Purification and Characterization of O_2-insensitive Pyruvate Decarboxylase from *Sulfolobus* species

by

Faisal Alharbi

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2020

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Pyruvate decarboxylase (PDC) is a key enzyme in a two-step pathway for the production of ethanol. It catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde in many mesophilic organisms. No conventional PDC has been found in hyperthermophiles, a group of microorganisms growing optimally at 80°C and above; however, bifunctional PDC/pyruvate ferredoxin oxidoreductase (POR) activities have been found to be present in several hyperthermophilic bacteria and archaea, but most of them are oxygen-sensitive and CoA-dependent. It was reported that the CoA is not required for a recombinant PDC/POR from *Sulfolobus tokodaii* (T_{opt} = 80ºC) and it is oxygen insensitive, but it's not known why it has a much lower activity compared to other PDCs/PORs. Since PORs from hyperthermophilic crenarchaea *Sulfolobus solfataricus* (T_{opt} = 80ºC) and *Sulfolobus acidocaldarius* (T_{opt} = 80°C) are not oxygen-sensitive, it was hypothesized that they might be the most thermostable O_2-insensitive PDCs. PDCs/PORs enzymes from *S. solfataricus* (Ss) and *S. acidocaldarius* (Sa) were purified using a fast performance liquid chromatography system (FPLC) anaerobically. POR activity was measured by monitoring the pyruvate-dependent reduction of benzyl viologen at 578 nm. PDC activity was measured by monitoring the pyruvate-dependent production of acetaldehyde. The acetaldehyde production was determined by using 2,4-dinitrophenylhydrazine (DNPH) derivatization method followed by high performance liquid chromatography (HPLC). Both enzymes from *S. solfataricus* and *S. acidocaldarius* were purified and SDS-PAGE showed that each heterodimeric enzyme had two subunits with a molecular mass of 37±3 kDa and 65±2 kDa respectively. *S. solfataricus* PDC and POR activities present in its cell-free extract (CFE) were determined to be 0.0027±0.0003 U/mg and 0.18±0.01 U/mg, respectively. Similarly, *S.*

acidocaldarius PDC and POR activities present in its CFE were determined to be 0.0011±0.0004 U/mg and 0.10±0.01 U/mg, respectively. The enzyme from *S. solfataricus* was purified approximately 42-fold with a recovery of 25%, while the enzyme from *S. acidocaldarius* was purified approximately 70-fold with a recovery of 19%. Optimal pH for both *S. solfataricus* and *S. acidocaldarius* PORs were determined to be at pH 8.6, while optimal pH for their PDCs were 7.8. PDC/POR enzyme from *S. solfataricus* showed maximum activity at 80 ºC for PDC activity and at 90 ºC for POR activity; however, the optimum temperatures of PDC/POR from *S. acidocaldarius* were at 90 ºC for PDC activity and at 80 ºC for POR activity. PDCs/PORs enzymes from *S. solfataricus* and *S. acidocaldarius* were CoA dependent for both PDCs and PORs activities, a common feature of other PDCs/PORs except the one from *S. tokodaii*. Thermostability of the purified enzymes from *S. solfataricus* and *S. acidocaldarius* were determined by measuring the time required for losing 50% activity ($t_{1/2}$) at 80 °C, which were approximately 2.9 h and 1.1 h respectively. It was determined that PDCs/PORs from *S. solfataricus*, and *S. acidocaldarius* were not oxygen sensitive. These thermostable and oxygen-stable PDCs may have great potential in applications in developing a more efficient system for bioethanol fermentation at high temperatures.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Kesen Ma, for his consistent support and guidance during the running of this project. Dr. Ma continuously provided encouragement and was always willing and enthusiastic to assist in any way he could throughout the research project. I would also like to thank my committee members, Dr. Laura Hug and Dr. Todd Holyoak for their time and helpful comments for my project. Also, I would like to think Thomas Knura and Bettina Siebers for their help.

Special thanks must go to my colleagues for their support and help during the past years: Sarah Kim, Navin Asokumar, Justin Pham, Edicon Chan, Mobolaji Adegboye, Olayinka Aiyegoro.

Finally, I express my most sincere thanks to my grandmother AL Anoud for her continued support and encouragement. My parents, Abdul-Aziz and Latifa, my sister and brothers, deserve special thanks for their love and support.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AcDH	Acetaldehyde dehydrogenase
AHAS	Acetohydroxyacid synthase
BV	Benzyl viologen
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CFE	Cell-free extract
CoA	Coenzyme A
DTT	Dithiotheritol
EPPS	N-(2-hydroxyethyl)-peperazine-N'-(3-propanedulfonic acid)
FPLC	Fast Performance Liquid Chromatography
HPLC	High performance liquid chromatography
MV	Methyl viologen
PDC	Pyruvate decarboxylase
POR	Pyruvate ferredoxin oxidoreductase
Sa	*Sulfolobus acidocaldarius*
SaPOR	*Sulfolobus acidocaldarius* pyruvate ferredoxin oxidoreductase
SDT	Sodium dithionite
Ss	*Sulfolobus solfataricus*
SsPOR	*Sulfolobus solfataricus* pyruvate ferredoxin oxidoreductase
TPP	Thiamine pyrophosphate
Tris	Tris (hydroxymethyl) aminomethane

Chapter 1 Introduction

1.1. Microbial production of ethanol

As the demand for energy increases and fossil energy resources irreversibly decreases, it gives rise to the development of efficient pathways for the production of fuels and chemicals (Bothast and Schilcher, 2005; Lin and Tanaka, 2006). Ethanol is one of the environmentally friendly energy sources used instead of fossil fuels. One of the main advantages of utilizing bio-ethanol is that bio-mass is renewable and can potentially provide long term sustainable fuel supply (Dale *et al.,* 2014; Olson *et al.,* 2015). Ethanol is naturally occurring alcohol. Moreover, it is predicted that the use of bio-ethanol leads to reduction in net greenhouse gas emissions (Dale *et al.,* 2014; Kumar *et al.,* 2015). Since the 1970s, interest and investment in the production of fuel ethanol have become widespread in the world.

Mesophilic microorganisms such as *Zymomonas mobilis, Saccharomyces cervisiae* and *Escherichia coli* are the best-known microorganisms for ethanol production (Bai *et al.,* 2008; Eram and Ma, 2013). Ethanol fermentation is a biological process in which enzymes such as cellulase, xylanase and amylase convert bio-mass materials into simple sugars which are converted to pyruvate by different metabolic pathways (Bai *et al.,* 2008). Pyruvate is then converted to ethanol by two different pathways. One of the main metabolic pathways involved in ethanol production is glycolysis which is used by *S. cervisiae* to produce two molecules of pyruvate from one molecule of glucose (Bai *et al.,* 2008). Pyruvate is then converted to ethanol under anaerobic conditions by pyruvate decarboxylase and alcohol dehydrogenase (Bai *et al.,* 2008). On the other hand, *Zymomonas mobilis* uses a the Entner–Doudoroff (ED) pathway to produce ethanol from glucose (Bai *et al.,* 2008).

There are three different groups of materials (simple sugar, starch, and lignocellulosic biomass) that are used as carbon sources to produce fuel ethanol. In a process that uses simple sugar such as sugarcane as a feedstock, sugar is directly fermented to ethanol followed by a distillation step to separate out the ethanol, however, using starch such as corn requires a saccharification step before the fermentation (Mussatto *et al.,* 2010). Production of fuel ethanol from agricultural wastes such as lignocellulosic materials needs an energy intensive pre-treatment step before the fermentation step.

Metabolic engineering has been focused on improving features of microorganisms to enhance the fermentation of ethanol (Zaldivar *et al.,* 2001; Yao *et al.,* 2010). To choose a microorganism for ethanol fermentation on an industrial scale, some metabolic traits have been taken into consideration, for instance, the ability to utilize various sugar sources, high yield of ethanol, minimum production of by-products, and tolerance to extreme conditions (Zaldivar *et al.,* 2001; Eram and Ma, 2013).

The most common pathway of producing ethanol in mesophilic organisms is through the pyruvate decarboxylation to acetaldehyde by pyruvate decarboxylase (PDC), which is subsequently reduced to ethanol. It has been found that hyperthermophilic microorganisms do not have conventional PDC. Hence, they utilize a bifunctional PDC/POR for this purpose. Hyperthermophiles are considered to be a source of valuable and essential biocatalysts for industrial applications (Huber and Stetter, 1998; Van Den Burg, 2003). They are potentially advantageous in comparison to mesophilic organisms in terms of reduced contamination during the processes conducted in industries, *in situ* distillation of the products, enhanced solubility, and

decrease in the viscosity of substrate (Egorova and Antranikian, 2005; Eram and Ma, 2013). Moreover, enzymes from hyperthermophiles are stable and active under conditions close to the growth conditions of the host organisms (Egorova and Antranikian, 2005; Albers *et al.,* 2011; Eram and Ma, 2013). Hyperthermophilic enzymes have the ability to resist sudden changes in temperature and pH, and highly concentrated solutions (Lamble *et al*., 2003). Also, they can be cloned and expressed in mesophilic hosts without losing their properties (Vieille and Zeikus, 2001).

Hyperthermophilic microorganisms are those that can grow optimally at 80ºC and above (Schonheit & Schafer, 1995; Eram and Ma, 2013). Their enzymes are thermostable and optimally active at high temperatures. They maintain their thermostability and catalytic properties when their genes are cloned and expressed in mesophilic hosts, for instance pyruvate ferredoxin oxidoreductase from *S. tokodaii* (Eram and Ma, 2013; Yan *et al.,* 2014). According to the comparisons of amino acids composition, sequence alignments, and protein crystal structures, it has been observed that there is a high degree of similarity between the enzymes of hyperthermophilic microorganisms and mesophilic homologs; however, The difference in their sequences was related to their flexibility and stability to function at high temperature (Vieille and Zeikus, 2001). The remarkable difference between the enzymes of hyperthermophilic microorganisms and mesophilic organisms is the thermostability of hyperthermophilic enzymes (Vieille and Zeikus, 2001).

Sulfolobus species are among the well characterized and studied hyperthermophiles. Initially, the *Sulfolobus* species were considered to be thermo-acidophilic bacteria; however, Carl Woese

and colleagues found that *Sulfolobus* belong to archaea, particularly crenarchaeota (Brouns *et al.,* 2005). *Sulfolobus* species can grow heterotrophically under aerobic conditions by oxidizing a variety of carbon sources and grow autotrophically using sulfur as electron donor (Schonheit & Schafer, 1995; Lamble *et al*., 2003; Nunn *et al*., 2010). *Sulfolobus solfataricus*, *Sulfolobus. acidocaldarius* and *Sulfolobus tokodaii* grow optimally at 75-80 ˚C and pH 2-3 (Kawarabayasi *et al*., 2001; Lamble *et al*., 2003; Nunn *et al*., 2010). *S. tokodaii* was previously named as *Sulfolobus* sp. Strain 7 (Suzuki *et al*., 2002). The complete genomes of *S. solfataricus*, *S. acidocaldarius* and *S. tokodaii* have been sequenced (Kawarabayasi *et al*., 2001; She *et al*., 2001; Chen *et al*., 2005). The genome of *S. solfataricus* has 2,992,245 bp and encodes 2,977 proteins, whereas *S. acidocaldarius* contains 2,225,959 bp and 2,292 protein-encoding genes (She *et al*., 2001; Chen *et al*., 2005). The genomic size of *S. tokodaii* is 2,694,756 bp long and consists of 2,826 protein-coding genes (Kawarabayasi *et al*., 2001)

Although *Sulfolobus* species are similar in optimal pH and temperature for their growth, they are different in genome aspects which lead to differences in growth substrates. The significant difference between *Sulfolobus* species is the metabolic potential of each organism (Nunn *et al.,* 2010; Chen *et al.,* 2005). *Sulfolobus* species catalyse D-glucose to pyruvate by non-phosphorylative Entner-Doudoroff pathway (Chen *et al.,* 2005; Nunn *et al.,* 2010). *S. solfataricus* grows in peptides, amino acids, and sugars including pentoses, polysaccharides, hexoses (Chen *et al.,* 2005; Quehenberger *et al.,* 2017). In contrast, *S. acidocaldarius* grows on a wide range of amino acids but a limited range of sugars such as sucrose, maltotriose, dextrin, starch, D-glucose, and D-fucose (Chen *et al.,* 2005; Nunn *et al.,* 2010). It was found that *S. acidocaldarius's* inability

to grow on ribose and fructose is caused by the absence of their corresponding sugar transporters (She *et al.,* 2001; Chen *et al.,* 2005).

S. acidocaldarius differs from other *Sulfolobus* in that it has special transporters for C4-dicarboxylates (She *et al.,* 2001; Chen *et al.,* 2005). It was found that when most of bacteria and eukaryotes grow in mixed sugars media, they utilize sugars in specific order based on the preference for growth by repressing the utilization of other sugars in mixed media (Chen *et al.,* 2005; Joshua *et al.,* 2011; Quehenberger *et al*., 2017). Unlike *S. solfataricus*, *S. acidocaldarius* has the ability to grow simultaneously on D-glucose and D-xylose because of the absence of carbon catabolite repression that was previously found in *S. solfataricus* (Chen *et al.,* 2005; Joshua *et al.,* 2011; Quehenberger *et al*., 2017). Growing simultaneously on mixed sugars is preferable for the production of biofuel from cellulosic biomass (Chen *et al.,* 2005; Joshua *et al.,* 2011; Quehenberger *et al*., 2017). It has been reported that *S. solfataricus* metabolizes both glucose and galactose by the partial phosphorylative or the non- phosphorylative Enter-Doudorff pathway (Joshua *et al.,* 2011; Quehenberger *et al*., 2017). In addition, *S. cervisiae* and *E. coli* metabolize glucose and xylose (5-carbon and 6-carbon sugars) sequentially; however, the hyperthermophilic microorganism *S. acidocaldarius* is able to utilize both sugars simultaneously (Joshua *et al.,* 2011; Quehenberger *et al*., 2017). The ability to utilize 5-carbon and 6-carbon sugars simultaneously can exclude the need for isolating the two sugars during the biofuel production in the pre-treatment process (Chen *et al.,* 2005; Joshua *et al.,* 2011; Quehenberger *et al*., 2017). As a result, the time required for the fermentation of both sugars and the cost of biofuel production would be reduced (Joshua *et al.,* 2011; Quehenberger *et al*., 2017). Similar to other hyperthermophilic

microorganisms, *Sulfolobus* species is suggested to be beneficial in the production of biofuel (Quehenberger *et al*., 2017).

1.1.1. Pathways of ethanol production

Pyruvate, an intermediate in the central metabolism of carbohydrates, is converted into acetaldehyde, which is then reduced to ethanol by two different pathways (**Fig. 1**). In organisms such as *S. cerevisiae* and *Z. mobilis*, ethanol is produced by a two-step metabolic pathway (Bai *et al*., 2008), which is catalyzed by PDC and alcohol dehydrogenase (ADH) respectively (Eram and Ma, 2013). Pyruvate is decarboxylated to acetaldehyde by a non-oxidative reaction, which is catalyzed by PDC. Acetaldehyde is subsequently reduced to ethanol by ADH (Bai *et al*., 2008; Eram and Ma, 2013). In this pathway, it is observed that when 25 mM pyruvate is added into culture medium, ethanol synthesis was improved and the ratio of carbon partitioning to ethanol increases (Luan *et al.,* 2015). However, it has been also reported that when 50 mM pyruvate added into the medium, it inhibits the growth of the cell and the synthesis of ethanol significantly (Luan *et al.,* 2015). This indicates that it is important to maintain a balance between the carbon source and chemicals added.

In most of the thermophilic organisms, pyruvate is converted to ethanol using a three-step pathway (Straub *et al.,* 2017). The catalysis of the oxidative decarboxylation of pyruvate is conducted by POR or pyruvate formate lyase (PFL), producing acetyl-CoA (Ragsdale, 2003; Eram *et al*., 2014). The acetyl-CoA is then converted to acetaldehyde, which is catalyzed by the CoA-dependent acetaldehyde dehydrogenase (AcDH). The enzyme catalyzing the conversion of acetaldehyde to ethanol is ADH (Eram *et al*., 2014).

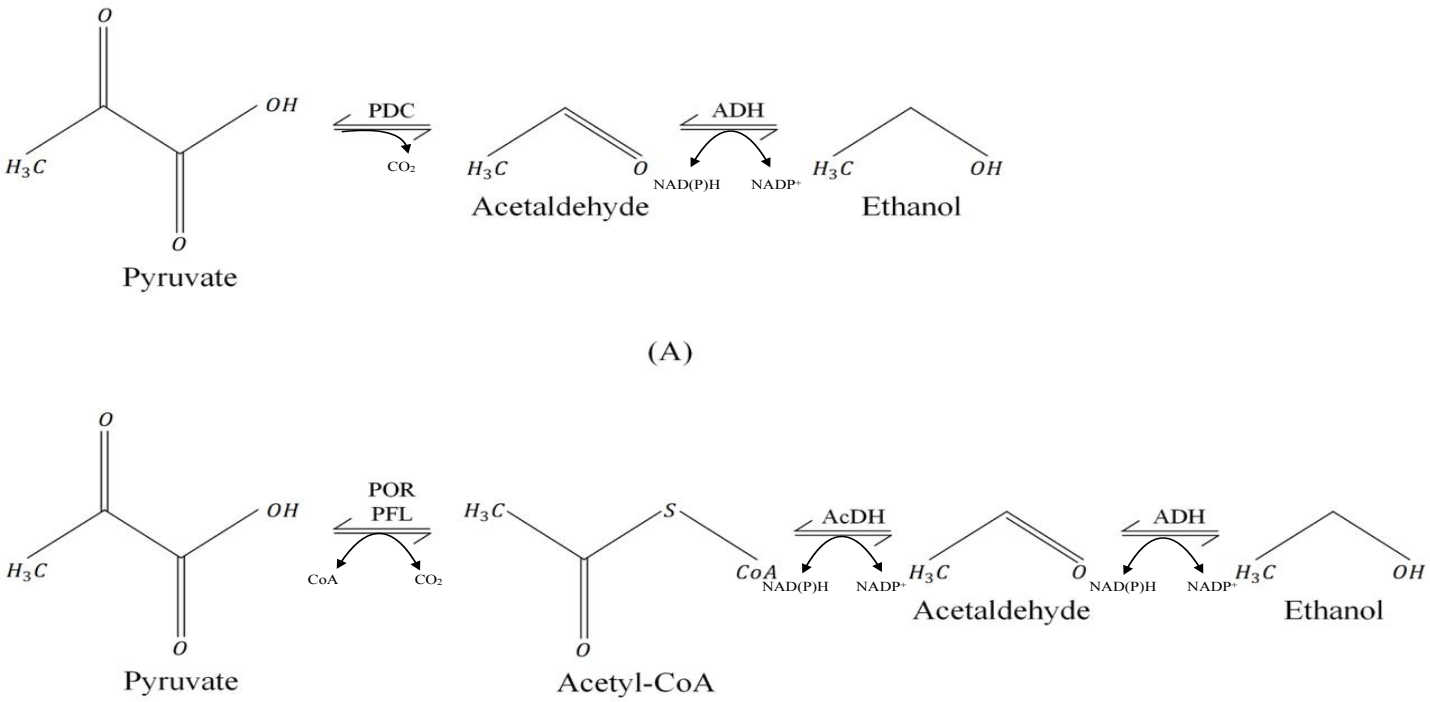

Figure 1: Pathways for ethanol production from pyruvate.

(A) Two-steps pathways, (B) Three-steps pathways. POR; Pyruvate ferredoxin oxidoreductase; PFL; Pyruvate formate lyase, AcDH; CoA-dependent acetaldehyde dehydrogenase, ADH; Alcohol dehydrogenase, PDC; pyruvate decarboxylase (Bai *et al*., 2008; Eram & Ma, 2013).

1.2. Enzymes involved in acetaldehyde production

1.2.1. Pyruvate decarboxylase

PDC is a key enzyme in the ethanol-production pathway and catalyzes the non-oxidative decarboxylation reaction of pyruvate to produce acetaldehyde and carbon dioxide using thiamine diphosphate and Mg^{2+} ion as cofactors (Eram & Ma, 2013). Non-oxidative decarboxylation of pyruvate to acetaldehyde was found in *S. cerevisiae* in 1911 by Neuberg and Karczag (Iding *et al*., 1998; Eram & Ma, 2013). PDC has been found in many organisms including *S. cerevisiae* (Table 1). Also, the enzyme is present in plants, for instance it is found in sweet potato, wheat, and cottonwood. PDC is present rarely in prokaryotes including *Z. mobilis*, and *Sarcina ventriculi* (Hoppner and Doelle 1983; Talarico *et al.,* 2001). This enzyme is also present in several fish species like carp and goldfish (Fagernes *et al.,* 2017). It is because of the presence of this enzyme, fish can perform the fermentation of ethanol when oxygen is present in limited quantity. Even though PDCs have been found in a wide range of organisms, no PDC homologs have been found in thermophilic and hyperthermophilic bacteria or archaea (Eram and Ma, 2013).

PDC is tetrameric enzyme that consists of four identical or non-identical subunits with a relative molecular mass of approximately 60 kDa (Berlowska *et al.* 2009). Each subunit binds to a thiamine diphosphate and a Mg^{2+} ion, and two subunits form a dimer with two active sites (Berlowska *et al.,* 2009; Eram and Ma, 2013). Thiamine diphosphate is an essential cofactor for PDC to catalyze the formation of acetaldehyde in the ethanol synthesis pathway (Iding *et al*., 1998; Pei *et al.,* 2010). In the non-oxidative decarboxylation reaction, pyruvate added to the C2 atom of the cofactor thiazolium ring and CO_2 is released to produce a carbanion/enamine intermediate (Iding *et al.,*

2010; Eram and Ma, 2013). Then, the carbanion/enamine intermediate is protonated to give hydroxyethyl diphosphate. Finally, the reaction is completed by releasing acetaldehyde, followed by the reduction of acetaldehyde to ethanol by ADH (Berlowska *et al.,* 2009; Pei *et al.,* 2010; Eram and Ma, 2013).

Table 1: Properties of PDCs characterized from mesophilic organisms

Organism	Specific activity (U/mg)[a]	Optimal Temperature ºC	Optimal pH	References
Zymomonas mobilis	120	60	6.0	Gocke *et al.,* 2009
Saccharomyces cerevisiae	40	43	6.0	Gocke *et al*., 2009
Acetobacter pasteurianus	71	65	5.0-5.5	Raj *et al*., 2002
Sarcina ventriculi	67	42	6.3-7.6	Raj *et al*., 2002
Gluconacetobacter diazotrophicus	20	45-50	5.0-5.5	Van Zyl *et al*., 2014
Torulopsis glabrata	40	-	6.0	Wang *et al.,* 2004

[a], one unit was defined as the production of 1 μmol of acetaldehyde per min.

-, not available.

1.2.2. Pyruvate ferredoxin oxidoreductase

POR is a member of the superfamily 2-oxoacid oxidoreductases that are able to catalyze coenzyme A- and TPP-dependent oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2, and reduce the electron acceptor ferredoxin or flavodoxin (Ragsdale, 2003; Eram *et al*., 2013). POR is considered to be an ancient molecule that is found in all three domains of life. In most of the anaerobic organisms, the catalysis of the oxidative decarboxylation of pyruvate to carbon dioxide and acetyl Co-enzyme A is performed through POR. POR is divided into three types based on the quaternary structures as follows: (1) homodimeric enzymes (mesophilic organisms); (2) heterodimeric enzymes (*Sulfolobus solfataricus* and *Sulfolobus tokodaii*); (3) heterotetramic enzymes (*Pyrococcus furiosus*) (Blamey and Adams, 1993; Zhang *et al*., 1996; Chabriere *et al*., 2011; Eram and Ma, 2013). PORs have been isolated from *Halobacterium salinarium* (Kerscher and Oesterhelt, 1981), *Clostridium* species (Wahl and Orme-Johnson, 1987), *P. furiosus* (Blamey & Adams, 1994), *S. tokodaii* (Iwasaki *et al*., 1994), *Methanosarcina barkeri* (Bock *et al.,* 1997), *S. solfataricus* (Park *et al*., 2006), *Thermotoga hypogea* (Eram *et al.,* 2015), and *Thermotoga maritima* (Eram *et al.,* 2015) (Table 2).

POR consists of a thiamine pyrophosphate (TPP) and at least one iron-sulfur cluster (Ragsdale, 2003; Eram and Ma, 2013). POR is a TPP-dependent enzyme that also known as pyruvate synthase in the reverse reaction where acetyl-CoA and carbon dioxide are converted to pyruvate which is a carbon dioxide fixation mechanism in autotrophic microorganisms (Eram and Ma, 2013; Yan *et al*., 2016).

Table 2: Properties of PORs characterized from various microorganisms

Organism	Specific activity (U/mg)	Optimal Temperature (ºC)	Optimal pH	Thermostability ($t_{1/2}$, h at 80 ºC)	Reference
Archaeoglobus fulgidus	74[a]	> 90	7.5	-	Kunow *et al*., 1995
Trichomonas vaginalis	18.3[b]	-	7.0	-	Williams *et al*. 1987
Clostridium acetobutylicum	25[c]	60	7.5	-	Meinecke *et al*., 1989
Desulfovibrio africanus	70[d]	-	9.0	-	Pieulle *et al.,* 1995
Halobacterium salinarium	6[e]	52	9.0	-	Kerscher and Oesterhelt 1981
Methanosarcina barkeri	25[f]	60	7.0	-	Bock *et al.* 1997
Pyrococcus furiosus	23.6[g]	90	8.0	0.3	Ma *et al.,* 1997
Thermotoga hypogea	96.7[h]	90	8.4	3	Eram *et al.,* 2015
Thermotoga maritima	90[i]	95	8.4	11	Eram *et al.,* 2015
Thermococcus guaymasensis	20.2[j]	95	8.4	-	Eram *et al.,* 2014
Sulfolobus solfataricus	16[k]	70	7.0-8.0	-	Park *et al.,* 2005
Sulfolobus tokodaii	39[l]	90	8.5	-	Zhang *et al.,* 1996; Fukuda *et al.,* 2001

[a], one unit of enzyme activity was defined as the reduction of 2 µmol methyl viologen per min at 65ºC and pH 7.5.

[b], one unit of enzyme activity was defined as the reduction of 2 µmol methyl viologen per min at 30ºC and pH 7.0.

[c], one unit of enzyme activity was defined as the oxidation of 1 μmol of pyruvate per min using methylene blue at 25ºC and pH 6.9.

[d], one unit of enzyme activity was defined as the reduction of 2 μmol methyl viologen per min at 30ºC and pH 8.5.

[e], one unit of enzyme activity was defined as the reduction of 1 μmol cytochrome c per min at 25 ºC and pH 8.0.

[f], one unit of enzyme activity was defined as the reduction of 2 μmol benzyl viologen per min at 37ºC and pH 7.0.

[g], one unit of enzyme activity was defined as the reduction of 2 μmol methyl viologen per min at 80ºC and pH 8.0.

[h], one unit of enzyme activity was defined as the reduction of 2 μmol methyl viologen per min at 80ºC and pH 8.4.

[i], one unit of enzyme activity was defined as the reduction of 2 μmol methyl viologen per min at 80ºC and pH 8.4.

[j], one unit of enzyme activity was defined as the reduction of 2 μmol methyl viologen per min at 80ºC and pH 8.4.

[k], one unit of enzyme activity was defined as the reduction of 1 μmol cytochrome c per min at 55 ºC and pH 7.0.

[l], one unit of enzyme activity was defined as the reduction of 1 μmol cytochrome c per min at 50 ºC and pH 6.8.

-, not available.

The initiation of the POR reaction begins with C2's nucleophilic attack of TPP (Ragsdale, 2003; Chen *et al.,* 2018). This reaction takes place on the 2-oxo carbon of pyruvate forming an adduct named lactyl-TPP (Zhang *et al.,* 1996; Ragsdale, 2003). After this step, the CO_2 moiety is released by the lactyl-TPP adduct (**Fig. 2**). This then forms an anionic intermediate, which then moves an electron to a [4Fe-4S] clusters (Zhang *et al.,* 1996; Ragsdale, 2003). These steps result in a stable radical intermediate which can be seen by using electron paramagnetic resonance spectroscopy (Ragsdale, 2003; Chen *et al*., 2018). The reaction of radical intermediate with a CoA molecule results in the transfer of another electron to [4Fe-4S] clusters from the radical intermediate (Zhang *et al.,* 1996; Ragsdale, 2003; Chen *et al*., 2018). This results in the formation of acetyl-CoA (**Fig. 2**)

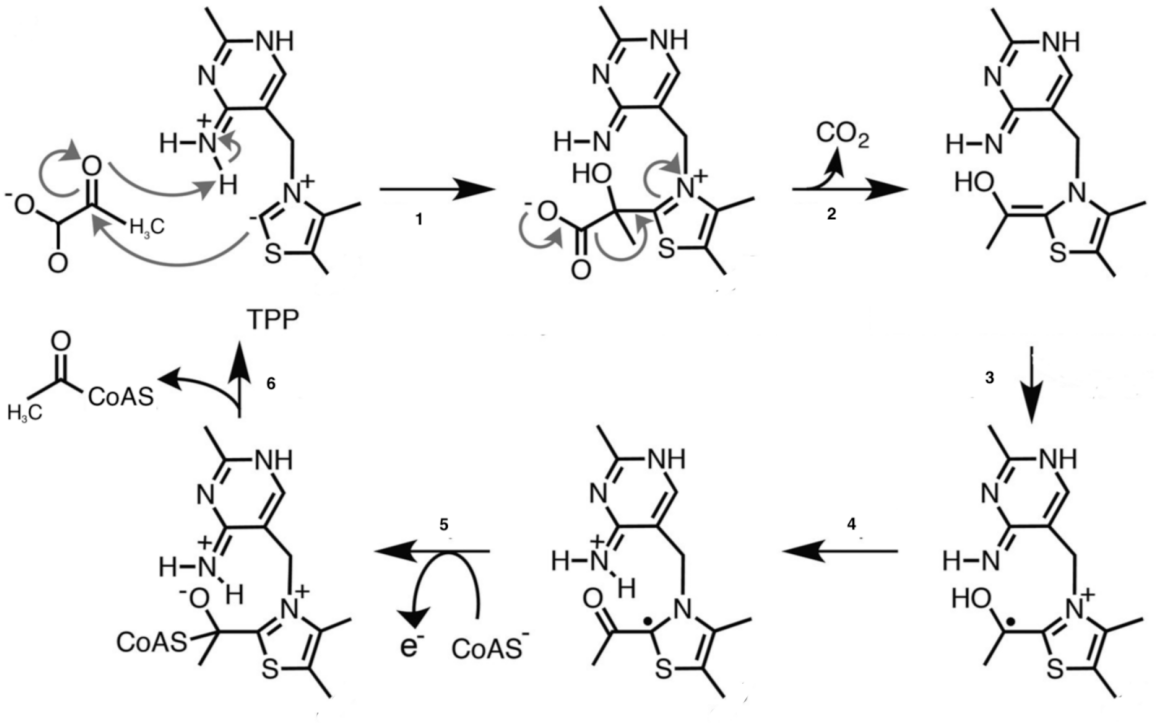

Figure 2: Catalytic mechanism of pyruvate ferredoxin oxidoreductase (Adopted from Chen *et al.,* 2018)

Most of the PORs are oxygen sensitive, suggested to be due to the structure of the enzyme (Eram and Ma, 2013; Yan *et al*., 2016). It was found that exposing cells to air causes the conversion of a stable $[4Fe\text{-}4S]^{2+}$ state to an unstable $[4Fe\text{-}4S]^{3+}$ form which inactivates the enzyme (Zhang *et al.,* 1996; Yan *et al*., 2016). According to a study conducted by Pieulle and colleagues in 1997, POR from *D. africanus* has an approximately 60 residue extension at the C-terminus of its polypeptide chain which protects the [4Fe-4S] clusters from oxidation. Additionally, some PORs are found to be oxygen insensitive such as those from *H. salinarium, S. tokodaii,* and *S. solfataricus* (Iwasaki *et al.,* 1994; Park *et al.,* 2006; Yan *et al*., 2016).

First crystal structures of POR was the homodimeric POR from *Desulfovbrio africanus* which consists of seven domains from I to V (Yan *et al*., 2014; Yan *et al*., 2016). Those domains included three [4Fe-4S] clusters and TPP (Zhang *et al*., 1996). Recent study of crystal structures of POR from *S. tokodaii* showed that it contains two subunits, α and β (heterodimeric enzyme) where α subunit corresponds to domains III-I-II, and β subunit corresponds to domain VI (Yan *et al*., 2014; Yan *et al*., 2016). Domain VII in POR from *D. africanus* and domain VI in POR from *S. tokodaii* were found to be similar where both form as arm that extended over the other subunits (Yan *et al*., 2016). Although there are similarities between PORs from *S. tokodaii* and *D. africanus*, sequence identity of each domain was approximately < 22% between these enzymes (Yan *et al*., 2016).

The intramolecular ferredoxin domain in PORs provide a pathway for the electron from pyruvate to an external ferredoxin (Yan *et al*., 2014; Yan *et al*., 2016). Since POR from *S. tokodaii* lacks domain V that contains the two-cluster intermolecular ferredoxin, domains III and VI surrounding large pocket found to be able to bind an external ferredoxin molecule (Yan *et al*.,

2014; Yan *et al*., 2016). POR from *S. tokodaii* considered to be a good model for studying the reaction mechanism of the POR because it is the smallest and simplest POR which contains only one [4Fe–4S] cluster (Fukuda *et al.,* 2001; Yan *et al*., 2014). Additionally, they showed a broad specificity for pyruvate and 2-oxoglutarate, indicating that they play important role in the TCA cycle metabolism (Fukuda *et al.,* 2001; Yan *et al*., 2014).

Heterodimeric PORs do not have a δ subunit/domain, dicluster-type ferredoxin carrying two [4Fe-4S] clusters, which is present in homodimeric and heterotetramic PORs (Iwasaki *et al.,* 1994; Zhang *et al.,* 1996; Park *et al.,* 2006). The structure of *S. tokodaii* POR indicates that the four loops covering the single [4Fe-4S] cluster could be the reason of the oxygen tolerance (Yan *et al.,* 2016). Besides the oxygen insensitivity, PORs from *H. salinarium* and *S. tokodaii* are found to be similar in terms of the enzyme structures (Iwasaki *et al.,* 1994; Zhang *et al.,* 1996; Iwasaki and Oshima, 2001). They are heterodimeric enzymes (αβ-type) consisting of two subunits, α and β, and using ferredoxin as electron acceptor (Zhang *et al.,* 1996; Iwasaki and Oshima, 2001). In addition, they contain one thiamin pyrophosphate (TPP), and one [4Fe-4S] (Zhang *et al.,* 1996; Iwasaki and Oshima, 2001). Despite the structural similarity, the sequence of α-subunit from *S. tokodaii* was 27.2% identity to α-subunit from *H. salinarium,* and it's β-subunit was 38.7% identity to β-subunit from *H. salinarium* (Zhang *et al.,* 1996)

PORs that are characterized from *Sulfolobus* are found to be thermostable and oxygen insensitive (Fukuda *et al.,* 2001; Park *et al.,* 2006). Moreover, the recombinant *S. tokodaii* POR is found to be almost identical to the native POR (Fukuda *et al.,* 2001). The native and recombinant POR share various characteristics for instance, the activity, oxygen insensitivity, optimum

temperature and optimum pH when methyl viologen was used as electron acceptor (Fukuda *et al.,* 2001; Yan *et al*., 2014). On the other hand, purified POR from *S. tokodaii* showed specific activity of 39 U/mg while recombinant POR found to have only 0.43 U/mg where 1 unit is defined as the reduction of 1 µmol of cytochrome c (Zhang *et al.,* 1996; Yan *et al*., 2014). *Sulfolobus* PORs would be preferable enzymes compared to other PORs from hyperthermophiles due to their oxygen resistance and the stability of recombinant PORs.

1.2.3. Acetaldehyde dehydrogenase (CoA acetylating)

CoA acetylating acetaldehyde dehydrogenase (AcDH) is a member of the superfamily aldehyde dehydrogenases. AcDH, a CoA dependent enzyme, has the ability to catalyze the production of acetaldehyde from acetyl-CoA which is produced by the oxidative decarboxylation of pyruvate (Sánchez 1998; Eram *et al*., 2014). The enzyme has been isolated and characterized from mesophilic bacteria including *E. coli* and *Clostridium kluyveri* (Smith & Kaplan 1980; Rodríguez-Zavala *et al*., 2006). AcDH can be divided into two forms based on the functionality of the enzyme: (1) a monofunctional enzyme with only AcDH activity; (2) a bifunctional enzyme with AcDH and ADH activities where the N-terminal domain carries AcDH and the C- terminal domain carries ADH (Sánchez 1998; Lo *et al.,* 2015). The bifunctional AcDH/ADH was purified and characterized from mesophiles such as *Giardia lamblia* and *Entamoeba histolytica* (Bruchhaus and Tannich 1994; Sánchez 1998). However, the AcDH/ADH have been found in thermophilic microorganisms like *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum*, and there is no monofunctional or bifunctional AcDH found in hyperthermophiles (Burdette and Zeikus 1994; Lo *et al*., 2015).

1.2.4. Bifunctional PDC/POR from the hyperthermophiles

The decarboxylation of pyruvate to acetaldehyde in hyperthermophilic microorganisms was first reported in anaerobic archaeon *P. furiosus* (Ma *et al.,* 1997). It is found that POR has the ability to catalyze both the non-oxidative decarboxylation and oxidative decarboxylation of pyruvate to produce acetaldehyde and acetyl-CoA, respectively (Ma *et al.,* 1997; Eram *et al.,* 2014; Eram *et al.*, 2015). Bi-functional PDC/POR enzymes have been reported in hyperthermophilic euryarchaeota (*P. furiosus* and *Thermococcus guaymasensis*) and hyperthermophilic bacteria (*T. maritima* and *T. hypogea*) (Table 3, Ma *et al.,* 1997; Eram *et al.,* 2014; Eram *et al.*, 2015). Most of PDC activities in hyperthermophiles are oxygen sensitive, however, PDC from mesophilic microorganisms are oxygen insensitive. Bifunctional PDC/PORs were TPP- and coenzyme A-dependent, although, CoA plays a structural and not catalytic role, ferredoxin is not required. Although Yan and his colleagues in 2014 reported that the CoA is not required for PDC activity from *S. tokodaii* PDC/POR and it is not oxygen sensitive (Yan *et al.,* 2014), CoA actually enhances the PDC activity by 20% (Yan *et al.,* 2014). The difference between bifunctional PDC/POR and conventional PDC can be seen in terms of oxygen sensitivity, dependency on coenzyme A, and lower catalytic activity (Eram *et al.,* 2014; Yan *et al.,* 2014).

Table 3: Kinetic parameters of PORs and PDCs from hyperthermophiles

Sources	Activity	Pyruvate		CoA		References
		K_m (mM)	V_{max} (U/mg)	K_m (µM)	V_{max} (U/mg)	
T. maritima	POR	0.4±0.1	81±6	63±6	94±2	Eram *et al*., 2015
	PDC	0.92±0.3	1.4±0.04	3.1±1.2	1.3±0.03	
T. hypogea	POR	0.13±0.03	99±3	21±2	73±4	Eram *et al*., 2015
	PDC	1.4±0.4	2.5±0.18	1.4±0.02	1.6±0.13	
T. guaymasensis	POR	0.53±0.03	18±0.23	70 ±10	21.8±0.8	Eram *et al.,* 2014
	PDC	0.25±0.05	3.8±0.14	20±1	3.3±0.09	
P. furiosus	POR	0.46	23.6	110	22	Ma *et al.,* 1997
	PDC[a]	1.1	4.3±0.3	110	4.3±0.3	

For POR assays, one unit was defined as 1 µmol of pyruvate oxidized or the reduction of 2 µmol methyl viologen per min at 80ºC and pH 8.4.

For PDC assays, one unit was defined as the production of 1 µmol of acetaldehyde per min at 80ºC and pH 8.4

[a], one unit was defined as the production of 1 µmol of acetaldehyde per min at 80ºC and pH 10.2

1.2.5. Bifunctional PDC/AHAS from the hyperthermophiles

Acetohydroxyacid synthase (AHAS), a member of the decarboxylase family, is a thiamine pyrophosphate (TPP) and Mg^{2+} dependent enzyme that catalyzes the production of acetolactate from pyruvate (Duggleby *et al*., 2008; Eram *et al.,* 2016). During the reaction, AHAS enzyme decarboxylates one molecule of pyruvate and adds a second molecule of pyruvate or 2-ketobutyrate, which leads to the production of acetolactate and 2-aceto-2-hydroxybutyrate respectively (Eram and Ma*,* 2015; Eram *et al*.*,* 2016). The enzyme consists of two subunits, and has been found in archaea, bacteria, fungi, algae, and plants (Duggleby *et al*., 2008; Eram and Ma*,* 2016). AHASs can be classified into two classes, anabolic and catabolic AHASs based on their metabolic roles, specificity of substrate and requirements of cofactor (FAD) (Eram *et al.,* 2015). The purified AHAS from the hyperthermophilic *T. maritima* shows the ability to catalyzes the production of acetolactate from pyruvate as well as catalyzes the production of acetaldehyde and CO_2 from pyruvate (Eram and Ma, 2016). This is the first time bifunctional PDC/AHAS is implicated in the production of acetaldehyde in hyperthermophiles.

1.3. Objectives of the present study

The aim of this study was to purify and characterize the bifunctional PDCs/PORs from *S. acidocaldarius* and *S. solfataricus*. It is hypothesized that PDC activity from both organisms is oxygen insensitive which could be beneficial for potential biofuel production on an industrial scale. The specific goals were as follows;

- To purify PDCs/PORs from *S. solfataricus* and *S. acidocaldarius*
- To determine the optimal temperature and pH for PDCs and PORs
- To measure the thermostability of PDC/POR enzymes
- To determine the CoA and pyruvate dependency of both PDCs and PORs
- To determine oxygen sensitivity of PDCs and PORs

Chapter 2 Materials and Methods

2.1 Microorganisms and Chemicals

S. solfataricus and *S. acidocaldarius* were grown in a 100 L fermenter as described (Brock *et al*., 1972) by Thomas Knura and Bettina Siebers (University of Duisburg-Essen, Germany). DNPH (2,4-dinitrophenylhydrazine) was obtained from Eastman Organic Chemicals (New York, USA), acetonitrile from Fisher Scientific (Canada), and coenzyme A from US Biological (USA). Sodium pyruvate, benzyl viologen, methyl viologen, acetaldehyde, dichloromethane, and hydrochloride acid were purchased from Sigma-Aldrich Canada Ltd (Canada). Dithiothreitol (DTT) was provided by Bio Basic Inc (Canada).

2.2 Enzyme purification

Cell-free extract (CFE) was prepared anaerobically from the frozen cells of *S. solfataricus* (Ss) and *S. acidocaldarius* (Sa). *S. solfataricus* cell pellets (approximately 5 g, wet weight) were transferred into a degassed serum bottle and suspended in the anaerobic buffer (30 ml) containing 10 mM sodium phosphate, 2 mM dithiothreitol (DTT) at pH 7.0. *S. acidocaldarius* cell pellets (approximately 5 g, wet weight) were transferred into a degassed serum bottle and suspended in the anaerobic buffer (30 ml) containing 50 mM Tris-HCl, 2 mM DTT at pH 7.3. The suspensions were stirred for 2 h at 30ºC. Cell suspensions were run through a French Pressure Cell (Thermo scientific, MA, USA) four times at 20,000 psi to break the cells. The obtained crude cell extracts were centrifuged at 20,000 × *g* for 30 min at 4ºC. The supernatant CFEs were transferred to anaerobic serum bottles for further use.

The enzyme purification was carried out at room temperature and under anaerobic conditions. Purification buffers in flasks were degassed and 2 mM DTT added to remove residual oxygen. The flasks were kept under a nitrogen positive pressure (3 psi). A Fast Performance Liquid Chromatography (FPLC) system with a P-920 pump (Amersham Pharmacia Biotech) was used. POR activity in each fraction was monitored, while PDC activity was measured in the CFE and after the final purification step. SDS-PAGE was used for determining the purity of the fractions according to Laemmli's method (Laemmli 1970).

CFE of *S. solfataricus* or *S. acidocaldarius* was diluted with buffer A (50 mM Tris-HCl pH 7.8 and 2 mM dithithreitol [DTT]) in a ratio 1:1 (v/v), and loaded onto a DEAE-Sepharose column

(2.6 cm × 11 cm) that was equilibrated with buffer A. The column was washed with one column volume (60 ml) using buffer A. The absorbed proteins were eluted with a gradient (300 ml, 0-1M NaCl) of buffer B containing 1 M NaCl, 50 mM Tris-HCl pH 7.8, and 2 mM DTT. The flow rate was 2 mL min^{-1}. The fractions with enzyme activities (33-75 mM NaCl for *S. Solfataricus* and 230-300 mM NaCl for *S. acidocaldarius*) were loaded onto a hydroxyapatite column (HAP, 2.6 cm x 9 cm) equilibrated with buffer A at a flow rate of 1 mL min^{-1}. After washing with buffer A for one volume of the column (50 ml), proteins bound to the column were eluted with a linear gradient of 0-0.5 M potassium phosphate using buffer A and buffer C containing 0.5 M potassium phosphate, 50 mM Tris-HCl pH 7.8, and 2 mM DTT. Fractions containing high activity of POR (125-310 mM potassium phosphate for *S. Solfataricus* and 280-350 mM potassium phosphate for *S. acidocaldarius*) were pooled and loaded onto a phenyl-Sepharose column (2.6 cm x 12 cm) equilibrated with buffer D containing 0.5 M ammonium sulfate in buffer A. After washing the column using buffer D (65 ml) at a flow rate of 2 mL min^{-1}, the enzyme was eluted out by applying a reverse linear gradient of 0.5 M to 0.0 M ammonium sulfate. Fractions containing the POR activity for *S. solfataricus* (110-55 mM ammonium sulfate) or *S. acidocaldarius* (165-110 mM ammonium sulfate) were combined and concentrated with ultrafiltration using a 30 kDa high-flow, polyethersulfone (PES) membranes (Sigma-Aldrish, Canada).

2.3 Enzyme assays

POR activity was determined by measuring the pyruvate and coenzyme A dependent reduction of benzyl viologen at 578 nm under anaerobic conditions at 80 ºC and pH 7.8 (Ma *et al.* 1997; Park *et al*. 2005). The assay mixture was transferred into degassed glass cuvettes sealed with rubber stopper. The cuvettes were kept under a nitrogen positive pressure (3 psi), after the oxygen was removed using the manifold. The assay mixture (2 mL) containing 100 mM sodium phosphate pH 8.0 (prepared at room temperature), 5 mM pyruvate, 1 mM benzyl viologen or methyl viologen, and approximately 50 µM sodium dithionite (SDT) in a glass cuvette with 1 cm light path was incubated for 4 min to reach 80 ºC. After the addition of the enzyme (3 µg SsPOR, 12 µg SaPOR, or an enzyme sample with less purity was used for some assays due to the insufficient purified enzyme available and then a relative activity was presented), 100 µM CoA was added to start the enzymatic reaction. The absorbance change at 578 nm was recorded using a Genesys 10Vis spectrophotometer (benzyl viologen ε_{578} = 8.65 mM^{-1} cm^{-1}, Mikoulinskaia *et al.* 1999; methyl viologen ε_{578} = 9.8 mM^{-1} cm^{-1}, Yoon *et al.,* 1997). One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate or the reduction of 2 µmol of benzyl viologen/methyl viologen per minute.

The activity of PDC was determined by measuring the acetaldehyde production using the 2,4-dinitrophenylhydrazine (DNPH) derivatization method followed by high performance liquid chromatography (HPLC) (Ma *et al.* 1997; Eram *et al.,* 2014). The reactions were carried out under anaerobic conditions at 80 ºC. The assay mixture (1 ml) contained 100 mM sodium phosphate pH 8.0 (prepared at room temperature), 10 mM pyruvate, 100 µM CoA in sealed 8 mL vials and was incubated in a waterbath (80 ºC) before adding the enzyme (in case of an enzyme sample with less

purity was used for some assays due to the insufficient purified enzyme available, a relative activity was presented), then incubated for 2 hours. Because SaPDC has lower activity than SsPDC, higher concentration of SaPDC enzyme was used for the assays. Enzymatic reaction was stopped by transferring the vials to an ice bath followed by adding 2 mL of saturated DNPH solution in 2 N HCl which derivatizes acetaldehyde, producing a yellow-reddish colored compound. The vials were shaken in the dark overnight at 315 rpm at room temperature. The extraction of acetaldehyde-DNPH derivative was performed twice by adding each time 1 ml of dichloromethane (DCM) in the vials and followed by shacking for 30 min. The lower organic phase was transferred to a new vial that was then covered by Parafilm M membrane punctured with a needle to create a few small holes and placed in a vacuum desiccator to evaporate the DCM. The resulting yellowish-red powder was dissolved in 4 ml of acetonitrile and incubated at 4 °C overnight, subsequently, filtered through 0.45 µm nylon syringe filter (Mandel Scientific, Canada). The filtered product was analyzed at room temperature using Perkin Elmer series 4 HPLC system equipped with a reversed-phase Allure C18 5µm column (150 x 4.6 mm). Samples (80 µl) were injected to the Rheodyne injection valve using 100 µl micro-syringe. The mobile phase of acetonitrile/water (80:20 v/v) was used at a flow rate of 1 mL. min^{-1}, and the acetaldehyde-DNPH was detected at 365 nm by a micro-metrics model 788 dual variable wavelength detector. The attenuation was set at 0.64 A. The concentration of acetaldehyde was measured based on acetaldehyde standard curve prepared under same conditions. One unit of activity was defined as the formation of 1 µmol of acetaldehyde per min.

2.4 Biophysical and biochemical characterization

Temperature dependence of PDC and POR for purified enzymes from *S. solfataricus* and *S. acidocaldarius* were determined anaerobically by performing the PDC and POR assays at temperatures ranging from 30ºC to 90ºC and 40ºC to 90ºC, respectively. Temperature dependence of PORs were measured using the standard assay mixtures (100 mM sodium phosphate pH 8.0 [prepared at room temperature], 5 mM pyruvate, 1 mM benzyl viologen, 100 µM CoA, approximately 50 µM SDT, and 3 µg SsPOR or 12 µg SaPOR). PDC assays were carried out in 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate, 100 µM CoA, and enzyme (25 µg for SsPDC and 50 µg for SaPDC).

To determine the thermostability of the enzymes, they were transferred into different anaerobic bottles and incubated for different periods of time at different temperatures (70 and 80 ºC) in a water bath. The activity was measured at different incubation time points selected, then compared to unheated enzyme as a control. The enzyme assays were carried out using the standard assay mixture as described above (see 2.3 Enzyme assays). All of the assays were measured in duplicate.

The oxygen sensitivity of PDCs and PORs activities for purified enzymes from *S. solfataricus* and *S. acidocaldarius* was investigated by exposing the purified enzymes to air at 4 ºC. Purified PORs from *S. solfataricus* and *S. acidocaldarius* were exposed to air for 48 h at 4 ºC, while the PDCs were exposed to air for 7 h at 4 ºC. Enzymes with less purity were used for POR assays (relative activity to the pure SsPOR and SaPOR were 61 % and 75 % respectively). The results

were compared with the control samples (anaerobic samples). All of the assays were carried out in duplicate.

The pH dependence of PDCs and PORs were determined by measuring PDC and POR activities of both purified enzymes from *S. solfataricus* and *S. acidocaldarius* at 80 ºC and various pH values (measured at room temperature) from 6.0-12.0 for PDC and 5.0-11.0 for POR, respectively. The assays were carried out under anaerobic conditions. The assay mixture of POR (2 mL) consists of 100 mM buffer, 5 mM pyruvate, 1 mM methyl viologen, 100 µM CoA, approximately 50 µM SDT, and enzyme (3 µg of SsPOR and 12 µg of SaPOR). Enzymes with less purity were used for POR assays (relative activity to the pure SsPOR and SaPOR were 45 % and 28 % respectively). The buffers used in this experiment for POR were sodium phosphate (Δ pK_a /ºC = −0.0028) for pH values of 6.0, 7.0, 8.0, EPPS (Δ PK_a /ºC = −0.015) for values of 7.0, 8.0, and 9.0, glycine (ΔpK_a/ºC = −0.025) for pH values of 9.0, 9.5, and 10.0, and CAPS (Δ pK_a /ºC = −0.009) for pH values of 10.0, 11.0, and 12.0. The assay mixture of PDC (1 ml) contains 100 mM buffer, 10 mM pyruvate, 100 µM CoA, and enzyme (25 µg of SsPDC and 50 µg of SaPDC). PDCs activities were measured using sodium phosphate buffer (pH 7.0, and 8.0), glycine buffer (pH 9.0, and 10), and CAPS buffer (pH 11.0, and 12.0). All of the assays were measured in duplicate. The buffers were prepared at room temperature.

The kinetic parameters of PDC and POR were measured anaerobically at 80ºC and pH 8.0 (prepared at room temperature) in duplicate. Various concentrations of pyruvate and CoA were applied to the standard assay mixtures for PDC and POR respectively. POR enzymes samples with less purity were also used (relative activity to the pure SsPOR and SaPOR were 45 % and 28 %

respectively). The concentrations of pyruvate and CoA in SsPOR assays were in ranges of 0.0-0.7 mM and 0.0-100 μM respectively, however, 0.0-0.6 mM of pyruvate and 0.0-100 μM CoA were used for determining kinetic parameters of SaPOR. To measure K_m and V_{max} for SsPDC and SaPDC, 0.0-10 mM of pyruvate and 0.0-100 μM CoA were used. The kinetic parameters were calculated by fitting the data using the Michaelis-Menten equation from KaleidaGraph (Synergy Software, US).

2.5 Protein determination

The protein concentrations were measured using the Bradford assay (Bradford, 1976). Assay mixture contained 200 µl of Bio-Rad reagent and 800 µl of protein solution (800 µl deionized water was replacing the protein solution in the controls samples). Bovine serum albumin (BSA) was used for preparation of the linear calibration curve. Samples were analyzed using a spectrophotometer at 595 nm.

Chapter 3 Results

3.1 Purification of PDC/POR from *S. solfataricus* and *S. acidocaldarius*

S. solfataricus and *S. acidocaldarius* POR activities present in CFEs were determined to be 0.18±0.01 U/mg and 0.10±0.01 U/mg, respectively, while their PDCs activities were determined to be 0.0027±0.0003 U/mg and 0.0011±0.0004 U/mg, respectively. For studying their biophysical and biochemical properties, both PDC/POR enzymes had to be purified.

The use of approximately 5 g of *S. solfataricus* and *S. acidocaldarius* resulted in about 2 mg purified SsPOR enzyme with specific activity of 7.5±0.05 U/mg (Table 4) and 0.6 mg purified SaPOR enzyme with specific activity of 7±0.02 U/mg (Table 5), respectively. The results showed that the recovery of the enzymes from SsPOR and SaPOR were 25% and 19% respectively, which were similar to those of SsPDC (24%) and SaPDC (14%) respectively (Table 4 & 5). SDS-PAGE was performed to determine the purity of the enzyme after each column, showing both SsPOR (**Fig. 3**) and SaPOR (**Fig. 4**) are heterodimeric enzymes with similar sized subunits (molecular mass of approximately 37 kDa and 66 kDa). Purities of SsPOR (**Fig. 3**) and SaPOR (**Fig. 4**) were estimated to be about 90% and 80%, respectively.

Table 4: Purification of the bifunctional PDC/POR from *S. solfataricus*

Step	Enzyme	Protein (mg)	Specific activity (U/mg)[a,b]	Total activity (U)	Fold	Recovery (%)
CFE	POR	354.2±2.5	0.18±0.01	63.8±0.15	1	100
	PDC	354.2±2.5	0.0027±0.0003	0.95±0.05	1	100
DEAE	POR	90±1	0.43±0.02	39±0.1	2.4	61.4
	PDC	nd	nd	nd	nd	nd
HAP	POR	63.1±0.5	0.6±0.02	37.9±0.05	3.3	59.3
	PDC	nd	nd	nd	nd	nd
Phenyl-Sepharose	POR	2.1±0.1	7.5±0.05	15.9±0.01	41.6	25
	PDC	2.1±0.1	0.11±0.004	0.23±0.005	40.7	24

[a], one unit of the POR activity was defined as micromole of pyruvate oxidized per min.

[b], one unit was defined as the production of 1 μmol of acetaldehyde per min.

nd, not determined

Table 5: Purification of the bifunctional PDC/POR from *S. acidocaldarius*

Step	Enzyme	Protein (mg)	Specific activity (U/mg)[a,b]	Total activity (U)	Fold	Recovery (%)
CFE	POR	231.8±2	0.1±0.01	23.2±1	1	100
	PDC	231.8±2	0.0011±0.0004	0.25±0.04	1	100
DEAE	POR	54.23±1.5	0.28±0.01	15.2±0.3	2.38	65.4
	PDC	nd	nd	nd	nd	nd
HAP	POR	22.6 ±0.4	0.45±0.03	10±0.2	4.5	39.4
	PDC	nd	nd	nd	nd	nd
Phenyl-Sepharose	POR	0.63±0.03	7±0.02	4.41±0.01	70	19
	PDC	0.63±0.03	0.055±0.003	0.035±0.001	50	14

[a], one unit of the POR activity was defined as micromole of pyruvate oxidized per min.

[b], one unit was defined as the production of 1 μmol of acetaldehyde per min.

nd, not determined

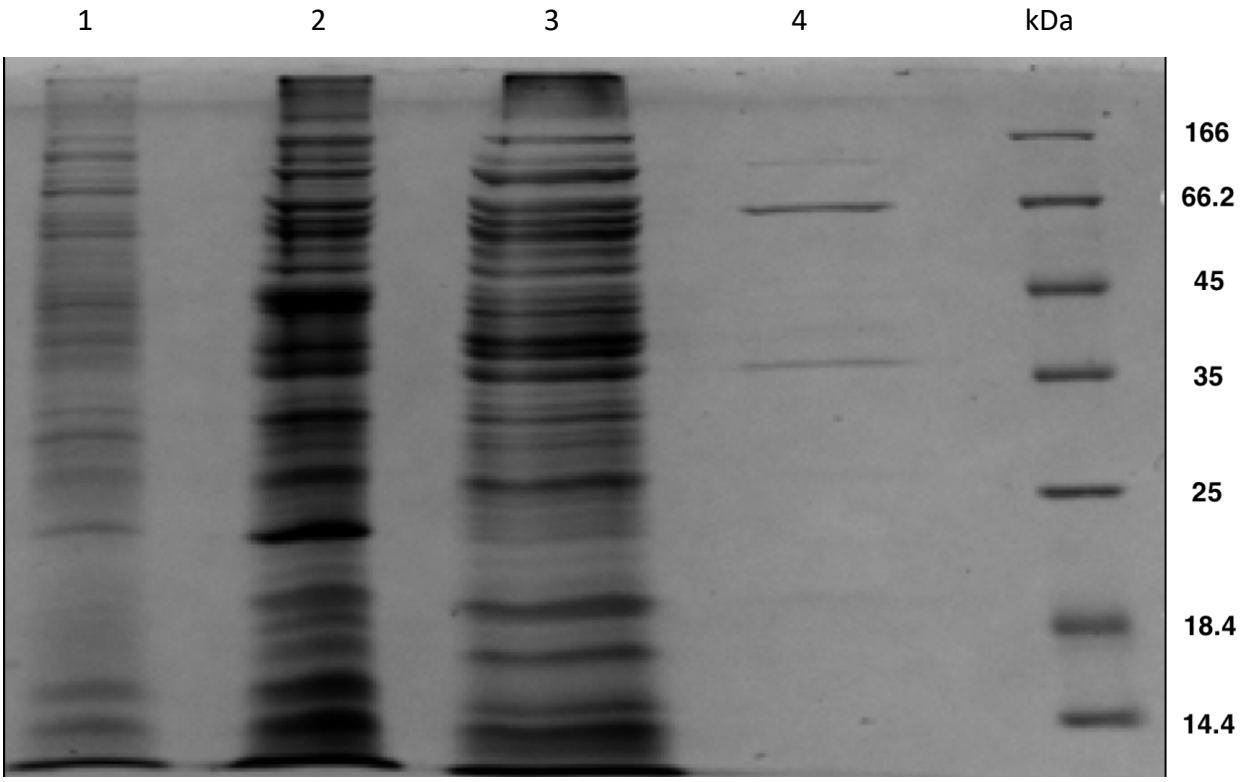

Figure 3: Analysis of purified enzyme from *S. solfataricus* using SDS-PAGE (12.5%). Lane 1, 10 µg of CFE; lane 2, 20 µg of DEAE fraction; Lane 3, 18 µg of HAP fraction; Lane 4, 1.5 µg of purified enzyme; Lane 5, BLUeye pre-stained protein ladder.

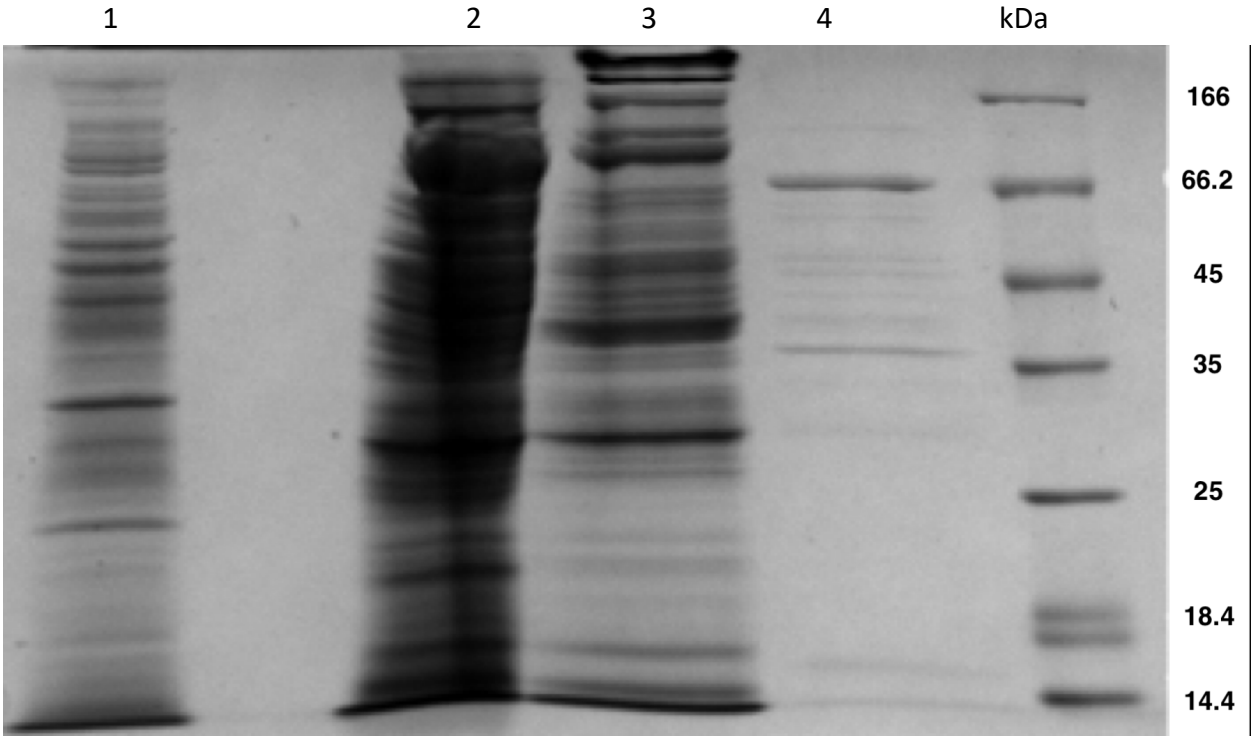

Figure 4: Analysis of purified enzyme from *S. acidocaldarius* using SDS-PAGE (12.5%). Lane 1, 12 µg of CFE; Lane 2, 33 µg of DEAE fraction; Lane 3, 22 µg of HAP fraction; Lane 4, 0.8 µg of purified enzyme; Lane 5, BLUeye pre-stained protein ladder.

3.2 Biophysical and biochemical properties

The time dependence of SsPDC and SaPDC showed that 2 hours incubation time was within a linear range for the enzyme activities (**Fig. 5**). It was observed that the detector of HPLC had an interference signal (area of approximate 900,000) corresponding to approximately 0.03 mM of acetaldehyde at zero incubation time, which was taken into consideration for calculating sample signals together with those from different controls (**Fig. 5**).

Known concentrations of acetaldehyde were chosen and prepared using 100 mM sodium phosphate and incubated anaerobically at 80°. Same procedures were followed to detect the signals of the acetaldehyde derivative using HPLC as described in section 2.3. A standard curve for the detection of acetaldehyde was obtained (**Fig. 6A**) and it was used for the determination of all PDC assay samples. It was thought that the lower range of the standard (**Fig. 6B**) may fit the detection range of PDC activity better, but both gave the same correlation equation (area = 3e+7 mM).

Oxygen sensitivity of PDCs and PORs from both *S. solfataricus and S. acidocaldarius* were determined by following residual activity after incubating enzyme in the presence and absence of air. Purified SsPOR and SaPOR in the absence of air had activities of 4.6 ± 0.2 U/mg and 5.5 ± 0.05 U/mg respectively, and after they were exposed to air for 48h at 4 ºC, their activities remained to be 4.6 ± 0.1 U/mg and 5.3 ± 0.2 U/mg respectively, indicating no loss of activity upon their exposure to air. The oxygen sensitivity of PDCs were also determined by comparing activities of SsPDC (0.1 ± 0.01 U/mg) and SaPDC (0.031 ± 0.005 U/mg) in the absence of air with those samples exposed to air for 7 h at 4 ºC, which were 0.083 ± 0.007 U/mg for SsPDC and 0.025 ±

0.002 U/mg for SaPDC. The results showed that both PDC and POR activities were not oxygen sensitive.

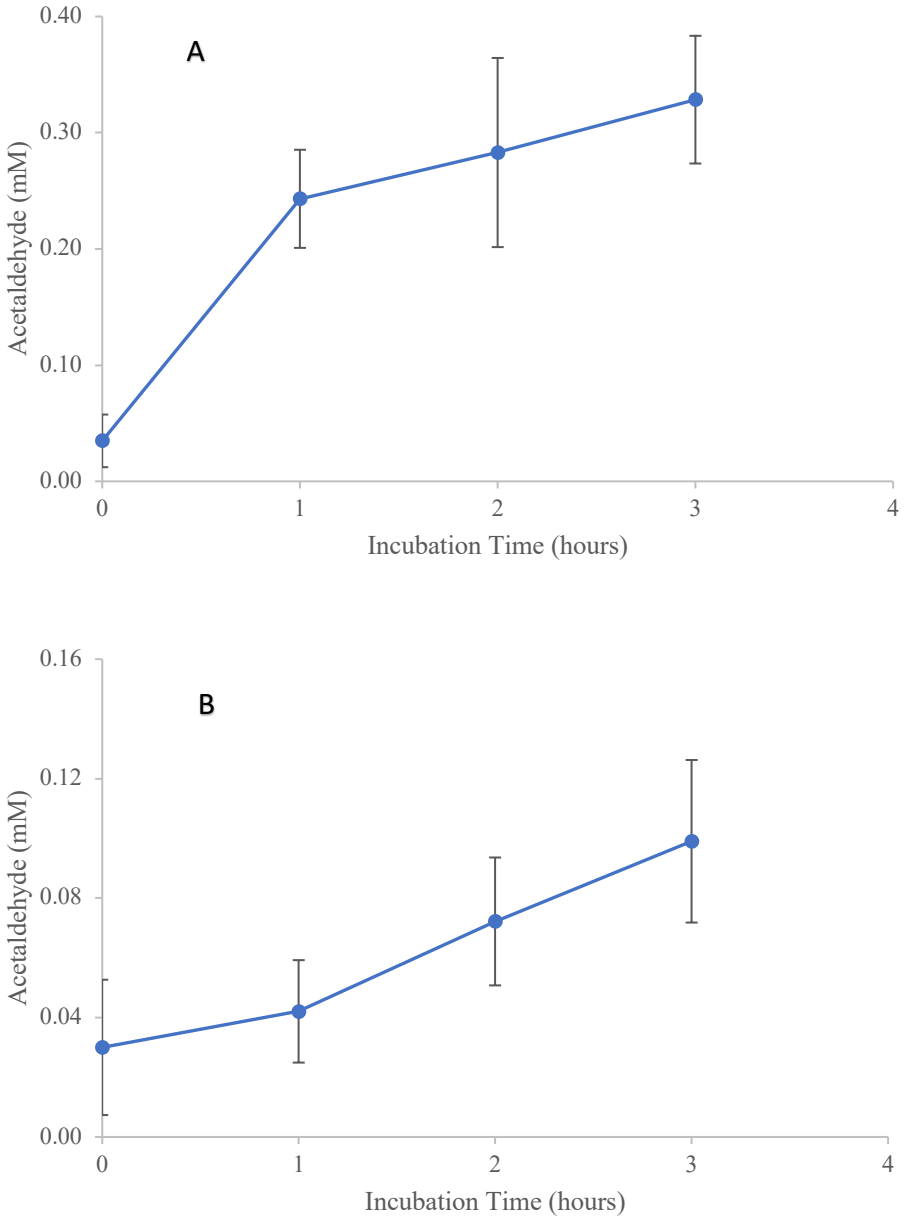

Figure 5: Time dependence of SsPDC (A) and SaPDC (B) activities. Assays were performed at 80 ºC and pH 8.0 (measured at room temperature) using purified enzymes (25 µg protein for SsPDC and 50 µg protein for SaPDC). Controls samples were contained 100 mM sodium phosphate, 10 mM pyruvate, and 100 µM CoA.

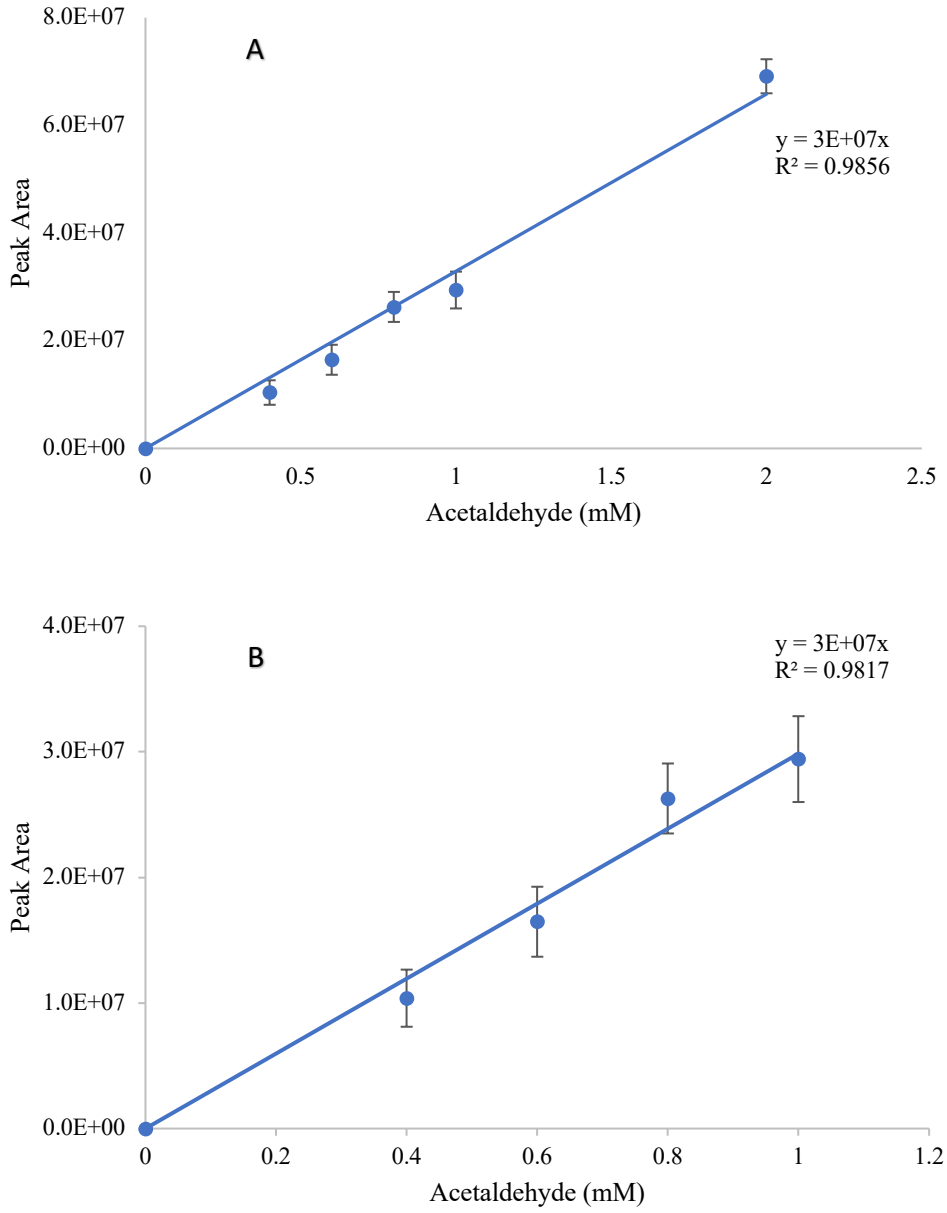

Figure 6: Acetaldehyde standard curves. A, plot range from 0-2 mM acetaldehyde; **B**, plot range from 0-1 mM acetaldehyde. Samples were prepared and measured under same conditions of the PDC assays at 80 ºC and pH 8.0 (measured at room temperature). The assay mixture was 100 mM sodium phosphate, 10 mM pyruvate and 100 µM CoA.

Since the assays were performed at 80 ºC and the buffers were prepared and measured at room temperature, actual pH value may be different from that measured at room temperature due to the temperature-dependent ΔpKa change (**Fig. 7-10)**. Therefore, estimated pH value of each buffer at different temperatures was calculated based on the ΔpKa change value of each buffer. A general trend was found to be that pH values at 80°C were smaller than those measures at room temperature (**Fig. 7-10**). The optimal pH values (at 80°C) for both PDCs and PORs from *S. solfataricus* and *S. acidocaldarius* were shown to be 7.8 (**Fig. 8B** & **Fig. 10B**) and 8.6 (**Fig. 7B** & **Fig. 9B**), respectively (**Fig. 7-10,** Table A1-A4).

The optimal temperatures of PDC and POR were determined using sodium phosphate (pH 8.0 measured at room temperature), and the results showed that the SsPOR activity increased continuously until 90 ºC with the highest activity of 12 U/mg (**Fig. 11A,** Table A7), while SsPDC activity showed no increase at 90 ºC compared to that at 80 ºC (**Fig. 11B,** Table A5). SaPOR activity increased along with the increase of the temperature until 80 ºC (8 U/mg), and remained approximately the same when the temperature was higher than 80 ºC (**Fig. 12A,** Table A8); however, SaPDC showed a continued increase up to 90 ºC (0.057 U/mg), the highest assay temperature achievable due to technical limitation (**Fig. 12B,** Table A6). The activation energy (E_{act}) for SsPOR and SaPOR, as calculated from the Arrhenius plots over the range of 60-90 ºC, were found to be 33.2 kJ/mol and 47 kJ/mol which is similar to POR from *T. hypogea* (34.8 kJ/mol range of 60-95ºC), while POR from *T. maritima* had lower E_{act} (23.6 kJ/mol range of 50-80ºC) comparing to other PORs (Eram *et al.,* 2015). On the other hand, POR from *A. fulgidus* had E_{act} of 75 kJ/mol for range of 30-70 ºC (Kunow *et al*., 1995). The activation energy for SsPDC and SaPDC over the range of 50-90 ºC were 44 kJ/mol and 70 kJ/mol, respectively (**Fig 11 & 12**).

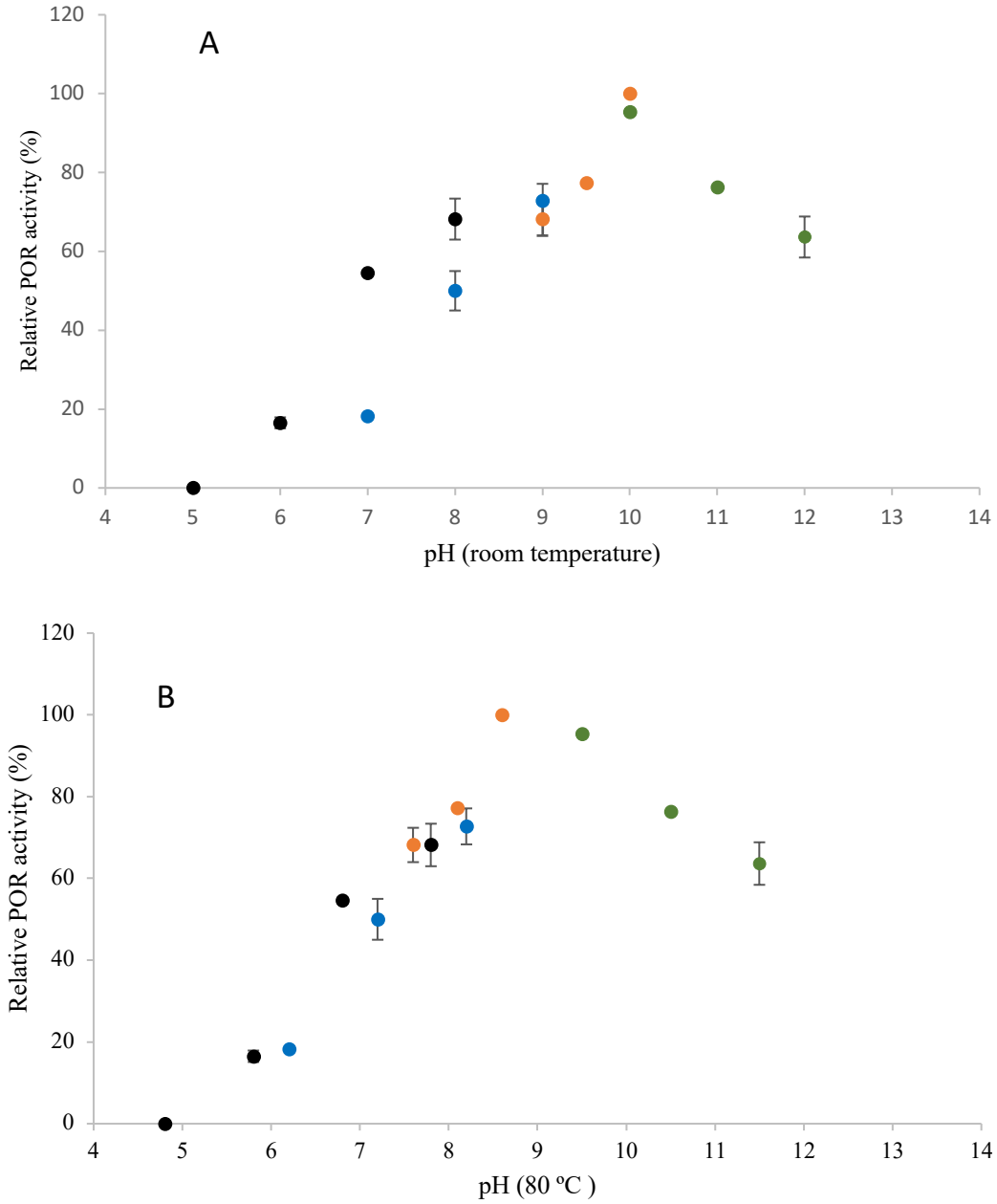

Figure 7: pH dependency of POR activity of *S. solfataricus*. POR activity was determined using 5 mM pyruvate, 1 mM methyl viologen, 100 µM CoA, approximately 50 µM SDT, and 3 µg purified SsPOR at 80ºC. At room temperature (A), the following buffers were used: sodium phosphate (•) (pH 5.0, 6.0, 7.0, and 8.0), EPPS (•) (pH 7.0, 8.0, and 9.0), glycine (•) (pH 9.0, 9.5 and 10), and CAPS (•) (pH10.0, 11.0, and 12.0). At 80 ºC (B), the pH values of the following buffers were estimated based on the pKa of each buffer at 80 ºC: sodium phosphate (•) (pH 4.9, 5.9, 6.9, and 7.9), EPPS (•) (pH 6.2, 7.2, and 8.2), glycine (•) (pH 7.6, 8.1 and 8.6), and CAPS (•) (pH 9.5, 10.5, and 11.5). The relative activities of 100% equals to the highest specific activities (2.2 U/mg).

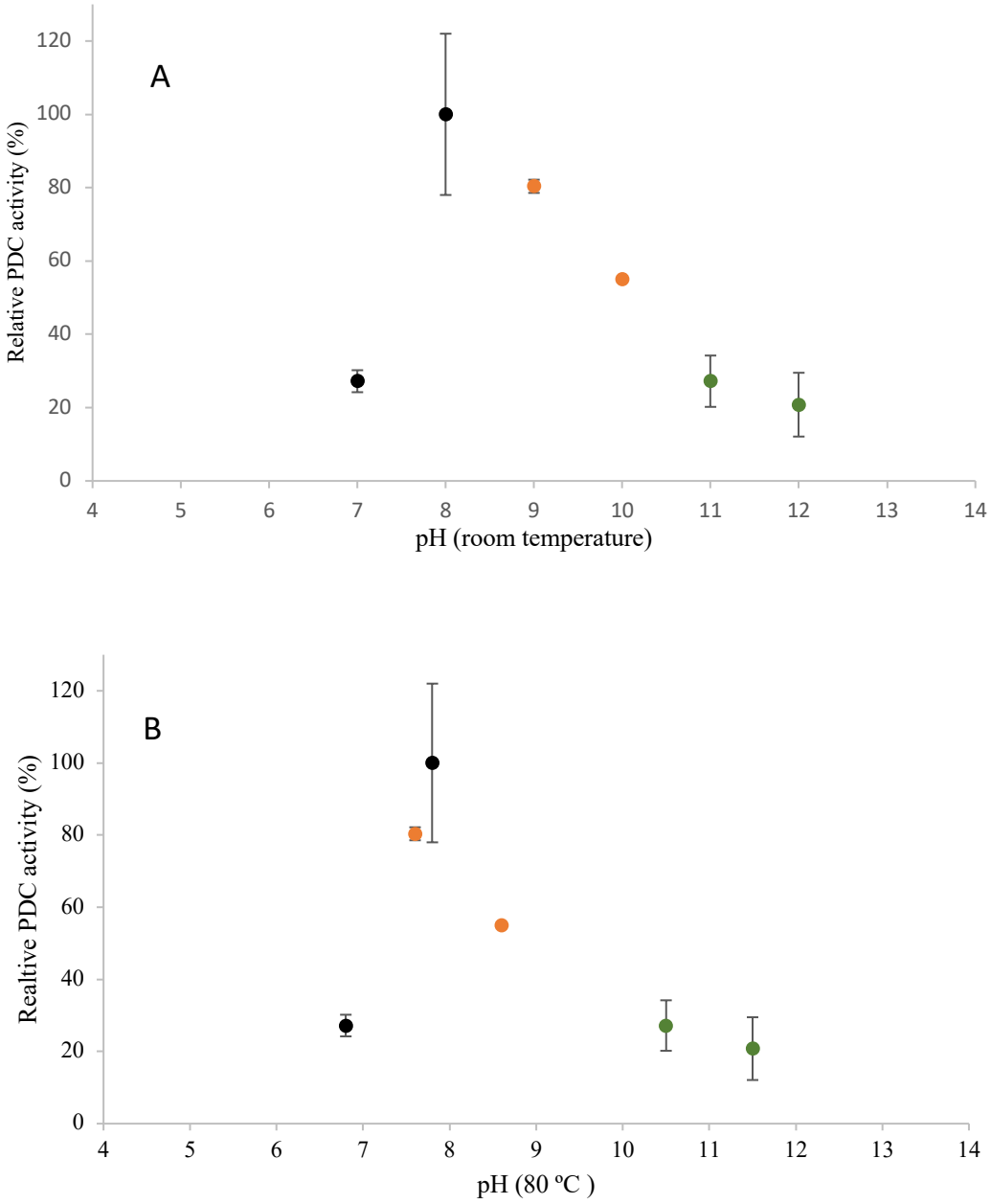

Figure 8: pH dependency of PDC activity of *S. solfataricus.* PDC activity was determined using 10 mM pyruvate, 100 µM CoA and 25 µg purified SsPDC at 80ºC. At room temperature (A), the following buffers were used: sodium phosphate (•) (pH 7.0, and 8.0), glycine (•) (pH 9.0, and 10), and CAPS buffer (•) (pH 11.0, and 12.0). At 80 ºC (B), the pH values of the following buffers were estimated based on the pKa of each buffer at 80 ºC: sodium phosphate (•) (pH 6.9, and 7.9), glycine (•) (pH 7.6, and 8.6), and CAPS (•) (pH 10.5, and 11.5). The relative activities of 100% equals to the highest specific activities (0.16 U/mg).

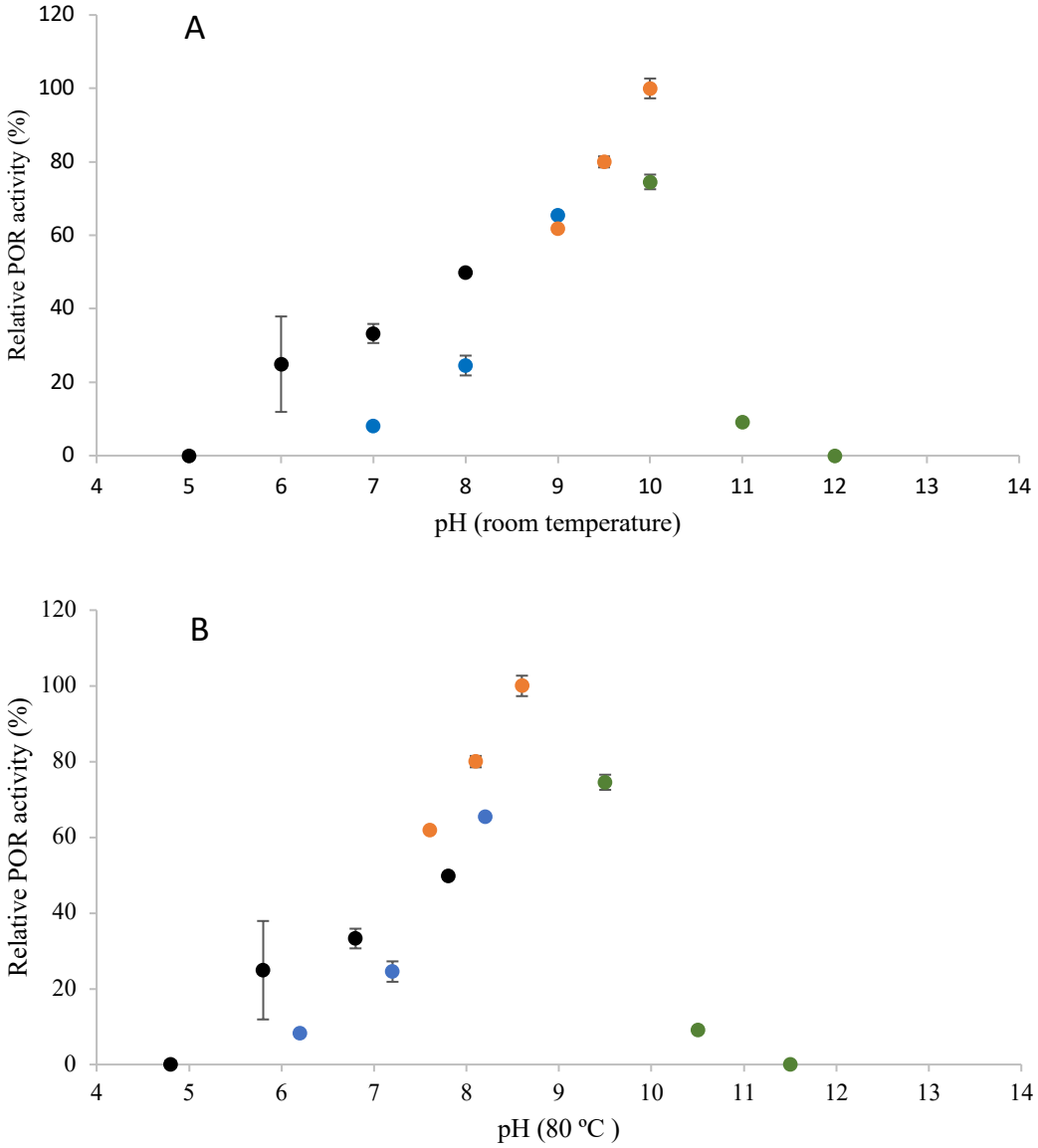

Figure 9: pH dependency of POR activity of the enzyme from *S. acidocaldarius.* POR activity was determined using 5 mM pyruvate, 1 mM methyl viologen, 100 µM CoA, approximately 50 µM SDT, and 12 µg purified SaPOR at 80ºC. At room temperature (A), the following buffers were used: sodium phosphate (•) (pH 5.0, 6.0, 7.0, and 8.0), EPPS (•) (pH 7.0, 8.0, and 9.0), glycine (•) (pH 9.0, 9.5 and 10), and CAPS (•) (pH10.0, 11.0, and 12.0). At 80 ºC (B), the pH values of the following buffers were estimated based on the p*Ka* of each buffer at 80 ºC: sodium phosphate (•) (pH 4.9, 5.9, 6.9, and 7.9), EPPS (•) (pH 6.2, 7.2, and 8.2), glycine (•) (pH 7.6, 8.1 and 8.6), and CAPS (•) (pH 9.5, 10.5, and 11.5). The relative activities of 100% equals to the highest specific activities (0.55 U/mg).

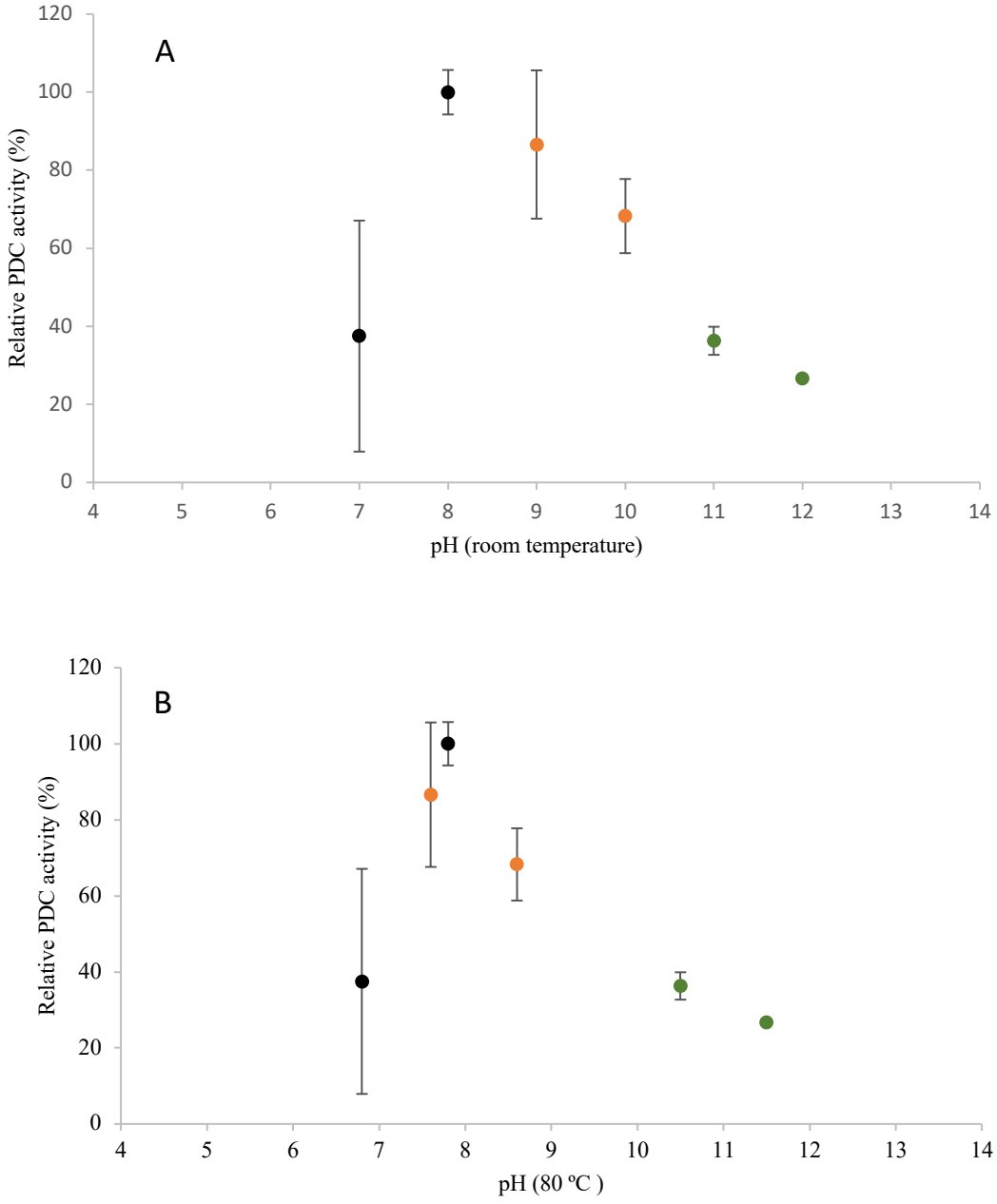

Figure 10: pH dependency of PDC activity of the enzyme from *S. acidocaldarius*. PDC activity was determined using 10 mM pyruvate, 100 µM CoA and 50 µg purified SaPDC at 80ºC. At room temperature (A), the following buffers were used: sodium phosphate (•) (pH 7.0, and 8.0), glycine (•) (pH 9.0, and 10), and CAPS buffer (•) (pH 11.0, and 12.0). At 80 ºC (B), the pH values of the following buffers were estimated based on the pKa of each buffer at 80 ºC: sodium phosphate (•) (pH 6.9, and 7.9), glycine (•) (pH 7.6, and 8.6), and CAPS (•) (pH 10.5, and 11.5). The relative activities of 100% equals to the highest specific activities (0.052 U/mg).

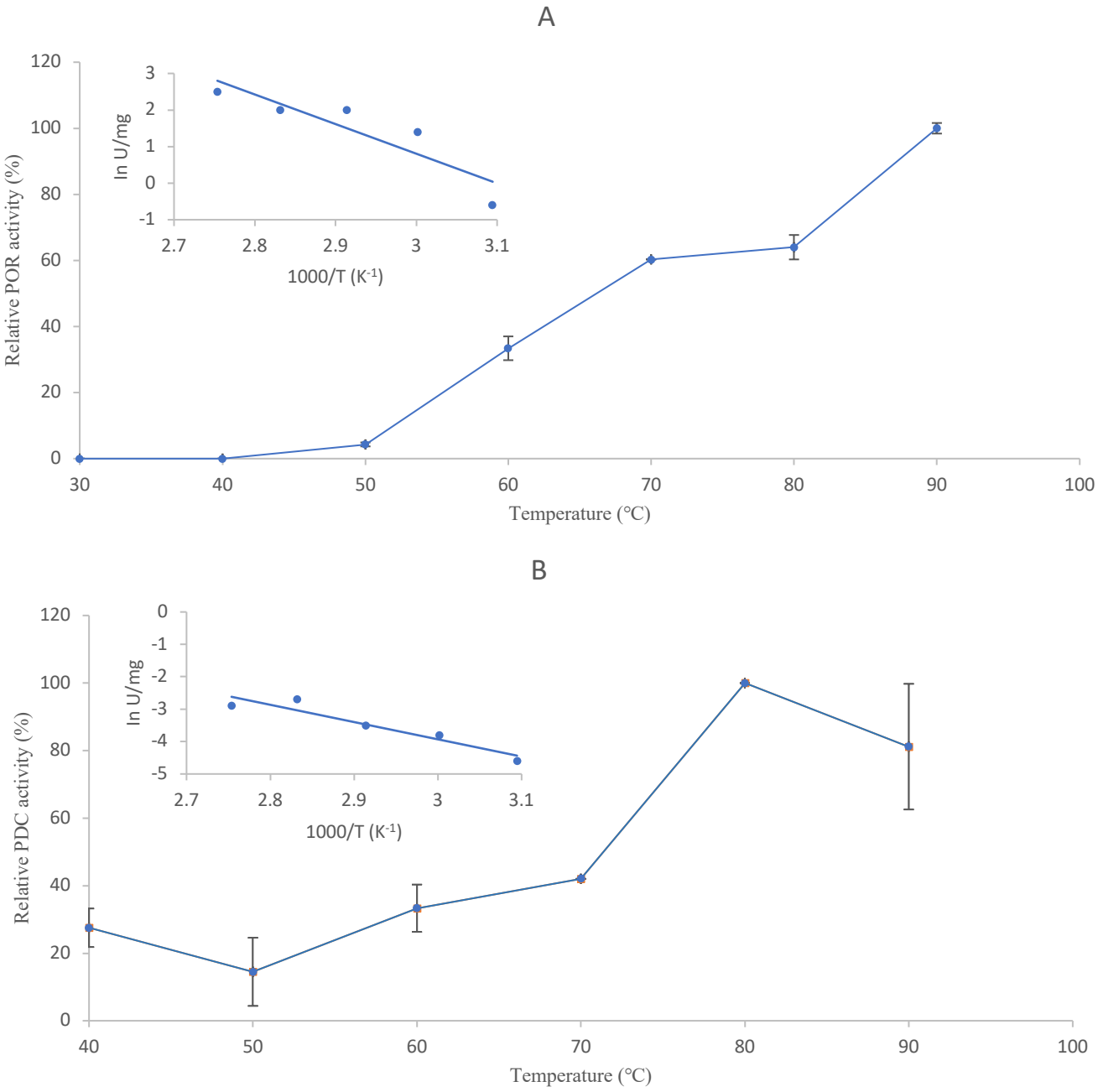

Figure 11: Temperature dependence of POR and PDC activities of the enzyme from *S. solfataricus*. Enzyme activity of POR (A) and PDC (B) were determined over a temperature range from 30 to 90 °C and 40 to 90 °C respectively. The insets show the Arrhenius plot based on the linear part of the plot A and plot B (from 50-90 ºC). Assay mixture of SsPOR contains 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate, 100 µM CoA, 1 mM benzyl viologen, approximately 50 µM SDT, and 3 µg purified SsPOR. SsPDC assay mixture was 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate, 100 µM CoA and 25 µg purified SsPDC. The relative activities of 100% equals to highest specific activities (12.1 U/mg and 0.069 U/mg for POR and PDC, respectively).

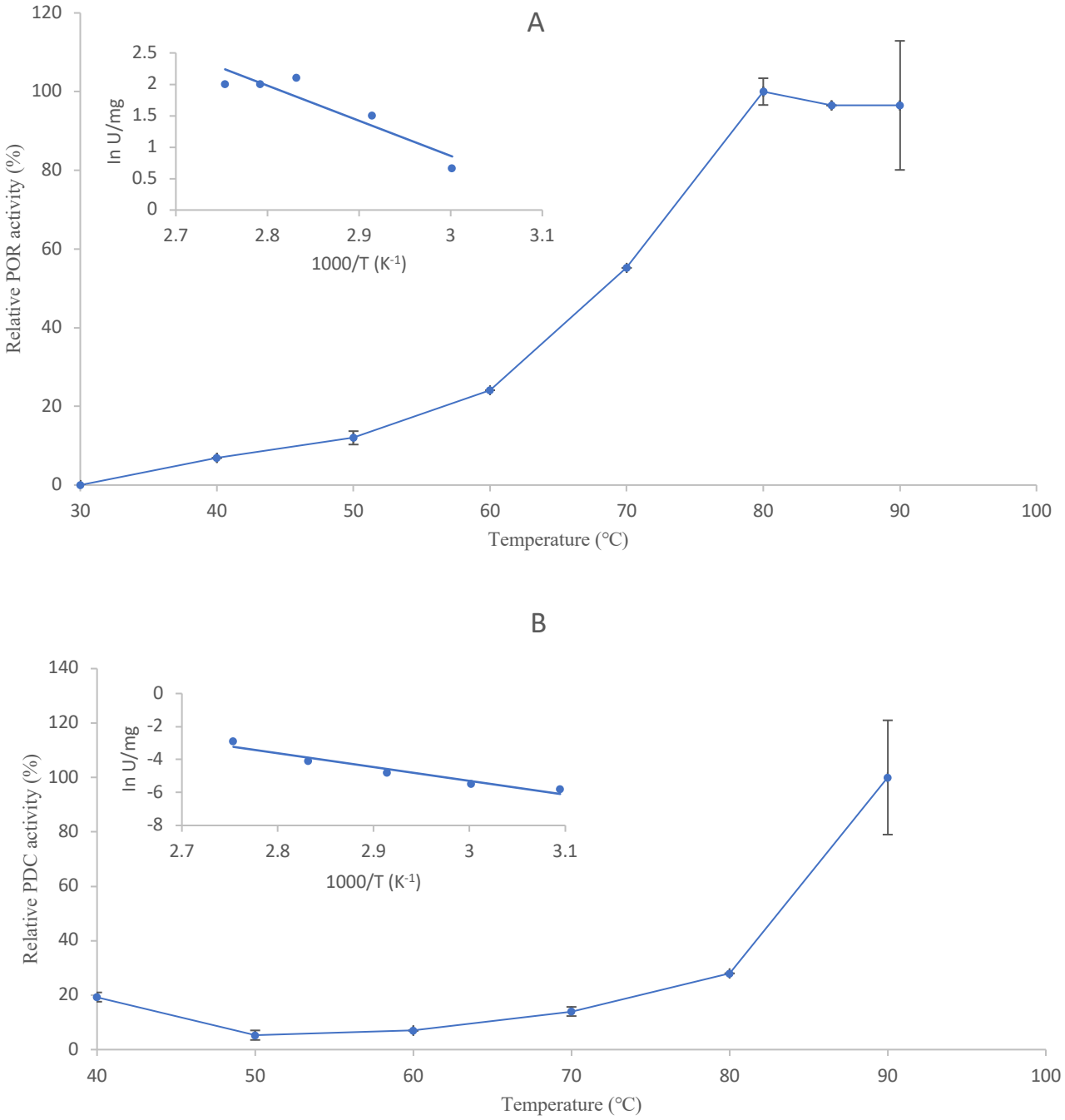

Figure 12: Temperature dependence of POR and PDC activities of the enzyme from *S. acidocaldarius.* Enzyme activity of POR (A) and PDC (B) were determined over a temperature range from 30 to 90 °C and 40 to 90 °C respectively. The insets show the Arrhenius plot based on the linear part of the plot A (from 60-90 ºC) and plot B (from 50-90 ºC). Assay mixture of SaPOR contains 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate, 100 µM CoA, 1 mM benzyl viologen, approximately 50 µM SDT, and 12 µg purified SaPOR. SaPDC assay mixture was 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate, 100 µM CoA and 50 µg purified SaPDC. The relative activities of 100% equals to highest specific activities (8 U/mg and 0.057 U/mg for POR and PDC, respectively).

Thermostability of PDC and POR enzymes were determined by measuring residual enzyme activities at different length of incubation time points. The time required for losing 50% of SsPOR activity ($t_{1/2}$) were found to be approximately 5.5 h at 70 °C and 2.9 h at 80 °C (**Fig. 13A**). The $t_{1/2}$ value for SaPOR activity was determined to be approximately 6.4 h at 70 °C and 1.1 h at 80 °C (**Fig. 13B**).

Both pyruvate and CoA dependence of POR and PDC activities of *S. solfataricus and S. acidocaldarius* were determined, and it was found that all activities were dependent on both pyruvate and CoA (**Fig. 14-17,** Table B1- B8). The enzyme kinetic parameters were calculated by fitting the Michaelis-Menten equation for pyruvate and CoA (Table 6). The apparent K_m values of SsPOR and SaPOR for pyruvate were 0.5 mM and 0.3 mM (Table 6) respectively, however, SsPOR and SaPOR showed apparent K_m values for CoA to be 10.7 µM and 21.5 µM respectively (Table 6). The enzyme kinetic parameters of SsPDC and SaPDC were also determined for pyruvate and CoA respectively. The apparent K_m values of SsPDC for pyruvate was 1.1 mM, while, the apparent K_m values of SaPDC for pyruvate was 0.9 mM (Table 6). Apparent K_m values of all enzymes for pyruvate were around 1 mM, while the apparent K_m values for CoA were much lower because the values for PORs were about 10.7 – 21.5 µM (Table 6). The apparent K_m values of SsPDC and SaPDC for CoA were not determined due to the testing concentrations of CoA used were probably much higher than the apparent K_m, resulting an atypical substrate-dependent curve of the Michaelis-Menten equation (**Fig. 15B** & **Fig. 17B**), from which a K_m value could not be estimated. However, the results showed that there were no PDC activity in the absence of CoA, and PDC activities were shown only in the presence of CoA, indicating both SsPDC and SaPDC were CoA dependent.

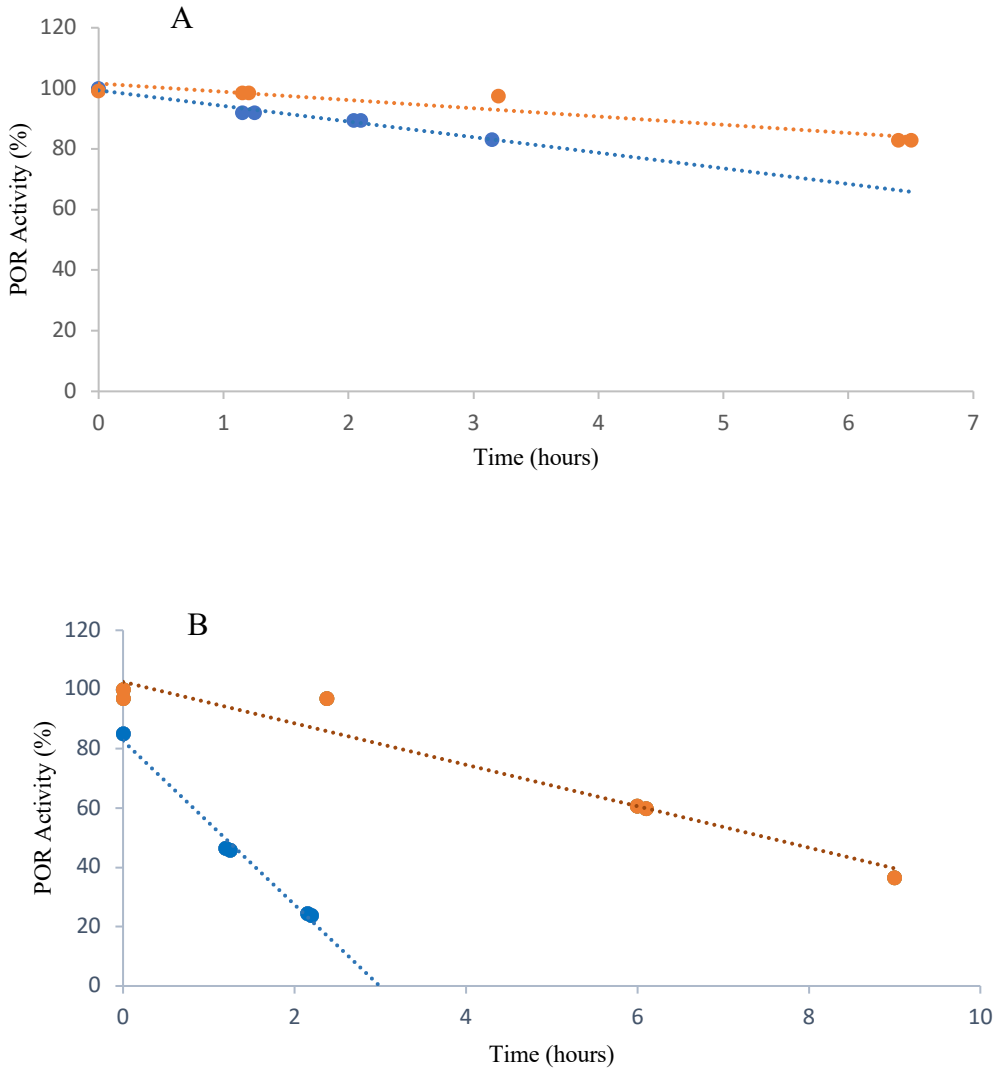

Figure 13: Thermostability of POR activity from *S. solfataricus* and *S. acidocaldarius*.

Temperature stability of POR enzymes was determined by the incubation of the enzymes at 70 °C (•) and 80 °C (•) respectively, and the enzyme activities were monitored at different time intervals. Assay mixture of PORs contain 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate, 100 µM CoA, 1 mM benzyl viologen, approximately 50 µM SDT, and 3 µg SsPOR or 12 µg SaPOR. The relative activities of 100% equals to 2 U/mg for SsPOR and 1.4 U/mg for SaPOR.

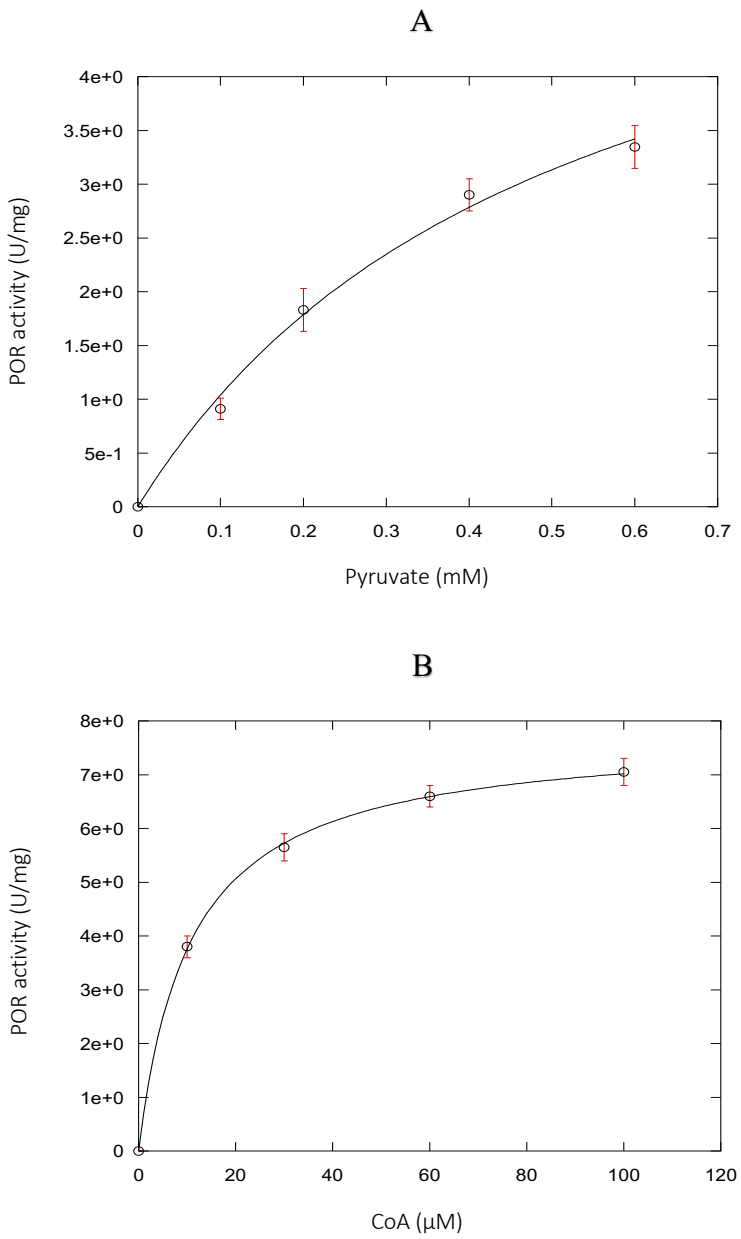

Figure 14: **Pyruvate and CoA dependency of POR from *S. solfataricus.*** Pyruvate (A, 0.0 to 0.6 mM in the presence of 100 mM sodium phosphate pH 8.0 (measured at room temperature), 100 µM CoA, 1 mM benzyl viologen, approximately 50 µM SDT and 3 µg purified SsPOR) and CoA (B, 0.0 to 100 µM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 5 mM pyruvate, 1 mM benzyl viologen, approximately 50 µM SDT and 3 µg purified SsPOR) dependent POR activities. Assays were performed at 80 ºC.

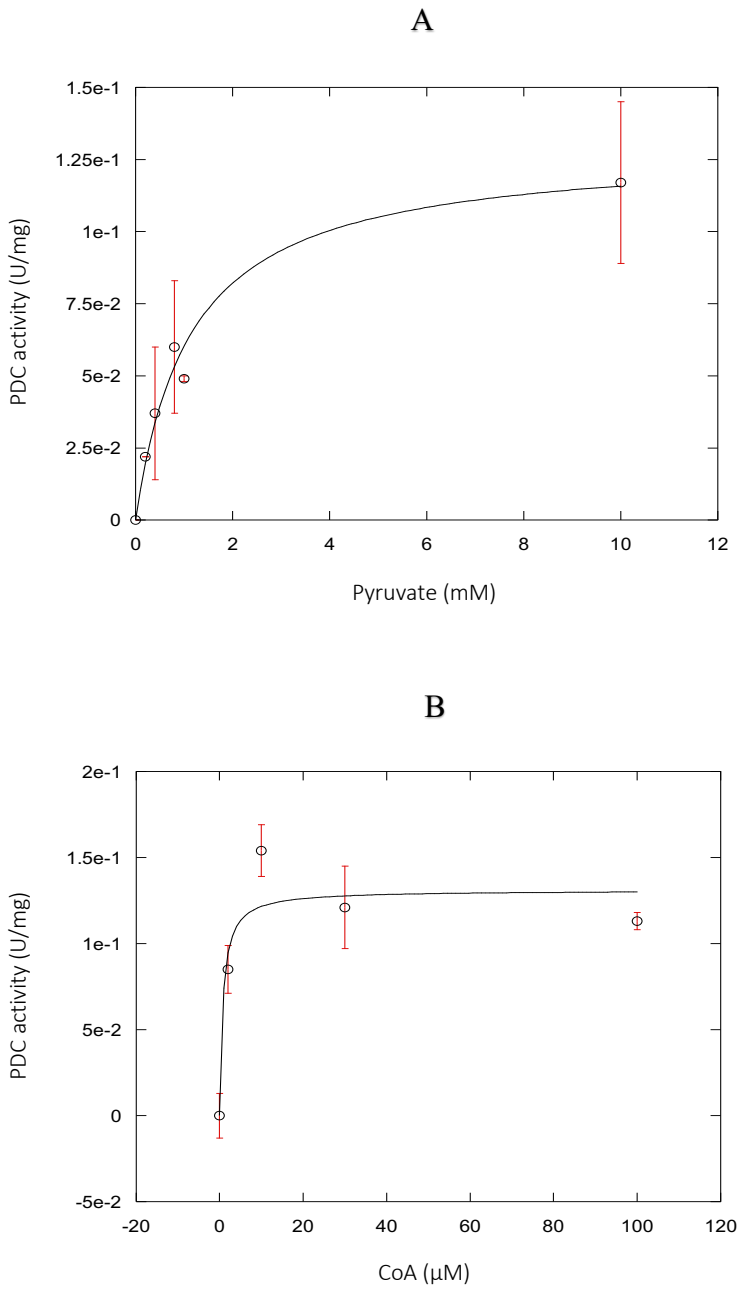

Figure 15: Pyruvate and CoA dependency of PDC from *S. solfataricus.* Pyruvate (A, 0.0 to 10 mM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 100 µM CoA and 25 µg purified SsPDC) and CoA (B, 0.0 to 100 µM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 10 mM pyruvate and 25 µg purified SsPDC) dependent PDC activities. Assays were performed at 80 ºC.

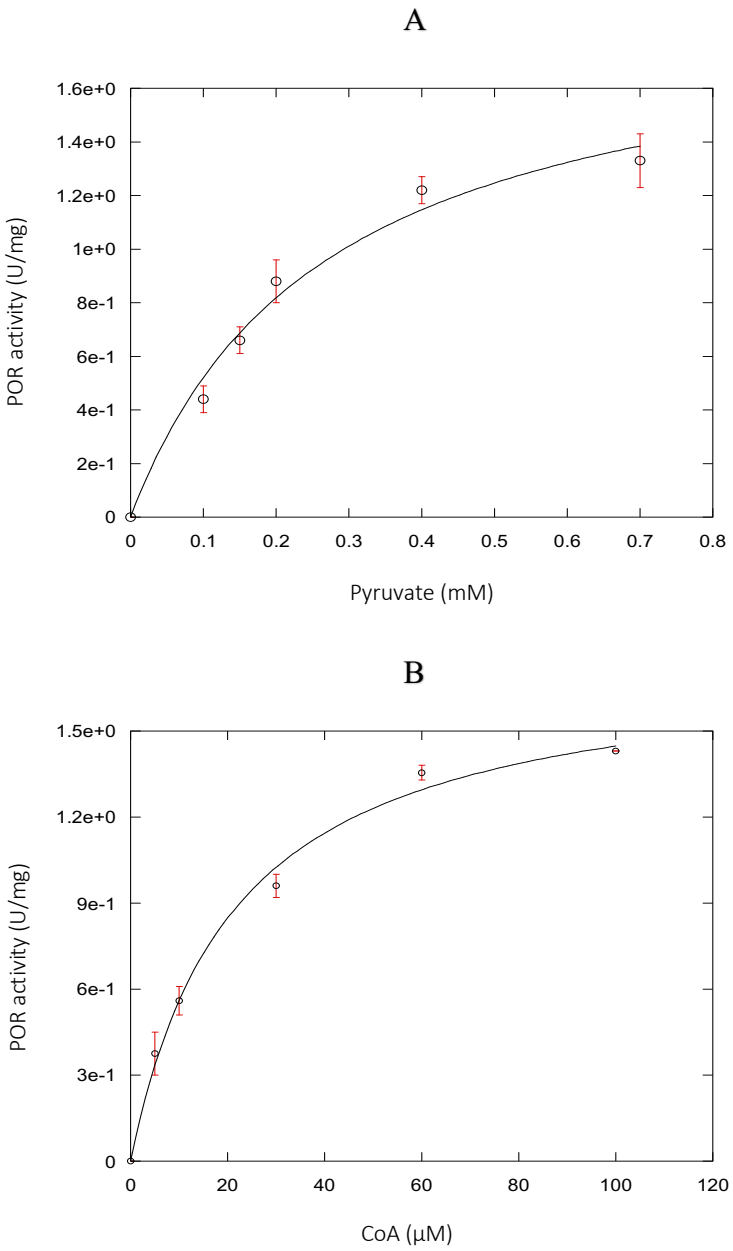

Figure 16: Pyruvate and CoA dependency of POR from *S. acidocaldarius.* Pyruvate (A, 0.0 to 0.7 mM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 100 µM CoA, 1 mM benzyl viologen, approximately 50 µM SDT and 12 µg purified SaPOR) and CoA (B, 0.0 to 100 µM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 5 mM pyruvate, 1 mM benzyl viologen, approximately 50 µM SDT and 12 µg purified SaPOR) dependent POR activities. Assays were performed at 80 ºC.

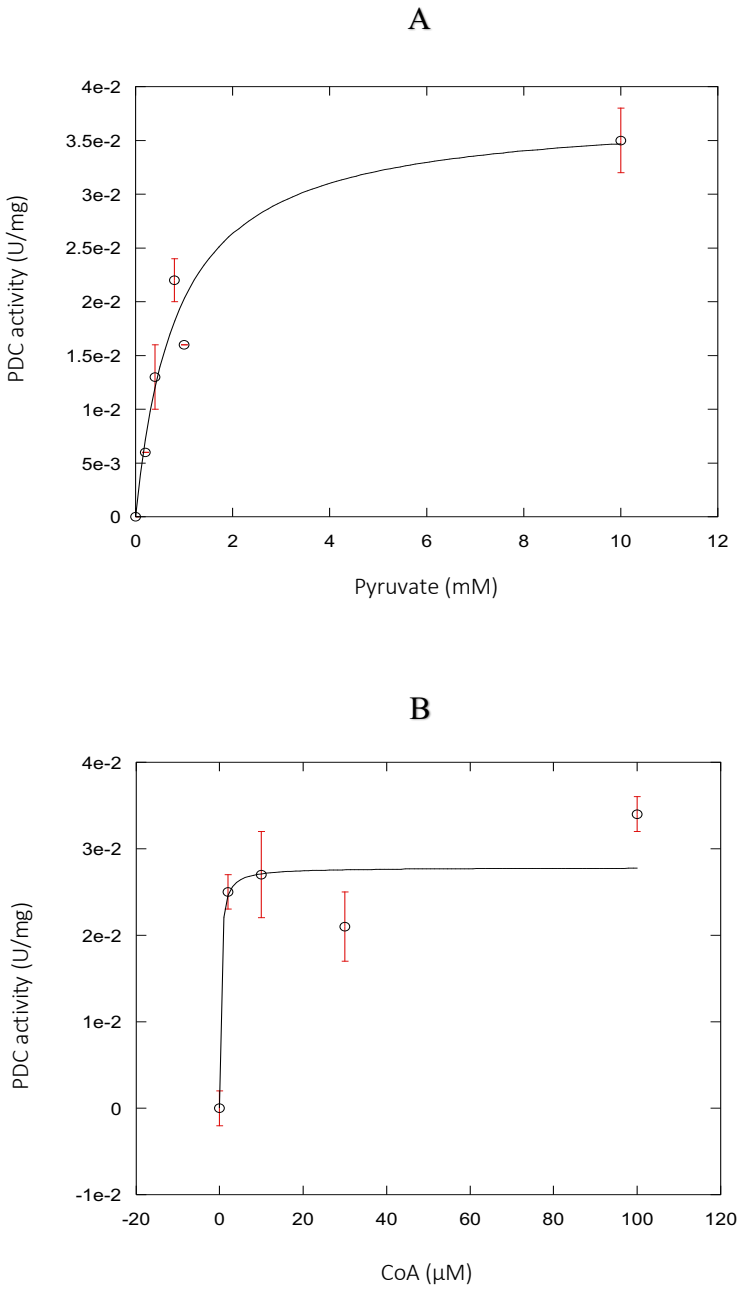

Figure 17: Pyruvate and CoA dependency of PDC from *S. acidocaldarius.* Pyruvate (A, 0.0 to 10 mM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 100 µM CoA and 50 µg purified SaPDC) and CoA (B, 0.0 to 100 µM in the presence of 100 mM sodium phosphate pH 8.0 [measured at room temperature], 10 mM pyruvate and 50 µg purified SaPDC) dependent PDC activities. Assays were performed at 80 ºC.

Table 6: Kinetic parameters of POR and PDC of *S. solfataricus* and *S. acidocaldarius*

Enzyme sources	Enzyme activity	[a]Pyruvate		[b]CoA	
		K_m (mM)	V_{max} (U/mg^{-1})	K_m (µM)	V_{max} (U/mg^{-1})
S. solfataricus	POR	0.5±0.1	6.3±0.7	10.7±0.4	7.7±0.07
	PDC	1.1±0.2	0.12±0.09	nd	nd
S. acidocaldarius	POR	0.3±0.05	1.9±0.2	21.5±3	1.7±0.08
	PDC	0.86±0.2	0.04±0.03	nd	nd

[a], POR activity was measured using 0.1 mM CoA, 1 mM BV, 50 µM SDT, 3 µg protein for SsPOR and 12 µg protein for SaPOR; and for PDC 0.1 mM CoA, 25 µg protein for SsPDC and 50 µg protein for SaPDC.
[b], POR activity was measured using 5 mM pyruvate, 1 mM BV, 50 µM SDT, 3 µg protein for SsPOR and 12 µg protein for SaPOR; and for PDC 10 mM pyruvate, 25 µg protein for SsPDC and 50 µg protein for SaPDC.
nd, not determined

Chapter 4 Discussion and Conclusions

4.1 Purification of the O_2-insensitive PDCs/PORs

POR and PDC are key enzymes for production of ethanol from pyruvate using a two-step pathway and a three-step pathway, respectively. POR has the ability to catalyze oxidative decarboxylation of pyruvate to acetyl-CoA which have been found in many organisms including hyperthermophiles. However, there is no conventional PDC has been found in hyperthermophiles. Bifunctional PDCs/PORs were discovered in several hyperthermophilic microorganisms which had the ability to catalyze both oxidative and non-oxidative decarboxylation of pyruvate. The serious challenge of the PDCs/PORs from hyperthermophiles was the oxygen sensitivity and CoA dependence of both PORs and PDCs (Ma *et al.,* 1997; Eram *et al.,* 2015). PORs from *Sulfolobus* species were found to be oxygen resistant and the bifunctional PDC/POR from *S. tokodaii* were reported to not be oxygen sensitive for both POR and PDC activities (Park *et al.,* 2005; Yan *et al.,* 2014). PORs from *Sulfolobus* showed a broad specificity for pyruvate, 2-oxoglutarate and 2-oxobutyrate (Park *et al.,* 2005; Yan *et al.,* 2014). Mutational analysis of POR from *S. tokodaii* provided valuable information about the residues that responsible for the broad 2-oxoacid specificity (Yan *et al.,* 2016).

The recombinant *S. tokodaii* POR was found to be similar to the native POR including enzyme activity and oxygen insensitivity (Fukuda *et al.,* 2001). Furthermore, the existence of only one [4Fe-4S] cluster in *Sulfolobus* PORs was advantageous to construct mutant PORs from *S. tokodaii* lacking the [4Fe-4S] cluster (Yan *et al.,* 2014). These mutants lost most POR activity compared to the recombinant POR, however, the recombinant and mutants showed similar PDC activity (approximately 0.07 U/mg), indicating that the [4Fe-4S] cluster is responsible for the oxidative

decarboxylation of pyruvate and does not affect the non-oxidative decarboxylation of pyruvate (Yan *et al.,* 2014).

The purification of PDCs/PORs from *S. solfataricus* and *S. acidocaldarius* was carried out successfully, showing that both are heterodimeric enzymes, which are in agreement with the reports of such enzymes from *S. solfataricus*, and *S. tokodaii* (Zhang *et al.,* 1996; Park *et al.,* 2005; Yan *et al.,* 2014). *S. solfataricus* and *S. acidocaldarius* POR activities in CFEs were found to be 0.18±0.01 U/mg and 0.10±0.01 U/mg, respectively, while their PDCs activities were 0.0027±0.0003 U/mg and 0.0011±0.0004 U/mg, respectively (Table 4 & 5). The purification achieved 41.6 fold for SsPOR and 70 fold increases for SaPOR via column purification using DEAE, HAP, and PS columns respectively, which were similar to those of SsPDC (40.7-fold, Table 4) and SaPDC (50-fold, Table 5). SaPDC (0.055±0.003) showed a less activity compared to SsPDC (0.11±0.004), but SsPDC is more than 50% higher activity than *S. tokodaii* PDC (Yan *et al.,* 2014). Although the purities of the purified enzymes reached approximately 90% for SsPDC/POR and 80% for SaPDC/POR, respectively, there was no indication of any interference to their characterization except their actual specific activities might be at least 10% higher than measured.

The determination of PDC and POR activities of the purified enzymes has proved that *S. solfataricus* and *S. acidocaldarius* have bifunctional PDC/POR enzymes similar to other hyperthermophiles. PDCs/PORs from both *S. solfataricus*, and *S. acidocaldarius* were not oxygen sensitive, a characteristic of *Sulfolobus* PORs which make these enzymes easy to handle and appropriate for further studies (Park *et al.,* 2005; Yan *et al*., 2014).

4.2 Optimal pH and temperature of POR and PDC activities

It was previously reported that PORs of *S. tokodaii* and *H. salinarium* showed similar optimal pH 8.5 and 9.0 pH respectively (Fukuda *et al.,* 2001; Kerscher and Oesterhelt 1981). PORs from *H. salinarium* and *Sulfolobus* are determined to be heterodimeric and oxygen insensitive enzymes which are similar to PORs from *S. solfataricus*, and *S. acidocaldarius* (Zhang *et al.,* 1996). POR from *S. solfataricus* was previously reported to have an optimum pH at 7.0-8.0 using different assay conditions (Park *et al.,* 2005). The difference between temperatures of preparing the buffers (25 ºC) and performing the assays (80 ºC) may cause a change in actual pH values due to the temperature-dependent change of pKa values. Hence, the temperature-dependent ΔpKa change of each buffer at high temperatures was taken into account when the optimal pH of PORs and PDCs from both *S. solfataricus*, and *S. acidocaldarius* were estimated. Although both SsPOR and SaPOR showed highest activity at pH 10 (measured at room temperature) using glycine buffer, the pK_a of glycine is temperature dependent (ΔpK_a/ºC = −0.025), which means the pH of glycine buffer at 80 ºC would be decreased by ~1.4 pH units. As a result, the optimal pH for PORs from *S. solfataricus*, and *S. acidocaldarius* were estimated to be 8.6 (**Fig. 7B** & **9B**), which is closer to that (pH 8.5) of PORs from *S. tokodaii*, *T. maritime,* and *T. hypogea* (Fukuda *et al.,* 2001; Eram *et al.,* 2015).

The optimum pH of PDCs/PORs from hyperthermophiles were previously reported to be higher than those (~pH 6) of PDCs from mesophilic organisms (Table 1), which is consistent with the results of SsPDC and SaPDC from this study. SsPDC and SaPDC showed optimal pH at 7.8 (**Fig. 8B** & **10B**, and sodium phosphate has a ΔpK_a/ºC of −0.0028), which is similar to the reported optimal pHs of some hyperthermophilic PDCs (Eram *et al.,* 2015). In most cases, the optimum pH

of PDCs from hyperthermophiles were reported to be equal or higher than their corresponding PORs from the same organisms (Ma *et al.,* 1997; Eram *et al.,* 2015); however, SsPDC and SaPDC have an optimum pH (7.8) that looks much lower than that (8.6) of their PORs (**Fig. 7-10**). This maybe a reflection of structural difference at their catalytic site, but exact mechanism for that is not known at this point. It is obvious that the optimum pH of hyperthermophilic PDCs were higher than the PDCs from mesophilic bacteria and fungi which prefer slightly acidic environments (Table 1), but so far, the optimal pH of SsPDC and SaPDC is found to be the lowest among hyperthermophilic PDCs (Ma *et al.,* 1997; Eram *et al.,* 2015).

The maximum activity of SsPOR were measured to be at 90 °C (highest testing temperature, **Fig. 11A**), which is similar to PORs from *P. furiosus, T. maritima,* and *T. guaymasensis*; however, SaPOR showed optimal temperature at 80 °C (**Fig. 12A**). There was no enzyme assay performed at temperatures higher than 90 ºC because of technical limitations. SsPOR in previous study displayed the highest activity at 70 °C, but no assay was performed at temperatures higher than 70 °C (Park *et al.,* 2005). POR from *S. tokodaii* showed an optimal temperature at 90 ºC (Fukuda *et al.,* 2001). However, optimal temperature for SsPDC was determined to be 80°C and decreased by ~20% at 90 °C (**Fig. 11B**), which is similar to PDCs from *T. hypogea* and *T. guaymasensis* (Eram *et al.,* 2015). It would be expected that such a thermoactivity of both SsPOR and SsPDC would be the same, so, such a difference might be caused by a much longer incubation time of PDC assay (~2 h) compared to that of POR (less than a min), during which (90°C) part of the enzyme might be inactivated by partially thermal denaturation. Unexpectedly, SaPDC activity was increased continually until 90 °C (**Fig. 12B**), which is similar to PDCs from *P. furiosus,* and *T. maritima* (Ma *et al.,* 1997; Eram *et al.,* 2015). However, SaPOR showed an optimal temperature at 80°C

that is lower than that of SaPDC, despite the POR assay time (less than a min) is much shorter than that of PDC (~2 h), which can not be explained by a partially thermal inactivation of POR activity. It might be possible that there were inconsistent measurement of the SaPDC, especially at 80 and 90°C (**Fig. 12B**) because of that an approximately 4 times difference in activity between 80 and 90 °C would not be anticipated. An approximately two-fold increase in activity would be possible when temperature rises 10 °C for an enzymatic catalyzed reaction. Therefore, such an abnormality requires a further investigation.

Comparing activation energy of corresponding PDC and POR from hyperthermophiles, it appears that activation energy of PDC is higher than that of POR. The activation energy of *Thermotoga* PDC is approximately double of its POR (Eram *et al.,* 2015). The activation energy of *Sulfolubus* PDCs is approximately 40-50% higher than that of their PORs (**Fig. 11**, 44 vs. 33.2 kJ/mol; **Fig. 12**, 70 vs. 47 kJ/mol). The activation energy values of conventional PDCs from mesophilic bacteria *Gluconacetobacter diazotrophicus* and *Zymobacter palmae* were determined to be 46 kJ/mol and 41 kJ/mol, respectively (Gocke, 2007; Van Zyl *et al.,* 2014), which is similar to that of SsPDC (44 kJ/mol). The optimal temperatures of PDCs from *G. diazotrophicus* and *Z. palmae* were determined to be 50 and 55 ºC respectively (Gocke, 2007; Van Zyl *et al.,* 2014). The activation energy of *Acetobacter pasteurianus* PDC was found to be significantly lower (27.1 kJ/mol with optimal temperature of 65 ºC) comparing to other PDCs (Gocke, 2007). In general, the activation energy of conventional PDCs appears to be lower than that of PDCs from hyperthermophiles.

Thermostability of *S. solfataricus* was determined under anaerobic conditions with a half-live of ~175 min at 80 ºC (**Fig. 13A**), which is similar to that from *T. hypogea* (Table 2). *S. acidocaldarius* enzyme was thermostable with a half-live of ~ 65 min at 80 ºC (**Fig. 13B**). POR from *P. furiosus* was found to be the lowest thermostable POR among hyperthermophiles with a half-live of 18 min at 80 ºC, however, the most thermostable POR was determined to be *T. maritima* POR with a half-live of 11 h at 80 ºC (Table 2).

4.3 Pyruvate and CoA dependence of POR and PDC activities

The bifunctional SsPDC/POR and SaPDC/POR had the ability to catalyze the oxidative decarboxylation and non-oxidative decarboxylation of pyruvate in the presence of CoA. Although there was no activity observed when the CoA was omitted from both assays, SsPOR had much lower K_m value for CoA (Table 6) than previously reported POR (Table 2). For steady-state kinetics study, enzyme concentration in the assay should be approximately the same as the concentration of enzyme-substrate complex, which should be determined by using an enzyme concentration-dependent specific activity. Since such a determination was not performed, an assumption was made so that the enzyme concentrations used in all assays would be within the linear range for achieving the same specific activity. This might be plausibly supported by the fact that both SsPDC/POR and SaPDC/POR activities from each indivisual assay were not too high, so, enzyme amount used in each assay would be within the linear range to show the same specific activity under the same assay conditions. Further experimental determination is needed.

The apparent K_m values for pyruvate of SsPOR and SaPOR were similar to PORs from other hyperthermophiles (Table 2 & Table 6). Although the K_m value for pyruvate of SsPOR was previously reported to be 0.27 mM (Park *et al*., 2005), in this study, it was found that the apparent K_m for pyruvate is 0.5 mM, which are similar, and closer to the apparent K_m for pyruvate of POR from *S. tokodaii* that was previously determined to be 0.32 mM (Yan *et al.* 2016).

SsPOR and SaPOR are found to be CoA dependent which is similar to PORs from other *Sulfolobus*. SsPOR showed lower apparent K_m value for CoA (10 μM) than other characterized

hyperthermophilic PORs (Table 2), however, the apparent K_m value for CoA of SaPOR (18.2 μM) was similar to that of the hyperthermophilic archaea *S. tokodaii* (17 μM) and hyperthermophilic bacterium *T. hypogea* (21 μM). The apparent K_m value for pyruvate of SsPOR (0.5 mM) and SaPOR (0.1 mM) were slightly lower than that of SsPDC (1.1 mM) and SaPDC (0.9 mM), which may indicate that under physiological conditions, POR is possibly the dominant catalytic activity, which maybe part of the reason that only lower concentrations of alcohols are produced by hyperthermophiles.

Although it was concluded that PDC from *S. tokodaii* was not CoA dependent measured by using different method and conditions, their results showed that the addition of CoA in the assay mixture enhanced the PDC activity by approximately 20% (Yan *et al.* 2014), indicating the CoA might still play an important role in the catalysis of its PDC activity. The results showed that the detected value at zero concentration of CoA was approximately 0.1 mM acetaldehyde for both SsPDC and SaPDC although these numbers are falling within the range of the interference peaks that the HPLC produced (Table B5 & Table B7). However, the addition of only small concentration of CoA (10 μM) showed a significant increase (maximum activity) in the enzyme's activities for both SsPDC and SaPDC (**Fig. 15B** & **Fig. 17B)**, indicating the requirement of CoA for achieving its highest PDC activity. The apparent K_m values for CoA have not been determined for SsPDC and for SaPDC because of incomplete data points that were unable to support a typical substrate-dependent Michaelis-Menten kinetics curve, however, the results proved that both PDCs were CoA dependent, which is different from PDC of *S. tokodaii* (Yan *et al.* 2016). It is likely that their K_m values for CoA maybe below 2 μM. To accurately determine the apparent K_m values for

CoA, smaller concentrations of CoA (a few concentrations lower than 2 μM) should be used for measuring CoA dependent activities of PDCs from both *S. solfataricus* and *S. acidocaldarius.*

The activities of both SsPDC and SaPDC were about 2-3% of the rate of the corresponding PORs, which are similar to enzymes from hyperthermophilic archaeal *S. tokodaii,* hyperthermophilic bacteria *T. hypogea* and *T. maritima* (Yan *et al.* 2014; Eram *et al* 2015). The activity of PDCs from *Sulfolobus* species were found to be lower than the PDCs from other hyperthermophiles which could be caused by the low activity of *Sulfolobus* PORs. Although the ratio of POR to PDC was similar, SsPOR and SaPOR activities (7.5 U/mg and 7 U/mg respectively) were much higher than the wild-type PORs (3.6 U/mg) from *S. tokodaii* (Yan *et al.* 2014). In addition, SsPDC showed more than 50 % higher activity (Table 4 & Table 5) than PDC from other *Sulfolobus* species (Yan *et al.* 2014). Such a CoA-dependent PDC activity is similar to other hyperthermophilic PDCs/PORs, indicating a structural role of CoA in the catalysis of the non-oxidative decarboxylation of pyruvate (Ma *et al.* 1997).

4.4 Conclusions

One of the challenges of studying the catalytic mechanisms of hyperthermophilic PDCs was the oxygen sensitivity of the enzymes. In contrast, the purification and characterization of PDCs/PORs from *S. solfataricus* and *S. acidocaldarius* proved that they are bifunctional, thermostable and oxygen insensitive enzymes. Also, it was anticipated that SsPDC and SaPDC would not be CoA dependent, similar to PDC from *S. tokodaii*, however SsPDC and SaPDC were shown to be CoA dependent.

The characterizations of SsPDC and SaPDC were accomplished for the first time including the kinetic parameters, optimum pH and optimum temperature. The optimal pH values for both PDCs and PORs from *S. solfataricus* and *S. acidocaldarius* were determined to be pH 7.8 and pH 8.6, respectively. The optimal temperatures for PDC/POR from *S. solfataricus* were found to be >90°C and 80°C, respectively; while, the optimal temperatures for PDC/POR from *S. acidocaldarius* were determined to be 80°C and >90°C, respectively. Although the great advantages of PDC/PORs from *Sulfolobus* species, the specific activity of PDCs from *Sulfolobus* species were found to be lower than the PDCs from other hyperthermophiles which might be caused by the lower PORs activities from *Sulfolobus*. These results provided further insights into investigating catalytic mechanisms of hyperthermophilic PDCs which would be valuable in developing a highly efficient bioethanol production system. The oxygen resistant PDCs from *Sulfolobus* have several features that may help overcome the obstacles of bioethanol production at industrial scales including the fermentation efficiency and the production costs.

4.5 Prospects for future research

The experiments of CoA dependence for PDCs were incomplete. The experiments should be re-designed using lower concentrations of CoA. Since, the recombinant *S. tokodaii* POR was found to be similar to the natural POR, it is anticipated that those features could be applicable for PORs from *S. solfataricus* and *S. acidocaldarius.* As a result, cloning and expressing oxygen insensitive PDCs/PORs from *S. solfataricus* and *S. acidocaldarius* in mesophilic host will help to study the catalytic mechanism and determine the amino acid residues that are responsible for PDC activities. It may be possible to further enhance the SsPDC and SaPDC specific activities using mutagenesis, providing a possibility for developing a more efficient system for bioethanol fermentation at high temperatures.

References

Abdel-Banat, B., Hoshida, H., Ano, A., Nonklang, S., & Akada, R. (2010). High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Applied Microbiology and Biotechnology, 85*(4), 861-867. doi:10.1007/s00253-009-2248-5

Albers, E., Johansson, E., Franzén, C., & Larsson, C. (2011). Selective suppression of bacterial contaminants by process conditions during lignocellulose based yeast fermentations. *Biotechnology for Biofuels, 4*(1), 59.

Atomi, H., Sato, T., & Kanai, T. (2011). Application of hyperthermophiles and their enzymes. *Current Opinion in Biotechnology, 22*(5), 618-626. doi:10.1016/j.copbio.2011.06.010

Bai, F. W., Anderson, W. A., & Moo-Young, M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances, 26*(1), 89-105. doi: http://dx.doi.org/10.1016/j.biotechadv.2007.09.002

Baross, A. J., & Holden, F. J. (1996). Overview of hyperthermophiles and their heat-shock proteins. *Advances in Protein Chemistry* 48: 1-34.

Blamey, J. M., & Adams, M. W.W. (1994). Characterization of an ancestral type of pyruvate ferredoxin oxidoreductase from the hyperthermophilic bacterium, *Thermotoga maritima. Biochemistry, 33*(4), 1000.

Blamey, J. M., & Adams, M. W. W. (1993). Purification and characterization of pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochimica Et Biophysical Acta (BBA)/Protein Structure and Molecular Enzymology, 1161*(1), 19-27. doi:10.1016/0167-4838(93)90190-3

Bock, A., Kunow, J., Glasemacher, J., & Schönheit, P. (1996). Catalytic properties, polecular composition and sequence alignments of pyruvate: ferredoxin oxidoreductase from the methanogenic archaeon *Methanosarcina Barkeri* (Strain Fusaro). *European Journal of Biochemistry, 237*(1), 35-44. doi:10.1111/j.1432-1033.1996.0035n.x

Bothast, R., & Schlicher, J. (2005). Biotechnological processes for conversion of corn into ethanol. *Applied Microbiology and Biotechnology, 67*(1), 19-25.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72(1): 248-254.

Brock, T., Brock, K., Belly, R., & Weiss, R. (1972). *Sulfolobus*: A new genus of sulfur- oxidizing bacteria living at low pH and high temperature. *Archiv Für Mikrobiologie, 84*(1), 54-68. doi:10.1007/BF00408082

Bruchhaus, I. and E. Tannich (1994) Purification and molecular characterization of the NAD(+)-dependent acetaldehyde/alcohol dehydrogenase from *Entamoeba histolytica*. Biochem. J. 303, 743-748.

Burdette, D. and J. G. Zeikus (1994) Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* 39E and characterization of the secondary-alcohol dehydrogenase (2 degrees Adh) as a bifunctional alcohol dehydrogenase--acetyl-CoA reductive thioesterase. *Biochemical journal*. 302, 163-0.

Candy, J. M., & Duggleby, R. G. (1998). Structure and properties of pyruvate decarboxylase and site- directed mutagenesis of the *Zymomonas mobilis* enzyme. *Biochimica Et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology, 1385*(2), 323-338. doi:10.1016/S0167-4838(98)00077-6

Chabriere, E., Cavazza, C., Contreras-Martel, C., & Fontecilla-Camps, J. C. (2007). Pyruvate Ferredoxin Oxidoreductase. *Handbook of Metalloproteins*. John Wiley & Sons, Ltd. doi:10.1002/0470028637.met220

Chabriere, E., Cavazza, C., Contreras-Martel, C., & Fontecilla-Camps, J. C. (2011). Pyruvate Ferredoxin Oxidoreductase. *Encyclopedia of Inorganic and Bioinorganic Chemistry*. John Wiley & Sons, Ltd. doi:10.1002/9781119951438.eibc0647

Chen, L. M., Brugger, K., Skovgaard, M., Redder, P., She, Q. X., Torarinsson, E., Greve, B., Awayez., M., Zibat, A., Klenk, H, P., & Garrett, R, A. (2005). The genome of *Sulfolobus acidocaldarius,* a model organism of the Crenarchaeota. Journal of Bacteriology 187, 4992-4999.

Chen, P. Y. T., Aman, H., Can, M., Ragsdale, S. W., & Drennan, C. L. (2018). Binding site for coenzyme A revealed in the structure of pyruvate: ferredoxin oxidoreductase from *Moorella thermoacetica.* Proceedings of the National Academy of Sciences, 115(15), 3846-3851.

Costelloe, S., Ward, J., & Dalby, J. (2008). Evolutionary analysis of the TPP-dependent enzyme family. *Journal of Molecular Evolution, 66*(1), 36-49.

Dale, B. E., Anderson, J. E., Brown, R. C., Csonka, S., Dale, V. H., Herwick, G., ... & Lynd, L. R. (2014). Take a closer look: biofuels can support environmental, economic and social goals.

De Rosa, M., Gambacorta, A., Nicolaus, B., Giardina, P., Poerio, E., & Buonocore, V. (1984). Glucose metabolism in the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus. The Biochemical Journal, 224*(2), 407.

Dobritzsch, D., & Kö. (1998). High resolution crystal structure of pyruvate decarboxylase from *Zymomonas mobilis.* Implications for substrate activation in pyruvate decarboxylases. *The Journal of Biological Chemistry,273*(32), 20196.

Duggleby, R. G., McCourt, J. A., & Guddat, L. W. (2008). Structure and mechanism of inhibition of plant acetohydroxyacid synthase. *Plant Physiology and Biochemistry*, *46*(3), 309-324.

Egorova, K., & Antranikian, G. (2005). Industrial relevance of thermophilic Archaea. *Current Opinion in Microbiology, 8*(6), 649-655. doi:10.1016/j.mib.2005.10.015

Eram, M. S., & Ma, K. (2014). The bifunctional pyruvate decarboxylase/ pyruvate ferredoxin oxidoreductase from *Thermococcus guaymasensis. Archaea, 2014* doi:10.1155/2014/349379

Eram, M. S., & Ma, K. (2013). Decarboxylation of pyruvate to acetaldehyde for ethanol production by hyperthermophiles. *Biomolecules, 3*(3), 578-596. doi:10.3390/biom3030578

Eram, M. S., & Ma, K. (2016). Pyruvate decarboxylase activity of the acetohydroxyacid synthase of *Thermotoga maritima. Biochemistry and Biophysics Reports,* doi:http://dx.doi.org/10.1016/j.bbrep.2016.07.008

Eram, M., Wong, A., Oduaran, E., & Ma, K. (2015). Molecular and biochemical characterization of bifunctional pyruvate decarboxylases and pyruvate ferredoxin oxidoreductases from *Thermotoga maritima* and *Thermotoga hypogea*. *The Journal of Biochemistry, 158*(6), 459-466.

Fagernes, C. E., Stensløkken, K. O., Røhr, Å. K., Berenbrink, M., Ellefsen, S., & Nilsson, G. E. (2017). Extreme anoxia tolerance in crucian carp and goldfish through neofunctionalization of duplicated genes creating a new ethanol-producing pyruvate decarboxylase pathway. *Scientific reports*, *7*(1), 7884.

Fukuda, E., Kino, H., Matsuzawa, H., & Wakagi, T. (2001). Role of a highly conserved YPITP motif in 2-oxoacid: ferredoxin oxidoreductase. *European Journal of Biochemistry, 268*(21), 5639-5646. doi:10.1046/j.1432-1033.2001.02504.x

Green, M. R. & Sambrook, J. (2012). *Molecular cloning: a laboratory manual* (4th edition). Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press

Gocke, D. (2007). *New and optimised thiamine diphosphate (ThDP)-dependent enzymes for carboligation* (Doctoral dissertation, Heinrich-Heine-Universität Düsseldorf, Germany).

Gocke, D., T. Graf, H. Brosi, I. Frindi-Wosch, L. Walter, M. Müller and M. Pohl (2009) Comparative characterisation of thiamin diphosphate-dependent decarboxylases. *Journal of Molecular Catalysis B: Enzymatic*. 61, 30-35.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G., & Zacchi, G. (2006). Bio-ethanol--the fuel of tomorrow from the residues of today. *Trends in Biotechnology, 24*(12), 549.

Huber, H., & Stetter, K. O. (1998). Hyperthermophiles and their possible potential in biotechnology. *Journal of Biotechnology, 64*(1), 39-52. doi:10.1016/S0168-1656(98)00102-3

Iding, H., Siegert, P., Mesch, K., & Pohl, M. (1998). Application of α-keto acid decarboxylases in biotransformations. *Biochimica Et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology, 1385*(2), 307-322.

Iwasaki, Wakagi, & Oshima, T. (1995). Ferredoxin-dependent redox system of a thermoacidophilic archaeon, *Sulfolobus* sp. strain 7. Purification and characterization of a novel reduced ferredoxin-reoxidizing iron-sulfur flavoprotein. *The Journal of Biological Chemistry, 270*(30), 17878-83.

Iwasaki, T., & Oshima, T. (2001). [1] Ferredoxin and related enzymes from *Sulfolobus*. *Methods in Enzymology, 334*, 3-22. doi:http://dx.doi.org/10.1016/S0076-6879(01)34453-1

Joshua, C. J., Dahl, R., Benke, P. I., & Keasling, J. D. (2011). Absence of diauxie during simultaneous utilization of glucose and xylose by *Sulfolobus acidocaldarius*. *Journal of bacteriology*, *193*(6), 1293-1301.

Kelly, R., & Adams, M. (1994). Metabolism in hyperthermophilic microorganisms. *Antonie Van Leeuwenhoek, 66*(1), 247-270. doi:10.1007/BF00871643

Kerscher, L. and D. Oesterhelt (1981) Purification and properties of two 2 oxoacid: ferredoxin oxidoreductases from *Halobacterium halobium*. Eur. J. Biochem. 116, 587-594.

Kumar, A., Bhansali, S., Gupta, N., & Sharma, M. (2019). Bioenergy and Climate Change: Greenhouse Gas Mitigation. In *Prospects of Renewable Bioprocessing in Future Energy Systems* (pp. 269-289). Springer, Cham.

Kunow, J., D. Linder and R. K. Thauer (1995) Pyruvate: ferredoxin oxidoreductase from the sulfate reducing *Archaeoglobus fulgidus:* molecular composition, catalytic properties, and sequence alignments. Arch. Microbiol. V163, 21-28.

Lamble, H. J., Heyer, N. I., Bull, S. D., Hough, D. W., & Danson, M. J. (2003). Metabolic pathway promiscuity in the archaeon *Sulfolobus solfataricus* revealed by studies on glucose dehydrogenase and 2- keto- 3- deoxygluconate aldolase. *The Journal of Biological Chemistry, 278*(36), 34066.

Lin, Y., & Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology and Biotechnology, 69*(6), 627-642. doi:10.1007/s00253-005-0229-x

Littlechild, J. A. (2015). Archaeal enzymes and applications in industrial biocatalysts. Archaea 2015:e147671. doi:10.1155/2015/147671

Lo, J., Zheng, T., Hon, S., Olson, D. G., & Lynd, L. R. (2015). The bifunctional alcohol and aldehyde dehydrogenase gene, adhE, is necessary for ethanol production in *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum*. Journal of bacteriology, 197(8), 1386-1393.

Lobell, M., & Crout, D. H. G. (1996). Pyruvate decarboxylase: a molecular modeling study of pyruvate decarboxylation and acyloin formation. *Journal of the American Chemical Society, 118*(8), 1867.

Luan, G., Qi, Y., Wang, M., Li, Z., Duan, Y., Tan, X., & Lu, X. (2015). Combinatory strategy for characterizing and understanding the ethanol synthesis pathway in cyanobacteria cell factories. *Biotechnology for biofuels*, 8(1), 184.

Ma, K., Hutchins, A., & Sung, S. S. (1997). Pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Pyrococcus furiosus*, functions as a CoA- dependent pyruvate decarboxylase. *Proceedings of the National Academy of Sciences of the United States of America, 94*(18), 9608-9613.

Meinecke, B., Bertram, J., & Gottschalk, G. (1989). Purification and characterization of the pyruvate-ferredoxin oxidoreductase from *Clostridium acetobutylicum. Archives of Microbiology*. *152*(3), 244-250. doi:10.1007/BF00409658

Mussatto, S. I., Dragone, G., Guimarães, P. M. R., Silva, J. P. A., Carneiro, L. M., Roberto, I. C., Vicente, A., Domingues, L., & Teixeira, J. A. (2010). Technological trends, global market, and challenges of bio-ethanol production. *Biotechnology Advances,28*(6), 817-830. doi:10.1016/j.biotechadv.2010.07.001

Nunn, C. E. M., Johnsen, U., Schönheit, P., Fuhrer, T., Sauer, U., Hough, D. W., & Danson, M. J. (2010). Metabolism of pentose sugars in the hyperthermophilic archaea *Sulfolobus solfataricus and Sulfolobus acidocaldarius*.*The Journal of Biological Chemistry, 285*(44), 33701. doi:10.1074/jbc.M110.146332

Olson, D. G., Sparling, R., & Lynd, L. R. (2015). Ethanol production by engineered thermophiles. *Current opinion in biotechnology*, *33*, 130-141.

Park, Y. J., Yoo, C. B., Choi, S. Y., & Lee, H. B. (2006). Purifications and characterizations of a ferredoxin and its related 2-oxoacid: ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Sulfolobus solfataricus* P1. BMB Reports, 39(1), 46-54. ISO 690

Pal-Bhowmick, I., Sadagopan, K., Vora, H., Sehgal, A., Sharma, S., & Jarori, G. (2004). Cloning, over-expression, purification and characterization of *Plasmodium falciparum* enolase. *European Journal of Biochemistry, 271*(23-24), 4845-4854.

Pei, J., Q. Zhou, Y. Jiang, Y. Le, H. Li, W. Shao and J. Wiegel (2010) *Thermoanaerobacte*r spp. control ethanol pathway via transcriptional regulation and versatility of key enzymes. Metab. Eng. 12, 420-428.

Pieulle, L., Guigliarelli, B., Asso, M., Dole, F., Bernadac, A., & Hatchikian, E. C. (1995). Isolation and characterization of the pyruvate- ferredoxin oxidoreductase from the sulfate- reducing bacterium *Desulfovibrio africanus*. *Biochimica Et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology, 1250*(1), 49-59. doi:10.1016/0167-4838(95)00029-T

Quehenberger, J., Shen, L., Albers, S. V., Siebers, B., & Spadiut, O. (2017). *Sulfolobus*–a potential key organism in future biotechnology. *Frontiers in microbiology*, *8*, 2474.

Ragsdale, S. W. (2003). Pyruvate ferredoxin oxidoreductase and its radical intermediate. *Chemical reviews*, *103*(6), 2333-2346.

Raj, K. C., L. A. Talarico, L. O. Ingram and J. A. Maupin-Furlow (2002) Cloning and characterization of the *Zymobacter palmae* pyruvate decarboxylase gene (pdc) and comparison to bacterial homologues. Appl. Environ. Microbiol. 68, 2869-2876.

Radianingtyas, H., & Wright, P. C. (2003). Alcohol dehydrogenases from thermophilic and hyperthermophilic archaea and bacteria. Oxford, UK: doi:10.1016/S0168-6445(03)00068-8

Reed, G. H., Ragsdale, S. W., & Mansoorabadi, S. O. (2012). Radical reactions of thiamin pyrophosphate in 2-oxoacid oxidoreductases. *Biochimica Et Biophysica Acta (BBA) – Proteins and Proteomics, 1824*(11), 1291-1298. doi:http://dx.doi.org.proxy.lib.uwaterloo.ca/10.1016/j.bbapap.2011.11.010

Reid, M., & Fewson, C. (1994). Molecular characterization of microbial alcohol dehydrogenases. *Critical Reviews in Microbiology, 20*(1), 13-56.

Rodríguez-Zavala, J. S., Allali-Hassani, A., & Weiner, H. (2006). Characterization of *E. coli* tetrameric aldehyde dehydrogenases with atypical properties compared to other aldehyde dehydrogenases. *Protein Science*, 15(6), 1387-1396.

Sánchez, L. B. (1998). Aldehyde dehydrogenase (CoA-acetylating) and the mechanism of ethanol formation in the amitochondriate protist, *Giardia lamblia*. *Archives of biochemistry and biophysics*, 354(1), 57-64.

Schönheit, P., & Schäfer, T. (1995). Metabolism of hyperthermophiles. *World Journal of Microbiology and Biotechnology, 11*(1), 26-57. doi:10.1007/BF00339135

Smith, L. T., & Kaplan, N. O. (1980). Purification, properties, and kinetic mechanism of coenzyme A-linked aldehyde dehydrogenase from *Clostridium kluyveri*. *Archives of biochemistry and biophysics*, 203(2), 663-675.

She, Q., Singh, R. K., Confalonieri, F., Zivanovic, Y., Allard, G., Awayez, M. J., Clausen, I. G., Curtis, B. A., De Moors, A., Erauso, G., Fletcher, C., Jong, I. H., Jeffries, A. C., Kozera, C. J., Medina, N., Peng, X., Thi-Ngoc, H. P., Redder, P., Schenk, M. E., Theriault, C., Tolstrup, N., Charlebois, R. L., Doolittle, W. F., Duguet, M., Gaasterland, T., Garrett, R. A., Ragan, M. A., Sensen, C. W., & Van Der Oost, J.(2001). The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *Proceedings of the National Academy of Sciences of the United States of America, 98*(14), 7835-7840.

Straub, C. T., Zeldes, B. M., Schut, G. J., Adams, M. W., & Kelly, R. M. (2017). Extremely thermophilic energy metabolisms: biotechnological prospects. *Current opinion in biotechnology*, *45*, 104-112.

Stetter, K. O. (2006). Hyperthermophiles in the history of life. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences, 361*(1474), 1837.

Suzuki, T., Iwasaki, T., Uzawa, T., Hara, K., Nemoto, N., Kon, T., ... & Oshima, T. (2002). *Sulfolobus tokodaii sp*. nov.(f. *Sulfolobus* sp. strain 7), a new member of the genus *Sulfolobus* isolated from Beppu Hot Springs, Japan. *Extremophiles*, 6(1), 39-44.

Taylor, M. P., Eley, K. L., Martin, S., Tuffin, M. I., Burton, S. G., & Cowan, D. A. (2009). Thermophilic ethanologenesis: future prospects for second- generation bioethanol production. *Trends in Biotechnology, 27*(7), 398-405. doi:10.1016/j.tibtech.2009.03.006

Van den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Current Opinion in Microbiology, 6*(3), 213-218. doi:http://dx.doi.org.proxy.lib.uwaterloo.ca/10.1016/S1369-5274(03)00060-2

Van Zyl, L. J., Schubert, W. D., Tuffin, M. I., & Cowan, D. A. (2014). Structure and functional characterization of pyruvate decarboxylase from *Gluconacetobacter diazotrophicus*. *BMC structural biology*, *14*(1), 21.

Vieille, C., & Zeikus, G. J. (2001). Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews, 65*(1), 1.

Wahl, R., & Orme-Johnson, W. (1987). Clostridial pyruvate oxidoreductase and the pyruvate-oxidizing enzyme specific to nitrogen fixation in *Klebsiella pneumoniae* are similar enzymes. *The Journal of Biological Chemistry, 262*(22), 10489-96.

Wang, Q., P. He, D. Lu, A. Shen and N. Jiang (2004) Purification, characterization, cloning and expression of pyruvate decarboxylase from *Torulopsis glabrata* IFO005. *Journal of biochemistry*, 136, 447-455.

Williams, K., P. N. Lowe and P. F. Leadlay (1987) Purification and characterization of pyruvate: ferredoxin oxidoreductase from the anaerobic protozoon *Trichomonas vaginalis*. *The Biochemical journal*. 246, 529-0.

Yan, Z., Maruyama, A., Arakawa, T., Fushinobu, S., & Wakagi, T. (2016). Crystal structures of archaeal 2-oxoacid: ferredoxin oxidoreductases from *Sulfolobus tokodaii*. *Scientific reports*, *6*, 33061.

Yan, Z., Fushinobu, S., & Wakagi, T. (2014). Four Cys residues in heterodimeric 2-oxoacid: ferredoxin oxidoreductase are required for CoA-dependent oxidative decarboxylation but not for a non-oxidative decarboxylation. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1844*(4), 736-743.

Yao, S., & Mikkelsen, M. (2010). Metabolic engineering to improve ethanol production in *Thermoanaerobacter mathranii*. *Applied Microbiology and Biotechnology, 88*(1), 199-208. doi:10.1007/s00253-010-2703-3

Yoon, K. S., Ishii, M., Igarashi, Y., & Kodama, T. (1996). Purification and characterization of 2-oxoglutarate: ferredoxin oxidoreductase from a thermophilic, obligately chemolithoautotrophic bacterium, *Hydrogenobacter thermophilus* TK-6. *Journal of bacteriology*, 178(11), 3365-3368.

Zhang, Q., Iwasaki, T., Wakagi, T., & Oshima, T. (1996). 2-oxoacid: ferredoxin oxidoreductase from the thermoacidophilic archaeon, *Sulfolobus* sp. Strain 7. *Journal of Biochemistry*, *120*(3), 587–599. doi:10.1093/oxfordjournals.jbchem.a021454

Appendix A

Table A1. Determination of optimal temperature of SsPOR

Temperature ºC	U/mg[a]	U/mg (Avg.)
30	0.000	0.0±0.0
40	0.000	0.0±0.0
50	0.450	0.525±0.075
50	0.600	
60	3.600	4.050±0.450
60	4.500	
70	7.300	7.300±0.000
70	7.300	
80	7.300	7.750±0.450
80	8.200	
90	12.000	12.1±0.200
90	12.300	

[a], POR activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate 0.1 mM CoA, 1 mM BV, 50 µM SDT, and 3 µg SsPOR.

One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table A2. Determination of optimal temperature of SaPOR

Temperature ºC	U/mg[a]	U/mg (Avg.)
30	0.000	0.000±0.000
40	0.550	0.550±0.000
40	0.550	
50	0.820	0.960±0.140
50	1.100	
60	1.930	1.930±0.000
60	1.930	
70	4.410	4.410±0.000
70	4.410	
80	7.710	8.00±0.275
80	8.260	
85	7.710	7.710±0.000
85	7.710	
90	6.060	7.710±1.375
90	8.810	

[a], POR activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate 0.1 mM CoA, 1 mM BV, 50 µM SDT, and 12 µg SaPOR.
One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table A3. Determination of optimal pH of SsPOR

pH (room temperature)	pH (80 ºC)[a]	U/mg[b]	U/mg (Avg.)
Sodium phosphate			
5	4.8	0.000	0.000±0.000
5	4.8	0.000	
6	5.8	0.320	0.360±0.045
6	5.8	0.410	
7	6.8	1.200	1.200±.0.000
7	6.8	1.200	
8	7.8	1.380	1.500±0.115
8	7.8	1.610	
EPPS			
7	6.2	0.400	0.400±0.000
7	6.2	0.400	
8	7.2	0.980	1.100±0.120
8	7.2	1.220	
9	8.2	1.500	1.600±0.100
9	8.2	1.700	
Glycine			
9	7.6	1.400	1.500±0.100
9	7.6	1.600	
9.5	8.1	1.700	1.700±0.000
9.5	8.1	1.700	
10	8.6	2.190	2.200±0.010
10	8.6	2.210	
CAPs			
10	9.5	2.090	2.100±0.010
10	9.5	2.110	
11	10.5	1.680	1.680±0.000
11	10.5	1.680	
12	11.5	1.300	1.400±0.000
12	11.5	1.500	

[a], those values were calculated based on their ΔpKa values.

[b], POR activity was measured at 80 ºC using 100 mM buffer, 5 mM pyruvate 0.1 mM CoA, 1 mM Methyl viologen, 50 µM SDT and 3 µg SsPOR.

One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table A4. Determination of optimal pH of SaPOR

pH (room temperature)	pH (80 ºC)[a]	U/mg[b]	U/mg (Avg.)
Sodium phosphate			
5	4.8	0.000	0.000±0.000
5	4.8	0.000	
6	5.8	0.130	0.140±0.010
6	5.8	0.150	
7	6.8	0.160	0.180±0.025
7	6.8	0.210	
8	7.8	0.230	0.280±0.045
8	7.8	0.320	
EPPS			
7	6.2	0.040	0.050±0.005
7	6.2	0.050	
8	7.2	0.130	0.140±0.010
8	7.2	0.150	
9	8.2	0.340	0.360±0.020
9	8.2	0.380	
Glycine			
9	7.6	0.340	0.340±0.000
9	7.6	0.340	
9.5	8.1	0.420	0.440±0.020
9.5	8.1	0.460	
10	8.6	0.500	0.550±0.050
10	8.6	0.600	
CAPs			
10	9.5	0.400	0.410±0.010
10	9.5	0.420	
11	10.5	0.050	0.050±0.000
11	10.5	0.050	
12	11.5	0.000	0.000±0.000
12	11.5	0.000	

[a], those values were calculated based on their ΔpKa values.

[b], POR activity was measured at 80 ºC using 100 mM buffer, 5 mM pyruvate 0.1 mM CoA, 1 mM Methyl viologen, 50 µM SDT and 12 µg SaPOR.

One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table A5. Determination of optimal temperature of SsPDC

Temperature ºC	Acetaldehyde (mM)	U/mg	U/mg (Avg.)
40	0.067	0.023	0.019±0.004
40	0.042	0.015	
50	0.050	0.017	0.010±0.007
50	0.010	0.003	
60	0.042	0.014	0.019±0.005
60	0.067	0.023	
70	-	-	0.029±0.000
70	0.082	0.029	
80	-	-	0.069±0.000
80	0.199	0.069	
90	0.125	0.043	0.056±0.013
90	0.199	0.069	

Assay mixture was 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate, 0.1 mM CoA, 25 µg enzyme.

One unit was defined as the production of 1 µmol of acetaldehyde per min.

-, not available.

Table A6. Determination of optimal temperature of SaPDC

Temperature ºC	Acetaldehyde (mM)	U/mg	U/mg (Avg.)
40	0.073	0.012	0.011±0.001
40	0.062	0.010	
50	0.029	0.005	0.003±0.002
50	0.012	0.002	
60	0.085	0.014	0.004±0.000
60	-	-	
70	0.046	0.008	0.008±0.001
70	0.052	0.009	
80	0.095	0.016	0.016±0.001
80	0.091	0.015	
90	0.270	0.045	0.057±0.012
90	0.411	0.068	

Assay mixture was 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate, 0.1 mM CoA, 50 µg enzyme.

One unit was defined as the production of 1 µmol of acetaldehyde per min.

-, not available.

Table A7. Determination of optimal pH of SsPDC

pH (room temperature)	pH (80 ºC)[a]	Acetaldehyde (mM)	U/mg	U/mg (Avg.)
7	6.8	0.138	0.048	0.043±0.005
7	6.8	0.110	0.038	
8	7.8	0.352	0.122	0.158±0.037
8	7.8	0.561	0.195	
9	7.6	0.355	0.123	0.127±0.004
9	7.6	0.375	0.130	
10	8.6	0.254	0.088	0.087±0.001
10	8.6	0.246	0.085	
11	10.5	0.095	0.033	0.045±0.012
11	10.5	0.163	0.057	
12	11.5	0.134	0.046	0.033±0.014
12	11.5	0.055	0.019	

[a], those values were calculated based on their ΔpKa values.

Assay mixture was 100 mM buffer, 10 mM pyruvate, 0.1 mM CoA, 25 µg enzyme.

Assays were performed at 80 ºC.

One unit was defined as the production of 1 µmol of acetaldehyde per min.

Table A8. Determination of optimal pH of SaPDC

pH (room temperature)	pH (80 ºC)[a]	Acetaldehyde (mM)	U/mg	U/mg (Avg.)
7	6.8	0.118	0.020	0.033±0.014
7	6.8	0.281	0.047	
8	7.8	0.314	0.052	0.055±0.003
8	7.8	0.350	0.058	
9	7.6	0.272	0.045	0.035±0.010
9	7.6	0.150	0.025	
10	8.6	0.215	0.036	0.031±0.005
10	8.6	0.158	0.026	
11	10.5	0.115	0.019	0.017±0.002
11	10.5	0.089	0.015	
12	11.5	0.084	0.014	0.014±0.000
12	11.5	0.082	0.014	

[a], those values were calculated based on their ΔpKa values.

Assay mixture was 100 mM buffer, 10 mM pyruvate, 0.1 mM CoA, 50 µg enzyme.

Assays were performed at 80 ºC.

One unit was defined as the production of 1 µmol of acetaldehyde per min.

Appendix B

Table B1. CoA dependence of SsPOR

CoA (μM)	U/mg[a]	U/mg (Avg.)
0	0.000	0.000±0.000
0	0.000	
2	0.900	0.850±0.040
2	0.800	
10	4.000	3.800±0.200
10	3.600	
30	5.900	5.600±0.250
30	5.400	
60	6.800	6.600±0.200
60	6.400	
100	7.300	7.100±0.250
100	6.800	

[a], POR activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate, 1 mM BV, 50 μM SDT and 3 μg SsPOR

One unit of enzyme activity was defined as the oxidation of 1 μmol of pyruvate

Table B2. Pyruvate dependence of SsPOR

Pyruvate (mM)	U/mg[a]	U/mg (Avg.)
0	0.000	0.000±0.000
0	0.000	
0.1	0.900	0.910±0.010
0.1	0.920	
0.2	1.820	1.830±0.005
0.2	1.840	
0.4	2.880	2.890±0.010
0.4	2.900	
0.6	3.250	3.345±0.095
0.6	3.440	

[a], POR activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 0.1 mM CoA, 1 mM BV, 50 µM SDT and 3 µg SsPOR

One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table B3. CoA dependence of SaPOR

CoA (µM)	U/mg[a]	U/mg (Avg.)
0	0.000	0.000±0.000
0	0.000	
5	0.450	0.375±0.080
5	0.300	
10	0.610	0.560±0.050
10	0.510	
30	1.000	0.960±0.040
30	0.920	
60	1.380	1.355±0.020
60	1.330	
100	1.430	1.430±0.000
100	1.430	

[a], POR activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 5 mM pyruvate, 1 mM BV, 50 µM SDT and 12 µg SaPOR

One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table B4. Pyruvate dependence of SaPOR

Pyruvate (mM)	U/mg[a]	U/mg (Avg.)
0	0.000	0.000±0.000
0	0.000	
0.1	0.430	0.440±0.007
0.1	0.450	
0.15	0.650	0.660±0.005
0.15	0.660	
0.2	0.880	0.880±0.005
0.2	0.890	
0.4	1.220	1.210±0.008
0.4	1.200	
0.7	1.300	1.300±0.000
0.7	1.300	

[a], POR activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 0.1 mM CoA, 1 mM BV, and 50 µM SDT and 12 µg SaPOR

One unit of enzyme activity was defined as the oxidation of 1 µmol of pyruvate

Table B5. CoA dependence of SsPDC

CoA (μM)	Acetaldehyde (mM)	(mM) – (controls)	mM (Avg.)	U/mg[a]
0	0.146	0.042	0.000±0.042	0.000±0.015
0	0.067	-0.043		
2	0.352	0.245	0.245±0.000	0.085±0.000
2	-	-		
10	0.592	0.485	0.443±0.042	0.154±0.015
10	0.508	0.401		
30	0.524	0.417	0.349±0.068	0.121±0.024
30	0.388	0.281		
100	0.446	0.339	0.325±0.014	0.113±0.004
100	0.418	0.311		
Control 1[b]	0.059		0.007±0.010	-
Control 2[b]	0.079			
Control 3[c]	0.024		0.025±0.001	-
Control 4[c]	0.027			

[a]. PDC activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate and 25 μg SsPDC.

[b]. Control samples contained 100 mM sodium phosphate, 10 mM pyruvate, 100 μM CoA, no enzyme used.

[c]. Control samples contained 100 mM sodium phosphate, 100 μM CoA, and enzyme.

Samples with only buffer showed 0.01 mM acetaldehyde.

Enzyme alone was calculated to be giving Peak Area around 700,000 (0.0013 mM).

One unit was defined as the production of 1 μmol of acetaldehyde per min.

-, not available.

Table B6. Pyruvate dependence of SsPDC

Pyruvate (mM)	Acetaldehyde (mM)	(mM) – (controls)	mM (Avg.)	U/mg[a]
0	0.024	0.000	0.000±0.000	0.000±0.000
0	0.027	0.000		
0.2	0.089	0.063	0.063±0.001	0.022±0.000
0.2	0.09	0.064		
0.4	0.201	0.173	0.106±0.067	0.037±0.023
0.4	0.067	0.039		
0.8	0.136	0.106	0.173±0.068	0.060±0.023
0.8	0.271	0.241		
1	0.171	0.139	0.141±0.001	0.049±0.001
1	0.174	0.142		
2	0.125	0.086	0.093±0.006	0.032±0.002
2	0.138	0.099		
10	0.446	0.352	0.338±0.014	0.117±0.028
10	0.418	0.324		

[a]. PDC activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 0.1 mM CoA and 25 µg SsPDC.

One unit was defined as the production of 1 µmol of acetaldehyde per min.

Table B7. CoA dependence of SaPDC

CoA (μM)	Acetaldehyde (mM)	(mM) – (controls)	mM (Avg.)	U/mg[a]
0	0.118	0.000	0.000±0.012	0.000±0.012
0	0.093	-0.025		
2	0.255	0.149	0.149±0.000	0.025±0.000
2	-	-		
10	0.290	0.184	0.163±0.022	0.031±0.005
10	0.247	0.141		
30	0.255	0.149	0.128±0.021	0.021±0.004
30	0.213	0.107		
100	0.299	0.193	0.201±0.007	0.034±0.002
100	0.314	0.208		
Control 1[b]	0.059		0.070±0.010	-
Control 2[b]	0.079			
Control 3[c]	0.048		0.049±0.001	-
Control 4[c]	0.05			

[a]. PDC activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 10 mM pyruvate and 50 μg SaPDC.

[b]. Control samples contained 100 mM sodium phosphate, 10 mM pyruvate, 100 μM CoA, no enzyme used.

[c]. Control samples contained 100 mM sodium phosphate, 100 μM CoA, and enzyme.

Enzyme alone was calculated to be giving Peak Area around 550,000 (0.02 mM).

One unit was defined as the production of 1 μmol of acetaldehyde per min.

-, not available.

Table B8. Pyruvate dependence of SaPDC

Pyruvate (mM)	Acetaldehyde (mM)	(mM) – (controls)	mM (Avg.)	U/mg[a]
0	0.048	0.000	0.000±0.000	0.000±0.000
0	0.054	0.000		
0.2	0.086	0.033	0.034±0.000	0.006±0.000
0.2	-	-		
0.4	0.114	0.060	0.080±0.020	0.013±0.003
0.4	0.154	0.100		
0.8	0.177	0.120	0.131±0.010	0.022±0.002
0.8	0.198	0.141		
1	0.250	0.192	0.096±0.000	0.016±0.000
1	-	-		
2	0.127	0.062	0.075±0.013	0.013±0.002
2	0.153	0.088		
10	0.314	0.194	0.212±0.018	0.035±0.003
10	0.350	0.230		

[a]. PDC activity was measured at 80 ºC using 100 mM sodium phosphate pH 8.0 (measured at room temperature), 0.1 mM CoA and 50 µg SaPOR.

One unit was defined as the production of 1 µmol of acetaldehyde per min.

-, not available.

Appendix C

Table C1. Acetaldehyde standard curve

Sample	Acetaldehyde (mM)	Peak Area	Peak Area (Avg.)	(Peak Area) – (Peak Area of Control [a])
1	0	4.3E+06	4.3E+06±8.6E+04	0.000±8.6E+04
2	0	4.5E+06		
3	0	4.3E+06		
4	0.4	1.3E+07	1.4E+07±2.3E+06	1.0E+07±2.3E+06
5	0.4	1.7E+07		
6	0.4	1.4E+07		
7	0.6	1.9E+07	2.1E+07±2.8E+06	1.6E+07±2.8E+06
8	0.6	2.4E+07		
9	0.6	1.9E+07		
10	0.8	2.8E+07	3.1E+07±2.8E+06	2.6E+07±2.8E+06
11	0.8	3.3E+07		
12	0.8	3.2E+07		
13	1	3.7E+07	3.4E+07±3.4E+06	2.9E+07±3.4E+06
14	1	3.0E+07		
15	1	3.5E+07		
16	2	7.7E+07	7.3E+07±3.2E+06	6.9E+07±3.2E+06
17	2	7.3E+07		
18	2	7.1E+07		

[a], Samples with zero concentrations of acetaldehyde were used as control samples.

Samples were prepared under conditions same as the PDC assay samples (100 mM sodium phosphate pH 8.0 that was measured at room temperature). Samples (80 µl) were injected and the attenuation of the detector was set at 0.64 A.

Table C2. Controls for Acetaldehyde determination for SsPDC

Samples	Acetaldehyde (mM)	mM (Avg.)
Control 1[a]	0.01	0.01
Control 2[b]	0.0013	0.0013
Control 3[c]	0.059	0.07±0.010
	0.079	
Control 4[d]	0.024	0.025±0.001
	0.027	

[a], samples with only 100 mM sodium phosphate pH 8.0.

[b], sample contains enzyme alone.

[c], samples contain 100 mM sodium phosphate pH 8.0, 10 mM pyruvate, 100 µM CoA, no enzyme used.

[d]. samples contain 100 mM sodium phosphate pH 8.0, 100 µM CoA, and enzyme.

Table C3. Controls for Acetaldehyde determination for SaPDC

Samples	Acetaldehyde (mM)	mM (Avg.)
Control 1[a]	0.01	0.01
Control 2[b]	0.018	0.018
Control 3[c]	0.059	0.07±0.010
	0.079	
Control 4[d]	0.048	0.05±0.001
	0.05	

[a], samples with only 100 mM sodium phosphate pH 8.0.

[b], sample contains enzyme alone.

[c], samples contain 100 mM sodium phosphate pH 8.0, 10 mM pyruvate, 100 µM CoA, no enzyme used.

[d]. samples contain 100 mM sodium phosphate pH 8.0, 100 µM CoA, and enzyme.