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Alcohol Dehydrogenases Catalyzing the Production of Ethanol at High Temperatures

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Abstract

Alcohol dehydrogenases (ADHs) are a group of enzymes within the class of oxidoreductases that catalyze the interconversion of alcohols to their corresponding aldehydes and ketones, and can be categorized into three different types (type I, II, and III) depending on the molecular size and metal requirements. Hyperthermophilic ADHs have superior catalytic properties compared to their mesophilic counterparts, due to their high tolerance of extreme conditions such as high temperatures. There are 19 hyperthermophilic ADHs that have been characterized. This review focuses on understanding biochemical and biophysical properties of hyperthermophilic ADHs involved in ethanol production at high temperatures.

Keywords: Thermophilic; Hyperthermophilic; Alcohol dehydrogenase; Ethanol

Introduction

Alcohol production or oxidation is catalyzed by the group of oxidoreductases called alcohol dehydrogenase (ADH). These ubiquitous enzymes are responsible for catalyzing the interconversion of alcohols to their corresponding aldehydes and ketones, with the concomitant reduction of NAD^+ to $NADP^+$ [1]. Based on the specificity of electron carriers required, they can be dependent on a) NAD/NADP, b) pyrollo-quinoline quinone, heme or cofactor F_{420} and c) flavin adenine di-nucleotide (FAD) [2-4]. ADHs are commonly utilized enzymes in the production of alcohol and, namely, in industrial ethanol production. A suitable substrate is required to allow fermenting microorganisms to produce ethanol. Substrates typically used are plant-based and high in sugar content. High sugar concentration is ideal for the production of ethanol, which begins with the conversion of sugars (i.e. glucose, sucrose) through the process of glycolysis (i.e. the conversion of glucose into pyruvate). Under anaerobic conditions, pyruvate is converted into ethanol using either a two-step pathway or a three-step pathway, with ADH is a key enzyme in both pathways [5]. During this conversion, pyruvate is first converted to the intermediate product, acetaldehyde, which is then converted into ethanol, while releasing CO_2 as a by-product. A major obstacle diverting more popularity in bio-ethanol production involves the difficulties in finding a suitable microorganism that can convert biomass into ethanol for fuel [6]. This has garnered research interests in finding suitable microorganisms that can have pathways genetically engineered to selectively produce ethanol. Yeast and Gram-negative microorganisms *Escherichia coli* and *Zymomonas mobilis* are commonly used microorganisms in the industrial production of bioethanol, although *Z. mobilis* is limited to fermentation of glucose and fructose [6]. *Z. mobilis* is able to utilize the Entner-Doudoroff (ED) pathway, as opposed to the commonly utilized Embden-Meyerhof-Parnas (EMP) pathway in glycolysis for most eukaryotes and prokaryotes [7]. The benefit of the ED pathway is that more carbons are available for ethanol production, due to the fact that only one ATP molecule is produced from one glucose molecule compared to that in EMP where two ATPs are produced from one glucose molecule [7]. *E. coli* is a commonly used industrial microbe due to its non-complex growth requirements and ability to ferment a wide range of sugars [6]. Research efforts to maximize yield of ethanol have also shown that pathways can be engineered to divert the flux of sugars [7]. Diverting the flux of sugar from biomass to ethanol production has been shown to maximize the ethanol yield when tested in *S. cerevisiae* [7]. Similar to the mesophilic production of ethanol, hyperthermophiles start fermentation with the conversion of glucose to the intermediate pyruvate. Both two-step and three-step pathways are used for converting pyruvate to ethanol by mesophilic and some moderate thermophilic microorganisms. *S. cerevisiae* and some bacteria such as *Z. mobilis* utilize a two-step pathway which involves the non-oxidative decarboxylation of pyruvate to acetaldehyde, catalyzed by pyruvate decarboxylase (PDC), then further reduced to ethanol by ADH [5]. The other common pathway is the three-step pathway, utilized by most bacteria, which involves the oxidative decarboxylation of pyruvate to acetyl-coenzyme A (acetyl-coA) by pyruvate ferredoxin oxidoreductase (POR) then following, acetyl-CoA to acetaldehyde by CoA-dependent-acetylating acetaldehyde dehydrogenase (AcDH), and acetaldehyde to ethanol by ADH [5]. Hyperthermophiles, however, use only a two-step pathway for their ethanol production, with a thermostable ADH acting is the key enzyme catalyzing the production of ethanol from acetaldehyde [5,8].

With continual concerns of the depletion of petroleum fuels, in addition to its negative consequences on the environment, alternative sources of energy are highly sought after. Researchers have studied the benefits of using non-mesophilic microorganisms, specifically, thermophilic and hyperthermophilic microorganisms in the production of bio-ethanol [9]. Thermophiles (and hyperthermophiles) are groups of microorganisms that are able to withstand, and thrive, in extreme environments which may imply high temperatures, also salinities, and pHs, to name a few. Hyperthermophiles are ideal candidates for bio-ethanol production due to their ability to adapt to changing environmental conditions [5]. Additionally, hyperthermophilic enzymes are more stable whilst still being active at high temperatures in contrast to their mesophilic counterparts [5,10] thus, making them

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ideal for ethanol fermentation. In terms of production efficiency, the utilization of hyperthermophiles is beneficial as it reduces potential contamination, eliminates unwanted cooling in the fermenter, and decreases end-product inhibition [5,10].

To date, 19 hyperthermophilic ADHs have been characterized. Depending on molecular size and metal requirements, ADHs can be categorized into three different "types". Type I, also known as medium chain ADHs, contain approximately 370 amino acid residues per chain, can be dimeric (in higher eukaryotes) or tetrameric (in prokaryotes), and are zinc dependent. Type II contains short chain ADHs rarely containing metal ions and has approximate lengths of 250 amino acid residues per chain. Type III contains iron-dependent long chain ADHs which range from 380-900 amino acid residues per chain [1,2,11]. The most widely studied type I ADH is horse liver ADH [1,11], which is a homodimeric protein with two zinc atoms and a NAD-binding site in each subunit [12]. Well-known type II ADHs are those from *Drosophila melanogaster* [1,11], and have been investigated to understand the evolution of enzymes in eukaryotes. A well-known representative of type III ADHs is ADH2 from *Z. mobilis*, and is a homotetramer that requires Fe^{2+} for activity [13]. Iron-containing type III ADHs have been found to be from hyperthermophiles [14,15].

Hyperthermophilic ADHs have been identified and characterized in their relation to ethanol production [5,9,16]. In order to have a better understanding of hyperthermophilic ADHs, general properties including thermostability, substrate specificity, X-ray crystallography structures, to name a few, are required. This review focuses on providing a general overview of the function, biophysical, and biochemical properties of ADHs in hyperthermophilic microorganisms, while providing future perspectives and outlooks on their utilization in the production of ethanol.

Thermostable Alcohol Dehydrogenases

Biophysical properties

Temperature-dependent activity: Hyperthermophilic ADHs show high activity at high-temperatures with extraordinary thermostability [1]. In general, they have an optimal temperature above 85°C (Table 1), for example, the activity of *Pyrococcus furiosus* AdhD and ADH from *Thermococcus sibiricus* increase along with the temperature up to 100°C [17-19] while the activities of ADHs from *Thermococcus guaymasensis* and *Thermococcus* strain ES1 increase at least up to 95°C [20,21]. ADHs of *Pyrobaculum aerophilum*, *Aeropyrum pernix,* and *Thermococcus kodakarensis* KOD1 show increasing activity up to 90°C [22-24]. ADHs of *Thermococcus litoralis*, *Thermococcus zilligii* and *Sulfolobus tokodaii* have an optimal temperature of 85°C [25-29].

It should be noted that ADHs from a single organism may have different optimal temperatures for their activities [1]. For instance, *P. furiosus* AdhD has an optimal temperature of activity of 100°C while AdhA and AdhC have 90°C and above 90°C, respectively [17,18,30,31]. This could possibly mean that one form of ADH is more dominant over the other and could play different physiological roles in the organism [1].

Thermostability: Although both mesophilic and hyperthermophilic ADHs are composed of commonly known amino acid residues, one could wonder how hyperthermophilic ADHs are resilient to and functional at high temperatures while the other is not. The answer lies in the sequence and structure of the protein. One major factor that increases thermostability is the composition of amino acids [32]. Changes such as: substitution of Gly or Lys with Arg for forming more α-helix structures, increased number of charged residues such as Glu, increased number of hydrophobic residues such as Val, Ala, and Pro and other changes to strengthen the interface between the subunits all play a role in increasing the thermostability [1,32]. For example, analysis of the primary structure of *T. guaymasensis* ADH showed an increase in Ala, Arg, Glu, Lys, and Pro which could have led to the increased thermostability [33]. An increase in small hydrophobic amino acid residues was also observed in *P. aerophilum* ADH which is considered to play a role in thermostability [22]. This phenomenon is also observed in the ADH of *S. solfataricus* strain 1617 where Glu substitutes Cys and twice the amount of Arg to Lys is seen, compared to mesophilic ADHs [34].

Other factors such as large hydrophobic cores, increased ionic interactions, polymeric structures, hydrogen bonds, disulfide bridges etc. have been identified in proteins of thermophilic and extremely thermophilic microorganisms but further studies using detailed structures of hyperthermophilic ADHs are required to identify if these adaptations are also seen in them [1,23].

Metal and cofactor specificity: So far, only a few hyper/thermophilic ADHs have been studied structurally. Approximately 15 ADH structures are solved from thermophilic organisms, including 5 from hyperthermophiles [35-39]. Therefore, most of the structural information about hyperthermophilic ADHs is based on the sequence and structures of their mesophilic counterparts.

Type I ADHs, comprised of zinc containing ADHs, have been extensively studied compared to type II and III ADHs. The widely known horse liver ADH (HlADH) belongs to this group. These ADHs usually, but not always, have two tetrahedrally coordinated zinc ions per subunit; one for the catalytic function and is found in the active site while the other is for structural integrity and is located at a site which influences subunit interactions [40,41]. The catalytic zinc ion interacts with four ligands which can be a combination of Cys, His, Asp and Glu in microbial ADHs [42]. For example, in the ADH of *S. solfataricus* the catalytic zinc interacts with two Cys, one His and one Glu residue [35], while in the ADH of *A. pernix*, the zinc ion interacts with a Cys, a His, an Asp and a Glu residue [37].

The structural zinc ion also interacts with four amino acid residues in a tetrahedral fashion. In higher eukaryotes and bacteria, these four ligands would be Cys residue. In HlADH, the structural zinc coordinates with Cys97, Cys100, Cys103 and Cys111. Structural zinc ion in *P. aerophilum* coordinates with four Cys residues while in *S. solfataricus* three Cys and a Glu coordinate with zinc [22,35]. In *A. pernix* ADH the zinc ion coordinates with three Cys and an Asp residue [37]. Some hyper/thermophilic ADHs from *T. brockii*, *T. ethanolicus* and *T. guaymasensis* have been found to have only the catalytic zinc ion [20] while some others such as the one from *P. aerophilum* has only the structural zinc [22]. Usually zinc containing ADHs are active under aerobic conditions since the Zn^{2+} ion cannot be further oxidized. There are exceptions. The ADH of *T. guaymasensis* is oxygen sensitive even though it's a zinc containing enzyme. It is hypothesized that the oxidative degradation of certain amino acids (especially Cys) could be the reason [20].

The iron-dependent ADHs of type III are represented by the well-studied mesophilic ADH, *Z. mobilis* ADH2 (ZmADH). Structural studies of ZmADH showed that the iron ion coordinates with three highly conserved His residues and one acidic residue such as Asp [43]. Sequence studies of all type III hyperthermophilic ADHs have showed the presence of the three highly conserved His residues, but structural

Organism	T_{opt} [a] (°C)	Cofactor/ metal	Type	Sub-unit (kDa α_n)	Opt. temp[b] (°C)	Thermo-stability [$t_{1/2}$, h[c] (at °C)]	Oxidation of alcohols			Reduction of aldehydes/ketones			Ref.
							Opt. pH[d]	App. K_m (mM)[e]	App. k_{cat} (s^{-1})	Opt. pH[f]	App. K_m (mM)[g]	App k_{cat} (s^{-1})	
Thermococcus litoralis	88	NADP/Fe	III	48 (α_4)	85	5 (85), 0.3 (96)	8.8	11	26	nd	0.4	nd	[27], [28]
Thermococcus. ES-1	91	NADP/Fe	III	46 (α_4)	>95	35 (85), 4 (95)	10.3-10.5	10.4	84	7	1.0	23.6	[21]
Thermococcus kodakarensis KOD1	85	NADP/Fe	III	31 (α1)	90	16.2 (85), 4.5 (95)	9	61.3[1]	43.8	3	30.2[2]	13.7	[24]
Thermococcus zilligii	75-80	NADP/Fe	III	46 (α4)	85	0.27 (80)	6.8-7.0	10	nd	nd	nd	nd	[25], [26]
Thermococcus hydrothermalis	85	NADP/Fe	III	45 (α2/ α4)	80	0.5 (80), 2.5 (70)	10.5	2.0[3]	23	7.5	0.01[4]	1.7	[63]
Pyrococcus furiosus (AdhA)	100	NADP/ non-metal	II	26 (α2/ α4/ α6)	90	150 (80), 22.5 (90), 0.42 (100)	10-11	60.8[5]	18.24	7.5	3.3[6]	13.86	[17], [30]
Pyrococcus furiosus (AdhC)	100	NADP/Zn/Fe		48 (α6)	>90	160 (85), 7(95)	9.4-10.2	29.4	19.17	nd	0.17	5.48	[17], [31]
Pyrococcus furiosus (AdhD)	100	NAD/ non-metal	II	32 (α1)	>100	2.16 (100)	8.8	86.8[7]	60.76	6.1	6.5[8]	11.7	[17], [18]
Sulfolobus acidocaldarius (Adh1)	80	NAD/ non-metal	II	29 (α4)	75	0.5 (90)	8.2	6.4[9]	13.7	5.1	0.71[10]	22	[62], [64]
Sulfolobus acidocaldarius (Adh2)	80	NAD/ non-metal	II	27 (α4)	80	0.5 (88)	10	0.81[11]	16.6	5	0.11[12]	0.65	[62], [63]
Thermococcus sibiricus	78	NADP/ non-metal	II	26.2 (α2)	>100	2 (90), 1 (100)	10.5	54.4[13]	0.7	7.5	17.5[14]	2.04	[19]
Sulfolobus tokodaii	80	NAD/Zn	I	38 (α4)	85	2 (70)	10.5	9.08	0.849	7	29.7	10.20	[29]
Sulfolobus solfataricus 1617	78-87	NAD/Zn	I	37.5 (α4)	>95	5 (70), 3 (85)	8.8-9.6	0.7	0.5	8.8-9.6	nd	nd	[35], [64], [65]
Sulfolobus solfataricus MT4	80	NAD/Zn	I	35.5 (α2)	>95	20 (60), 5 (70)	8.5	0.26	1.833	7.5	0.0025[15]	0.933	[67]
Thermococcus guaymasensis	88	NADP/Zn	I	40 (α4)	>95	24 (95), 70 (80)	10.5	0.38[16]	833	7.5	0.21[17]	202	[20]
Thermotoga maritima	80	NAD/Zn	I	39.7 (α4)	80	7 (50)	7.9	39[18]	15.84	6	30[19]	8.866	[68], [69]
Aeropyrum pernix	90-95	NAD/Zn	I	39.5 (α4)	90	0.5 (90)	10.5	13.7	0.23	8	nd	nd	[23]
Pyrobaculum aerophilum	100	NADP/Zn	I	38.5 (α4)	90	nd	na	na	na	7.5	2.4[20]	1.7 x 10^2	[22]
Hyperthermus butylicus	95-106	NAD/Zn	I	33 (α4)	60	3 (60)	8.5	1.68[21]	4.36	5.0	0.41[22]	6.55	[32]

[a]Optimal temperature for organism growth
[b]Optimal temperature for enzyme activity
[c]Thermostability expressed as $t_{1/2}$, which is the time required to decrease 50% of its activity at the specified temperature
[d]Optimal pH for alcohol oxidation
[e]Apparent K_m values with ethanol, unless indicated with a note (see below)
[f]Optimal pH for aldehyde/ketone reduction
[g]Apparent K_m values with acetaldehyde, unless indicated with a note (see below)
[1](meso-2,3-butanediol); [2](acetoin); [3](benzylalcohol); [4](benzaldehyde); [5](2-pentanol); [6](pyruvaldehyde); [7](2,3-butanediol); [8](acetoin); [9]((S)-indanol); [10](isatin); [11](isoborneol); [12](2,2-dichloroacetophenone); [13](D-xylose); [14](pyruvaldehyde); [15](anisaldehyde); [16](2-butanol); [17](diacetyl); [18](glycerol); [19](DHA); [20](α-tetralone); [21](butanol); [22](propanal)
Opt.=optimal; App.=apparent; h=hour; Ref.=reference; nd=not determined; na=not applicable

Table 1: Biophysical and catalytical properties of well characterized hyperthermophilic ADHs.

studies are required to conclude that they play a role in metal ion coordination. The iron ion has catalytic and/or structural functions and iron-dependent hyperthermophilic ADHs are always oxygen sensitive since the Fe^{2+} ion gets oxidized to Fe^{3+} under oxidative conditions [1].

Cofactor specificity can be identified by the presence of certain highly conserved domains. One of them is the Rossmann fold domain which is composed of alternating beta strands and alpha helical segments and characteristic of nicotinamide cofactor-binding proteins [37]. This motif, annotated as $GXGX_2G$, can be seen in all type I ADHs [44]. The specificity of using NAD or NADP is conferred by other amino acid residues present in the chain. In type I ADHs the presence of an Asp residue at a certain position equivalent to Asp223 in HlADH makes the enzyme NAD(H) specific. The Asp residue binds to the adenosine ribose of NAD^+ *via* two hydrogen bonds with the hydroxyl groups. In NADP-dependent type I ADHs a Gly, Ser, Arg, and sometimes a Tyr residue can be found approximately at the position of the Asp residue as in an NAD specific enzyme [1]; This is be seen in the ADH of *T.*

guaymasensis [20].

In type III ADHs, a highly conserved glycine-rich motif equivalent to Gly96-Ser99 in ZmADH is required for accepting NAD^+ and $NADP^+$. The specificity between the cofactors is brought by many residues, but one specific residue, an Asp residue at the 39th position in ZmADH has been identified to be crucial in spatial determination of accepting NAD^+ over $NADP^+$ [43]. All type III hyperthermophilic ADHs are $NADP^+$ dependent and have a Gly instead of the Asp residue.

Biochemical properties

The substrate specificity and enzyme kinetic parameters are vital factors that can be used to deduce the physiological roles of the ADH in an organism and further elucidate possible industrial applications. The substrate specificities of ADHs in oxidation and reduction are tested using a range of primary and secondary alcohols, aldehydes and ketones including aliphatic, cyclic, branched and aromatic.

Even though most of the hyperthermophilic ADHs are capable of oxidizing some alcohols and reducing either aldehydes or ketones, only one of those reactions may take place within the organism, depending on the substrate concentrations and apparent K_m values towards certain substrate(s). Determination of the $K_m$values of an ADH sheds light on its physiological role and also aids in selecting possible candidates for industrial alcohol production. For example, ADH of *T. litoralis* has K_m values of 11.1 mM (in the presence of 0.4 mM NADP) and 33 μM (in the presence of 60 mM ethanol) for ethanol and NADP, respectively [26]. However, the K_m values for acetaldehyde and NADPH were 0.4 mM (in the presence of 0.3 mM NADPH) and 0.3 mM (in the presence of 4 mM acetaldehyde), respectively [26]. This indicates that the ADH has a lower $K_m$value towards acetaldehyde rather than ethanol and would have a physiological role in reducing acetaldehyde rather than oxidizing ethanol [26]. The same could be true for ADH of *Thermococcus* strain ES1 where the K_m value of NADPH and acetaldehyde was almost 10 times lower than that of NADP and ethanol while the catalytic efficiency (k_{cat}/K_m) for NADPH and acetaldehyde was approximately 3 times greater than that for NADP and ethanol [21]. ADH of *T. guaymasensis* also has lower K_m value towards ketone than alcohol [20]. The apparent K_m for diacetyl reduction was found to be 0.21 mM. Compared to other Zn containing ADHs, this enzyme exhibited the highest thermoactivity (1,149 U/mg) and thermostability ($t_{1/2}$ of 24 h at 95°C) [20]. Some other hyperthermophilic ADHs that efficiently catalyze alcohol production are those from *P. furiosus*, *T. hydrothermalis*, *A. pernix*, and *S. solfataricus* MT4 (Table 1).

The ADH of *P. aerophylum* is only active with α-tetralone as substrate [22]. The apparent K_m was found to be 2.4 mM and the catalytic efficiency was 716.4 $s^{-1}mM^{-1}$ [22]. The recombinant *H. butylicus* ADH showed a preference in reducing propanal to 1-propanol [44]. However, the optimal temperature for enzyme activity was found to be 60°C while the optimal temperature for growth of the organism was 95°C [44]. At 95°C the enzyme lost 85% of its activity. Therefore, it is hypothesized that this ADH characterized could not be responsible for the production of 1-butanol in *H. butylicus* since it takes place at 95°C [44]. ADH of *S. tokodaii* reacts towards broad substrates including primary alcohols, secondary alcohols and various aldehydes except formaldehyde [29]. However, it was found that the V_{max} values of reduction reactions are much higher than the oxidation reactions, but the K_m values for substrates such as 1-pentanol, 1-hexanol and benzyl alcohol are lower than those for the corresponding aldehydes [29]. Therefore, it indicates that ADH of *S. tokodaii* prefers catalyzing the oxidation of alcohol instead of the reduction of aldehydes/ketones.

Ethanol production at high temperature

Traditionally bioethanol is produced at temperatures between 25°C-37°C in order to maintain optimal growth of mesophilic ethanol producers. It has been reported that fermentation at elevated temperature provides many advantages including a considerable amount of cost reduction. According to Abdel-Banat et al. [45], increasing the fermentation temperature by 5°C could save approximately $800,000 per year, for a 30,000-kL scale ethanol plant, in terms of cooling, reducing contamination and simultaneous saccharification, and fermentation. When using elevated fermentation temperatures, thermotolerant ethanol producers can be employed instead of mesophilic organisms such as *S. cerevisiae* and *Z. mobilis*. Unlike mesophilic ethanol producers, many hyper/thermophilic microorganisms are known to utilize pentose sugars from the abundantly available lignocellulosic material. Using this as a substrate would make bioethanol production more economically viable [46,47].

An ideal ethanol producer is expected to produce high yields of ethanol with few or no by-products, have low inhibitor sensitivity and high ethanol tolerance [46,47]. By nature, hyper/thermophilic organisms do not carry out homo ethanol fermentation and do not exhibit high product tolerance [47]. For instance, less than 1 mM of ethanol was found in the tested cultures of *T. guaymasensis* and *H. butylicus* [20,44]. Strain development, often involving metabolic engineering, is used to address these deficiencies. Many thermophilic microorganisms have been metabolically engineered to give a high titer of ethanol. *Thermoanaerobacterium saccharolyticum* strain ALK2 which had acetate production eliminated by deletion of phospho-transacetylase and acetate kinase was reported to produce a titer of 33 g/l [48]. Some thermophilic *Clostridium* species like *C. thermocellum* and *C. thermohydrosulfuricum,* can withstand ethanol concentrations up to 40 g/l and deletion of a key hydrogenase maturation protein, *hydG*, increased ethanol yield by 53% of theoretical [49,50]. *Geobacillus thermoglucosidasius* can tolerate up to 10% ethanol (v/v) [46]. Eliminating lactate production, deleting pyruvate-formate lyase and overexpressing pyruvate dehydrogenase caused an increase in ethanol titer up to 15.9 g/l [51]. In the thermotolerant yeast *Ogataea polymorpha*, reducing the ethanol stress by overexpressing gamma glutamylcysteine synthetase resulted in a titer of 45 g/l [52].

Some work has been done by manipulating the ADH gene in hyper/thermophilic species. Overexpression of bifunctional alcohol and aldehyde dehydrogenase, *adhE*, in *Thermoanaerobater ethonolicus* JW200 resulted in a 3-fold increase in ALDH activity and a corresponding 40% increase in ethanol production [53]. The same technique was applied to the anaerobic thermophile *Thermoanaerobacter mathranii*. The resulting strain produced ethanol from xylose at a yield of 95% of theoretical [54]. The introduction of *adhE* from *C. thremocellum* in *Caldicellulosiruptor bescii*, an extremely thermophilic anaerobic bacterium, caused the strain to produce ethanol at 33% of the maximum theoretical yield [55]. The hyperthermophilic archaeon *P. furiosus* was engineered with *adhA* from *Thermoanaerobacter* sp. X514. The expression of this gene caused the strain to produce ethanol with a yield of 35% of theoretical [56]. It should be noted that so far no bifunctional alcohol and aldehyde dehydrogenase have been found in hyperthermophilic microorganisms.

The advancement of cloning and expression of genes has facilitated the expression of hyper/thermophilic ADHs in mesophilic hosts. This circumvents the difficulties in cultivating the thermophilic organisms in large scale, low basal level expression of the enzymes in native hosts, and complex purification processes [57]. *E. coli* seems to be the most preferred expression host but other mesophilic organisms such as

Bacillus subtilis, *Kluyveromyces lactis*, mycelial fungus *Trichoderma reesei* have also been used [33,58]. Comparison of the native and recombinant ADHs have showed that the recombinant enzyme almost always retains all of the native enzyme's biophysical and biochemical properties, such as proper folding, thermostability, substrate specificity, and optimal activity at high temperatures [59,60]. Moreover, structural studies have also showed that the recombinant protein show little to no difference compared to their native counterparts [61]. The first hyperthermophilic ADH to be cloned in *E. coli* was from *T. hydrothermalis* [62]. This has been followed by many other hyper/thermophilic ADHs such as *P. furiosus* [30], *T. maritima* [36], *A. pernix* [37], *T. guaymasensis* [Ma et al. unpublished] and *Thermococcus* strain ES1 [21].

Conclusion and Future Perspectives

ADHs are found in all three domains of life and play a vital role in many metabolic reactions including alcohol oxidation and aldehyde/ketone reduction. It is one of the major enzymes in the alcohol fermentation pathway, which makes it very important for the commercial production of bioalcohols. Traditionally, mesophilic organisms (or ADHs derived from them) are used for alcohol production but recently hyper/thermophilic organisms and their ADHs have come under scrutiny. Compared to their mesophilic counterparts, many hyperthermophilic microorganisms can utilize complex sugars such as cellulose, hemicellulose, and xylose as carbon source and grow optimally at elevated temperatures. Their enzymes, including ADHs, show activity at high temperatures, different pHs, and increased solvent concentrations [63-69].

Ethanol production at elevated temperatures using hyper/thermophilic organisms have been found to have many advantages including significant cost reductions. Although most hyper/thermophilic organisms do not naturally achieve an ethanol yield of >90% of theoretical, titer of >40 g/l, and productivity of >1 g/l/h [46,47], metabolic engineering has yielded very positive results. Studies on hyperthermophilic ethanol production are still at its infancy. Many hyperthermophilic species have had their genomes sequenced and potential ADHs have been identified, however, they are yet to be characterized. After characterization, the physiological role and levels of expression under certain conditions could be analyzed using techniques such as quantitative reverse transcription PCR. This information, combined with metabolic engineering could lead to the development of a consolidated bioprocessing hyperthermophile that could produce commercially viable amounts of ethanol using lignocellulosic carbon source.

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