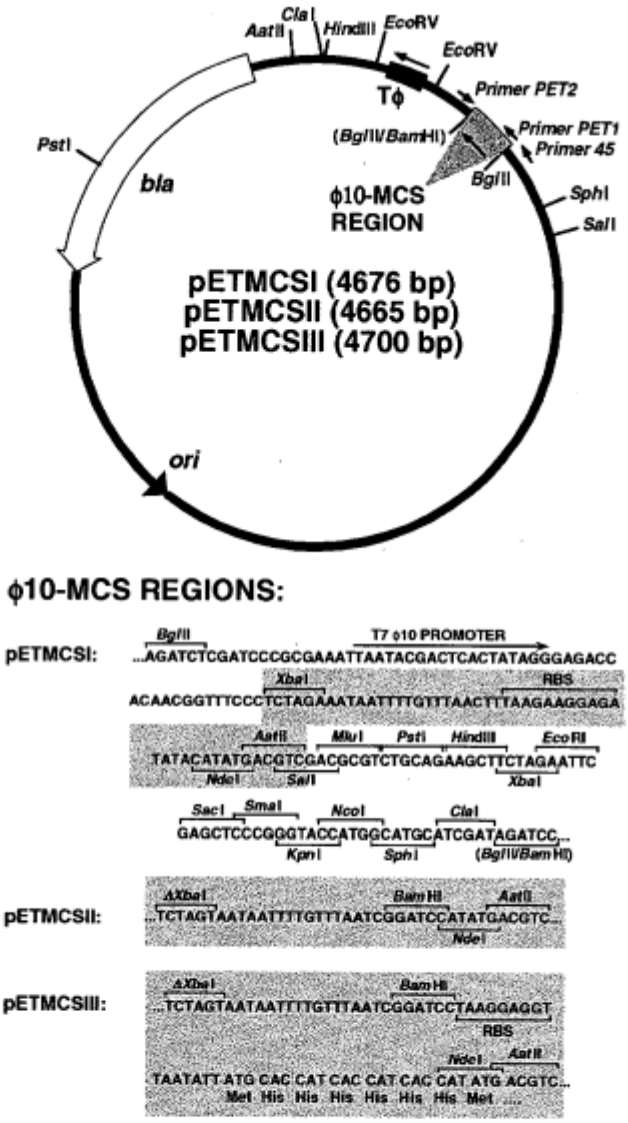
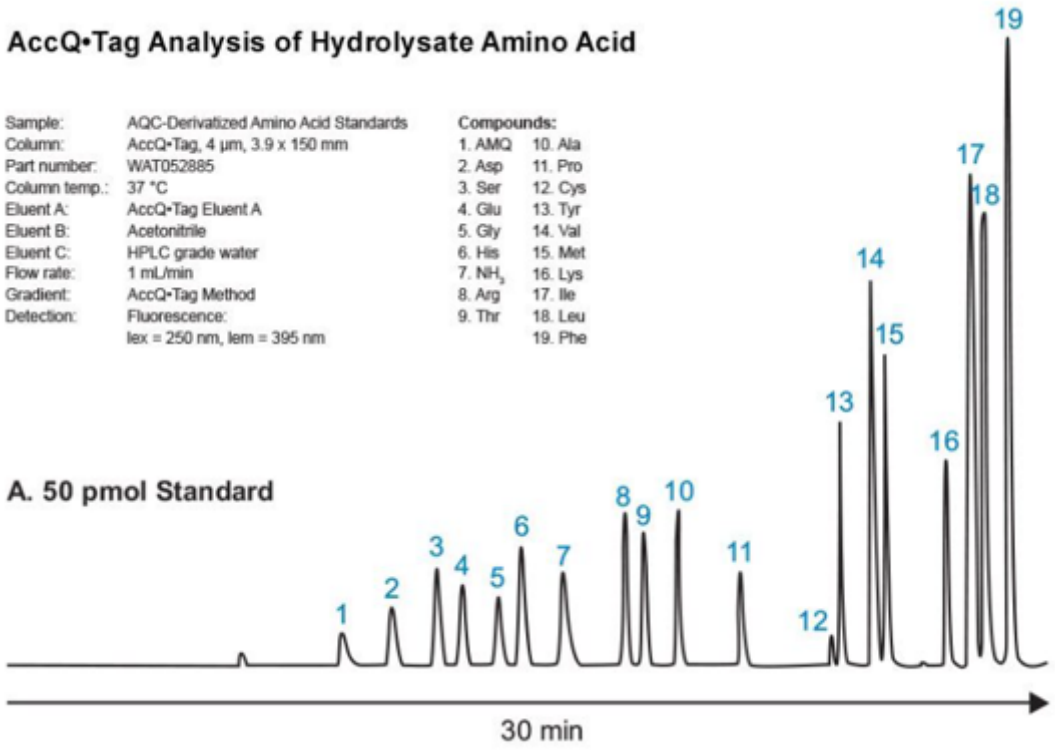


FIGURE S1: Physical and genetic maps of bacteriophage T7-promoter vectors. Restriction endonuclease sites in the T7 promoter-polylinker (ϕ10-MCS) region are shown in full for pETMCSI, below. Note that some sites also occur elsewhere in the vectors (above). Primers 45, PET1 and PET2, used for DNA sequence determination, are described in the text. Sequences in the ϕ10-MCS regions of the other vectors, including the strong ribosome-binding sites (RBS) positioned upstream of His_6 encoding sequence (pETMCSIII) or unique *NdeI* sites (pETMCSI and pETMCSIII) are shown insofar as they differ from pETMCSI (shaded boxes).

Derived from (Neylon et al., 2000)

Detection of aspartate using AccQ tag analysis by HPLC



pRSFDuet-1 cloning/ expression regions

pRSFDuet-1 cloning/expression regions

pRSFDuet-1 restriction sites

pRSFDuet-1 Restriction Sites

TB391 0903

Enzyme	# Sites	Locations				
AatII	1	346				
Acc65I	1	348				
AccI	2	138	411			
AclI	1	3674				
AfeI	1	1658				
AflII	1	163				
AflIII	2	1782	3334			
AgeI	1	566				
AloI	1	2420				
ApaI	1	3131				
ApaLI	2	2085	3354			
AscI	1	125				
AseI	6	213	732	921	2592	2651
		3812				
AsiSI	1	337				
AvaI	2	354	1246			
AvrII	1	433				
BaeI	1	365				
BamHI	1	106				
BanI	4	348	2565	2695	3414	
BanII	3	122	1471	3131		
BbeI	1	2699				
BbsI	2	2849	3188			
BceAI	4	211	801	2850	3477	
BcgI	2	162	3014			
BciVI	4	728	1604	1974	2882	
BclI	1	3320				
BfrBI	2	1008	1274			
BglII	1	305				
BlpI	2	451	2309			
Bme1580I	3	2089	3131	3358		
BmrI	3	2536	3176	3413		
BpmI	2	3013	3502			
Bpu10I	1	1102				
BpuEI	5	515	1862	2160	2340	2526
BsaHI	3	343	2696	3379		
BsaI	1	645				
BsaWI	8	551	566	983	1804	1977
		2124	2512	3015		
BsaXI	2	655	2666			
BseYI	3	2075	2800	2935		
BsgI	2	3289	3489			
BsiEI	9	153	199	325	337	636
		1124	1698	2111	2555	
BsiHKAI	3	122	2089	3358		
BsmAI	7	645	1102	1604	2719	3106
		3232	3637			
BsmBI	2	1102	2719			
BsmI	2	1163	1240			
Bsp1286I	5	122	1471	2089	3131	3358
BspCNI	6	443	530	1094	2059	2322
		2755				
BspHI	2	725	1602			
BspLU11I	1	1782				
BspMI	1	124				
BsrBI	5	13	243	723	1608	1715
BsrDI	2	2927	3293			
BsrFI	4	324	566	1164	3648	
BsrGI	1	190				
BssHII	2	125	2923			
BssSI	1	1944				
BstAPI	1	3658				
BstEII	1	3152				
BstXI	3	3288	3411	3540		
BstYI	5	106	305	869	2558	3770
Bsu36I	1	517				
BtgI	1	69				
BtsI	5	543	1176	1263	2607	2975
ClaI	1	1429				
DrdI	1	637				
EaeI	5	150	196	322	326	2660
EagI	3	150	196	322		

Enzyme	# Sites	Locations				
EarI	5	1306	1562	1666	2442	3717
EciI	3	1833	1979	3549		
Ecl136II	1	120				
Eco57I	1	2474				
Eco57MI	3	2474	3013	3502		
EcoICRI	1	120				
EcoNI	2	1209	3802			
EcoO109I	1	478				
EcoRI	1	112				
EcoRV	1	319				
FalI	1	671				
FseI	1	328				
HaeII	5	1660	2019	2699	2942	3723
HincII	3	139	2392	2632		
HindIII	1	143				
HpaI	1	2832				
KasI	1	2695				
KpnI	1	352				
MfeI	1	311				
MluI	1	3334				
MslI	3	2968	2998	3286		
NaeI	1	326				
NarI	1	2696				
NcoI	1	69				
NdeI	1	298				
NgoMIV	1	324				
NotI	1	150				
NruI	1	1465				
NsiI	2	1010	1276			
NspI	2	1654	1786			
PacI	1	429				
PciI	1	1782				
PflMI	3	401	862	3759		
PluI	1	3766				
PinAI	1	566				
PspOMI	1	3127				
PstI	1	135				
PvuI	1	337				
PvuII	2	2645	2738			
SacI	1	122				
SalI	1	137				
SapI	1	1666				
SbfI	1	135				
SfcI	4	29	131	226	3825	
SfoI	1	2697				
SmaI	1	1248				
SmlI	7	163	354	494	1877	2139
		2319	2541			
SphI	1	1654				
Sse8387I	1	135				
SspI	2	1197	1571			
StyI	4	69	433	473	2287	
TaqII	4	870	1684	2368	2541	
TatI	1	190				
TspGWI	3	1303	1315	2396		
Tth111I	2	637	2209			
XbaI	1	2414				
XcmI	3	2949	2967	3483		
XhoI	1	354				
XmaI	1	1246				
XmnI	1	733				
ZraI	1	344				

Enzymes that do not cut pRSFDuet-1:

AarI	AhdI	AleI	AlwNI	BbvCI
BglI	BmgBI	BmtI	BpiI	BsaAI
BsaBI	BseRI	BsiWI	BsmFI	BspEI
Bst1107I	BstBI	BstZ17I	BtrI	DraI
DraIII	FspAI	FspI	MscI	NheI
NspV	PmeI	PmlI	PpiI	PpuMI
PshAI	PsiI	PsrI	RsrII	SacII
SanDI	ScaI	SexAI	SfiI	SgrAI
SnaBI	SpeI	SrfI	StuI	SwaI