

Alcohol Dehydrogenases from Marine Hyperthermophilic Microorganisms and Their Importance to the Pharmaceutical Industry[‡]

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ABSTRACT

Alcohol dehydrogenases (ADHs) are enzymes capable of catalyzing the interconversion of alcohols to their corresponding aldehydes or ketones by using different redox-mediating cofactors. They are valuable tools for the biocatalytic synthesis of various chiral compounds, which are potential building blocks or intermediates in the organic synthesis of therapeutics in the pharmaceutical industry. In particular, ADHs from marine hyperthermophilic microorganisms have attracted special attention as catalysts in several industrial processes over the last two decades, mainly due to some unique properties, including their relatively broad specificity towards various carbonyl substrates, their high thermal and chemical stability, as well as their suitability for cofactor regeneration. Therefore, this review article describes ADHs from marine hyperthermophiles, including their activity screening, cloning, heterologous production, and characterisation. Their potential for application in the pharmaceutical industry is discussed.

List of Contents:

ABSTRACT.....	1
1. INTRODUCTION: A Brief Overview of ADHs.....	2
2. REACTION AND CLASSIFICATION OF ADHs.....	3
3. (META)GENOMIC-BASED SCREENING OF ADHs.....	4
3.1. Genomic-based Screening.....	4
3.2. Metagenomic-based Screening.....	6
4. HETEROLOGOUS EXPRESSION OF ADHs.....	7
4.1. Bacterial System.....	8
4.2. Archaeal System.....	9
5. PHYSIOCHEMICAL PROPERTIES OF ADHs.....	10
5.1. Structural Properties.....	11
5.2. Functional Properties.....	13
6. PHARMACEUTICAL POTENCY OF ADHs.....	14
6.1. Chiral Compound Preparation.....	14
6.2. Cofactor regeneration.....	16
7. CONCLUDING REMARKS.....	17
REFERENCES.....	18

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1. INTRODUCTION

Oceans are vast untapped reservoirs of highly diverse and unique natural products accumulated in marine organisms. Enzymes belong to the most interesting natural products from both scientific and industrial perspectives. They are enjoying increasing popularity in the chemical and pharmaceutical industries as environmental friendly, economical, and clean catalysts (Wahler and Reymond, 2001). By employing recombinant DNA technology, enzymes can be produced in a relatively short time in quantities that would allow large-scale applications. Using a recently-developed, holistic molecular approach called metagenomics, they can even be accessed from the unculturable microbial world with a genetic diversity of more than 99.8% in most environments, including marine environment (Handelsman, 2004). For such reasons, it appears that enzymes are gradually taking over from chemical catalysis in many industrial applications (Van der Oost and De Graaff, 2001).

In recent years, enzymes belonging to the oxidoreductase group such as alcohol dehydrogenases (ADHs) have caught much scientific attention in the pharmaceutical industries. They are highly desirable as biological catalysts in the production of chiral pharmaceutical intermediates or building blocks in the organic synthesis of drugs (Patel, 2004; Clark and Ensign, 2002, D'Auria, 2003). Use of such enzymes as biocatalysts offers many advantages over chemical catalysts, including their high enantioselectivity and regioselectivity, their high catalytic activity, the easy and reproducible up-scaling of the process, the lower energy consumption due to reduced process temperature, the utilization of low cost raw materials (such as aldehydes and ketones), and the reduced costs due to reduced application of organic solvents (Julich Chiral Solutions-Germany, <http://www.julich.com/>). In addition, enzymes (including ADH) can be immobilized and reused for many enzymatic cycles, and they can be over-produced to make catalytic processes more economically efficient (Patel, 2004). This trend plays an important role in the use of chiral drugs (D'Auria, 2003).

Many ADHs of microbial origin have previously been explored for industrial applications. However, the majority of them originate from the microorganisms living at relatively low and moderate temperatures, which are referred to as mesophiles. Mesophilic ADHs in general suffer some disadvantages, such as their instability at high temperatures, their relatively narrow substrate specificity, their poor stereospecificity, their rather high sensitivity to organic solvents, and their tendency to lose their activity during immobilization (Hummel and Kula, 1989; Inoue, *et al.*, 2005). For such reasons, there has been considerable interest in ADHs from heat-loving microorganisms over the last two decades. They are not only active at higher temperatures, but also more stable at moderate temperatures and in the presence of organic solvents (Hummel and Kula, 1989; Yoon, *et al.*, 2002). In addition, many of them can perform activity towards a wide range of substrates such as aliphatic, cyclic and aromatic alcohols, diketones, and keto acid esters (Yoon, *et al.*, 2002; Guagliardi, *et al.*, 1996; Van der Oost, *et al.*, 2001; Uria, 2004).

There are two related terms used to describe heat-loving microorganisms: (i) thermophiles referring to those having the optimal growth between 60-80°C and (ii) hyperthermophiles for those with the optimal growth of >80°C (Kristjánsson and Ásgeirsson, 2003). In relation to that, ADHs from

hyperthermophiles are very desirable in many biotechnological and industrial processes which are frequently carried out at high temperatures. There are some benefits of operating such processes at higher temperatures including decreased viscosity, increased diffusion rates, solubility of most organic compounds, an increase in reaction rates, and reduced risk of microbial contaminations (Kristjánsson and Ásgeirsson, 2003).

To meet the industrial needs for a variety of hyperthermophilic ADHs with specific enantiomeric activities, it is necessary to do extensive screening from various temperature-elevated marine biotopes, such as hydrothermal vents, volcanic marine sediments, coastal hot springs, and deep sea chimneys. Further studies on how they can be recombinantly expressed and purified are very useful to provide them in large amount for generating chiral pharmaceutical compounds. Subsequently, detailed understanding on their properties can give insight about how to employ them optimally in the industrial scale. Therefore, this review article will deal with ADHs from marine hyperthermophilic, starting from their screening from marine nature, via their heterologous production and characterisation, towards their application potency in the pharmaceutical industry.

2. REACTION AND CLASSIFICATION OF ADHs

Alcohol dehydrogenases (ADHs; EC 1.1.1) are enzymes capable of catalyzing the reversible conversion of alcohols to their corresponding aldehydes or ketones by using a variety of cofactors. They can be found both in prokaryotes and eukaryotes. They vary with respect to their structural organization and their catalytic mechanism (Reid & Fewson, 1994). ADHs are oxidoreductases that are dependent on a variety of different electron-mediating cofactors. Therefore, they can be classified on the basis of their cofactor specificity (Reid & Fewson, 1994; Radianingtyas & Wright, 2003): (i) NAD(P)-dependent alcohol dehydrogenases (ADHs), (ii) F₄₂₀-/Zn-/PQQ-dependent alcohol dehydrogenases, and (iii) FAD-dependent alcohol oxidases. Of the three ADH classes, the NAD(P)-dependent ADHs appear very abundant in the Thermococcales. This group uses NAD(P)⁺ and NAD(P)H as the electron donor and acceptor for catalyzing the interconversion of alcohols with the corresponding aldehydes or ketones. In particular, it converts reversibly primary (1°) alcohols into aldehydes, and secondary (2°) alcohols into ketones (Fig. 1). In methanogenic archaea such as *Methanoculleus thermophilicus* an ADH has been identified that uses the F₄₂₀ cofactor (Radianingtyas & Wright, 2003).

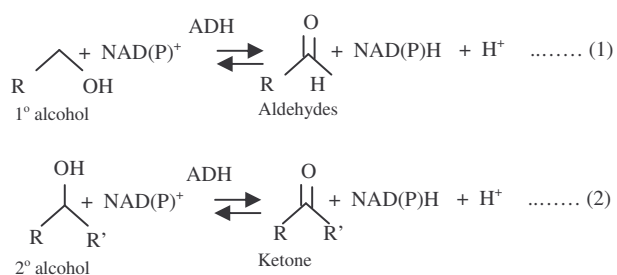


Fig. 1. The interconversion of alcohols into the corresponding aldehydes or ketones catalyzed by NAD(P)-dependent ADH (Benach, 1999).

The NAD(P)-dependent ADHs can be further subdivided according to the size of their amino acid sequences into three distinct classes (Reid & Fewson, 1994; Antoine, *et al.*, 1999; Van der Oost *et al.*, 2001; Klimacek, *et al.*, 2003; Guy, *et al.*, 2003), namely: (i) type I, medium-chain dehydrogenase/reductases (MDR, ~350 amino acids per subunit); (ii) type II, short-chain dehydrogenase/reductases (SDR, ~250 amino acids); and (iii) type III, long-chain dehydrogenases (LDH, ~ 360-550 amino acids, but often as many as 900 residues). The ADHs belonging to type I may be dimeric in higher eukaryotes (Guy, *et al.*, 2003) or tetrameric forms in bacteria and yeast (Esposito, *et al.*, 2002), represented by horse liver ADH and *Saccharomyces cerevisiae* ADH I, II, and III (Antoine, *et al.*, 1999). This type mostly contains zinc (Guy, *et al.*, 2003; Esposito, *et al.*, 2002); so it is widely known with the name, zinc-dependent medium chain ADHs. The ADHs of type II have been characterized from both prokaryotes and eukaryotes (Reid & Fewson, 1994). Since the type II rarely contain metals, including zinc (Guy, *et al.*, 2003), it is commonly called zinc-independent short chain ADHs. The type III ADHs seem to be exclusively of microbial origin. Since they show little sequence homology with type I or II and are frequently activated by iron, they are also referred to as iron-activated long chain ADHs (Reid & Fewson, 1994).

A recently identified class of NAD(P)-dependent ADHs that is not closely related to one of the above mentioned groups, has resulted in a different superfamily called the aldo-keto reductases (AKRs; Jez, *et al.*, 1997). All of the enzymes belonging to AKRs are NAD(P)-dependent. So far this AKR superfamily is divided into 14 families, named AKR1-AKR14, found in both prokaryotes and eukaryotes (Hyndman, *et al.*, 2003). The majority of mammalian enzymes belongs to the AKR1 family, but there is diversity between the microbial enzymes, which are distributed in 10 families: AKR2 (xylose reductases), AKR3, AKR5, and AKR8-AKR14 (Ellis, 2002). Most of the known AKRs are monomeric ($\alpha\beta$)₈-barrel proteins, about 320 amino acids in length (Jez, *et al.*, 1997; Jez and Penning, 2001). However, recent evidence suggests multimeric forms in AKR2, AKR6, and AKR7 families (Hyndman, *et al.*, 2003). They metabolize a wide range of substrates, including aldehydes, monosaccharides, prostaglandins, and polycyclic aromatic hydrocarbons (Jez, *et al.*, 1997).

3. (META)GENOMIC-BASED SCREENING OF ADHs

3.1. Genomic-based Screening

Screening of ADHs from marine hyperthermophilic microbes is normally done by isolating such microbes in pure cultures from temperature-elevated marine environments, followed by isolating ADHs of interest. However, this way in most cases is hampered by the difficulty to prepare the ADHs in large amount as well as in pure form. An alternative way is finding genes potentially encoding ADHs in microbial genomes, and then cloning them in bacterial production strains, aiming at producing recombinant ADHs. In the absence of a genome sequence, finding a specific ADH gene is a difficult and challenging task. In this condition, total DNA is normally prepared from a microbe, and then digested into fragments. All resulting fragments can be cloned into an appropriate expression

vector, and transformed to a bacterial host; subsequently each clone from this so-called expression library can be screened for ADH activity.

Recent advances in DNA sequencing techniques during the last decade have led to the increasing accumulations of complete genome sequences of many microbes in public databases (for the update please refer to <http://wit.integratedgenomics.com/GOLD/>). This makes it significantly easier to identify and analyse all genes on a genome, including ADH genes, by using various existing computational software tools. Subsequently target genes are generally isolated from the genome by either Polymerase Reaction Chain (PCR), and then over-expressed in suitable laboratory hosts, thereby producing recombinant enzymes (Fig. 2).

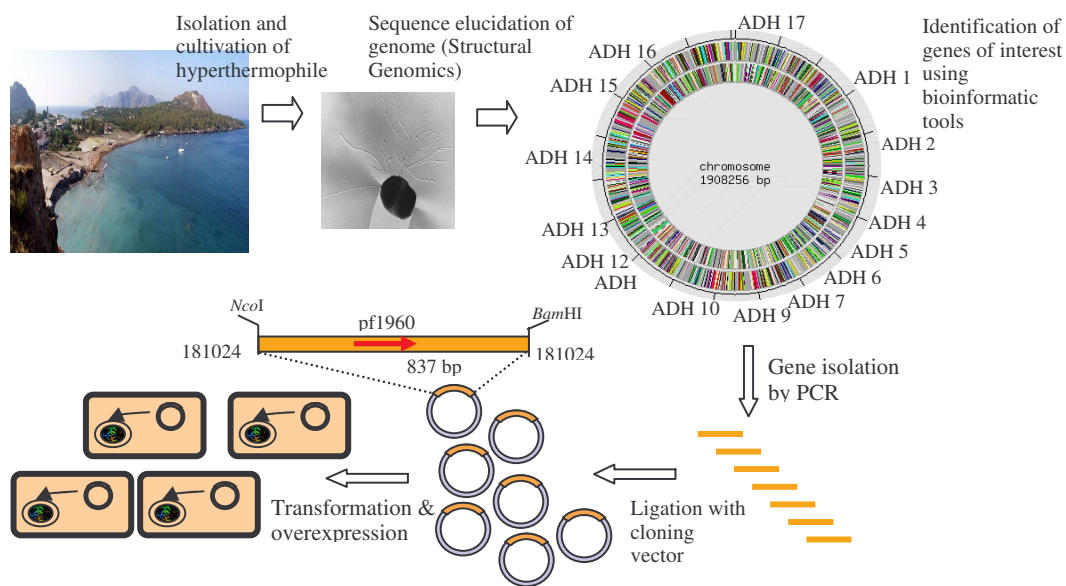


Fig. 2. Genomic-based screening of ADHs from culturable hyperthermophiles

Such genome based screening has been demonstrated in the search for alcohol dehydrogenases from the marine hyperthermophilic archaeon, *Pyrococcus furiosus*. This member of the order Thermococcales was isolated from geothermally heated marine sediments at Vulcano island in Italy and grows optimally at 100°C by the fermentation of peptides and carbohydrates to produce acetate, CO₂, alanine and H₂, together with minor amounts of ethanol. The organism will also generate H₂S if elemental sulfur is present (Fiala and Stetter, 1986; Kengen, *et al.*, 1994; Kengen, *et al.*, 1996). Three different alcohol dehydrogenases were previously identified in *P. furiosus*: (i) a short-chain AdhA and (ii) an iron-containing AdhB encoded by the *lamA* operon (Van der Oost *et al.*, 2001) and (iii) an oxygensensitive, iron-and zinc-containing alcohol dehydrogenase that has been purified from cell extracts of *P. furiosus* (Ma and Adams, 1999).

Automated function predictions for all genes in the genomes of the related marine species *P. abyssi*, *P. furiosus* and *P. horikoshii* were carried out by Pfam (*Protein Family*) annotation and COG (*Cluster of Orthologous Groups of Genes*) annotation. The results were presented in a web-accessible database, Pyrobases (<http://www.cmbi.ru.nl/pyrobases/>). Pyrobases database was scanned for homologs

of domains relevant for the conversion between alcohols and aldehydes or ketones. Four promising candidate alcohol dehydrogenase families were selected, containing 17 genes in *P. furiosus*: iron-containing alcohol dehydrogenase (Fe-ADH), short chain dehydrogenase (*adh_short*), zinc-dependent dehydrogenase (*adh_zinc*) and UDP-glucose/GDP-mannose dehydrogenase family (UDPG_MGDP_dh).

Table 1. Putative alcohol dehydrogenases in the marine hyperthermophile, *Pyrococcus furiosus*

Pfam domain	COG	Gene	Plasmid	Annotation
<i>adh_short</i>	COG1028	PF0577	pWUR75	Alcohol dehydrogenase (EC 1.-.-.-)
		PF1899	pWUR84	3-oxoacyl-ACP reductase (EC 1.1.1.100)
		PF0149	pWUR73	Enoyl-ACP reductase (EC 1.3.1.9)
		PF0074 (AdhA)	pWUR106	Alcohol dehydrogenase (EC 1.1.1.1) (Van der Oost, <i>et al.</i> , 2001)
	COG0451	PF1853	pWUR83	Glucose 1-dehydrogenase (EC 1.1.1.47)
		PF1788	pWUR82	UDP-glucose 4-epimerase (EC 5.1.3.2)
		PF0402	pWUR74	UDP-glucose 4-epimerase (EC 5.1.3.2)
	PF1357	pWUR79	dTDP-glucose 4,6-dehydratase (4.2.1.46)	
<i>adh_zinc</i>	COG1063	PF0991 (PfTDH / AdhC)	pWUR78	L-threonine dehydrogenase (EC 1.1.1.103) (Machielsen and Van der Oost, 2006)
Fe-ADH	not assigned	PF0075 (AdhB)	pWUR72	Alcohol dehydrogenase (EC 1.1.1.1) (Van der Oost, <i>et al.</i> , 2001)
	COG1454	PF0608	pWUR76	Alcohol dehydrogenase (1.1.1.1)
	COG0371	PF1382	pWUR80	Glycerol-1-phosphate dehydrogenase (EC 1.1.1.261)
UDPG_MDPG_dh	COG0677	PF0765	pWUR102	NDP-sugar dehydrogenase
		PF0799	-	Gene fragment
		PF1631	pWUR81	N-acylmannosamine dehydrogenase (EC 1.1.1.233)
	COG1004	PF0771	pWUR77	UDP-glucose dehydrogenase (EC 1.1.1.22)
		PF1355	pWUR103	UDP-glucose dehydrogenase (EC 1.1.1.22)
Aldo/keto reductase	COG0656	PF1960 (AdhD)	pWUR85	Alcohol dehydrogenase (EC 1.1.1.1) (Machielsen, <i>et al.</i> , 2006)

In addition, the genome of *P. furiosus* was searched for homologs of canonical NAD(P)-dependent alcohol dehydrogenases by BLAST (*Basepair Length Algorithms Search Tools*) search analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>). This analysis of the *P. furiosus* genome for genes encoding putative alcohol dehydrogenases resulted in the identification of *adhA*, *adhB* and 16 genes that potentially encode alcohol dehydrogenases (Table 1). For all identified genes a more detailed level of function prediction was attempted using STRING (*Search Tool for the Retrieval of Interacting Genes/Proteins*) genomic context analysis (<http://string.embl.de/>) and Pfam and COG annotation. After successful production in *E. coli*, an initial screening for activity was performed in which two of the putative alcohol dehydrogenases showed relatively high activities. The two enzymes were selected for a more detailed study (*AdhD* – Machielsen, *et al.*, 2006; *AdhC/PfTDH* – Machielsen and Van der Oost, 2006). With respect to the other putative alcohol dehydrogenases, a more elaborate screening is required to obtain insight into their substrate specificity and possibly their physiological function.

3.2. Metagenomic-based Screening

For many years, discovery of new biocatalysts, including ADHs from microorganisms, were mostly based on cultivation-dependent methods, in which microorganisms are enriched and isolated in

pure cultures, followed by extracting the biocatalysts of interest. However, these methods count only less than 1 % of the microbial diversity in most environments, including marine environment, since the majority (over 99%) can not be grown under laboratory conditions (Arnold, 2001).

A recent breakthrough in the area of Microbial Ecology has led to the birth of a powerful approach for gaining access to the enormous uncultured microbial diversity (Riesenfeld, *et al.* 2004). This cultivation-independent approach, popularly known as metagenomics, provides a faster way of discovering an unlimited pool of new potential biocatalysts, genes, and biosynthetic pathways from uncultivable marine microorganisms (Arnold, 2001; Daniel, 2001). More recently, many single genes, such as those coding for ADH (Knietsch *et al.*, 2003) and cellulolytic enzymes (Handelsman, 2004) have been successfully collected from environments and expressed by using metagenomic-based screening.

Metagenomic-based screening of enzymes, including ADHs, from the uncultured hyperthermophilic microorganisms may involve the following stages (Fig. 3A) (Uria, *et al.*, 2005): (i) construction of a metagenomic library, (ii) activity screening of the metagenomic library, (iii) sequence analysis of selected clones, and (iv) expression of ADH genes of interest in appropriate hosts, thereby producing the corresponding ADHs. The performance of the ADHs obtained through metagenomics can then be improved by mutagenesis, such as Laboratory Evolution (Fig. 3B). By this approach, a series of biocatalyst properties have been improved, in the absence of detailed structural and functional information, including stability, catalytic activity, and specificity towards new substrates (Arnold, 2001).

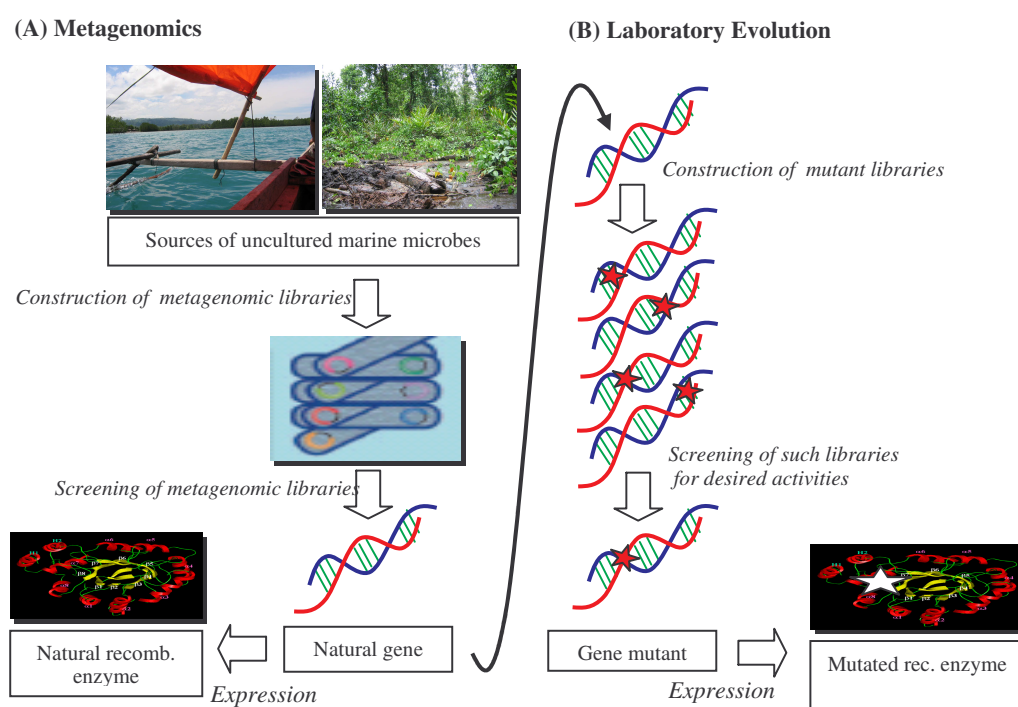


Fig. 3. Metagenomic-based screening of ADHs from uncultured hyperthermophiles, which can be coupled with laboratory evolution.

The metagenomic library is normally constructed by isolating total DNA from the obtained uncultivated cells, followed by its cloning into an easily cultivable bacterial host, thereby resulting in clones, which are collectively known as a metagenomic library. The DNA insert present in a positive clone may contain functional genes (encoding enzymes), phylogenetic genes (markers that give insight in the phylogenetic origin of the DNA fragment, such as rRNA gene), or both. Construction of metagenomic libraries from a marine environment for the discovery of biocatalysts can be performed by various molecular methods. The metagenomic library constructed needs to be screened in order to detect transformants containing functional genes (e.g. ADH genes) as well as phylogenetic markers. If the functional genes present in transformants are expressed, such screening can be done by assaying the activity of the expression products, including biocatalysts. However, in the case that the ADH genes are not expressed, thereby no corresponding enzymes are produced, employing *in situ* hybridization or PCR–amplification using specific primers might be the right choice. These methods enable the detection of target genes in each clone.

The clones with ADH activity, and hence with ADH genes of interest, are recovered from the transformants for sequence analysis. The sequence data are analyzed by using the existing bioinformatics tools and databases, which are freely available on internet. The analysis results can provide useful information on the functional and structural properties of the biocatalysts encoded by corresponding genes. If the properties are different from those of the known biocatalysts available in the databases, it means that the cloned genes and corresponding biocatalysts may have novel features. In the case that a functional gene is located in the same single DNA insert as a phylogenetic marker (e.g. 16S rRNA gene), sequence analysis of the marker can give a clue about the microbial source of the functional gene.

Metagenomic-based screening for obtaining ADHs from uncultured microorganisms has been successfully been demonstrated by Knietzsch *et al.* (2003). Total DNA was directly retrieved from an environmental sample without prior cultivation, which was then partially digested by using the restriction enzyme, *Bsp*1431. The resulting DNA fragments of > 2 kb were ligated into *Bam*HI-digested pBluescript SK⁺ vector (Stratagene), and the clones formed was transformed into *E. coli* DH5 α for clone amplification, which was followed by transformation into *E. coli* ECL707 for insert expression. The expression product was prepared from the bacteria at the stationary growth phase and assayed for ADH activity. The inserts from ADH-positive clones were subsequently sequenced and analyzed. Results of such sequence analysis indicated that three open reading frames (*orfs*) were identified as novel genes encoding presumptive ADHs, four *orfs* encoded putative oxidoreductases belonging to groups different from ADHs, one encoded putative glycerol kinase, and one coded for a protein unrelated to known gene products. Interestingly, a protein deduced from *orf* located on the positive clone, designated as pAK101, revealed no significant similarity to alcohol oxidoreductase available in the NCBI and POROSITE databases; whereas another positive clone, pAK110, harbored an *orf* which showed 90% identity to the putative iron-containing type III ADH of *Salmonella enterica*. Two specific fingerprints of this ADH were available on the encoded amino acid sequences.

4. HETEROLOGOUS EXPRESSION OF ADHs

The genes recovered from both culturable and uncultured hyperthermophiles need to be over-expressed in convenient microbial production systems. This step is crucial for producing enzymes in significant amount and in pure form for both for thorough analysis and eventually for its application. For such purposes, there are two microbial systems which will be considered here, *i.e.* a bacterial and an archaeal system.

4.1. Bacterial System, *Escherichia coli*

Among the microbial systems available for gene expression, the gram-negative bacterium, *E. coli* is still the most attractive and widely used host due to its ability to grow rapidly at high density on inexpensive substrates, its well-characterized genetics, the availability of a large number of cloning vectors, and the availability of a large set of host strains (Banex, 1999). The *E. coli* strains used as host generally lack restriction enzymes; in addition, they are *recA*⁻ to reduce the recombination between the introduced heterologous DNA and the host chromosome (Prescott *et al.*, 2002). To express a foreign gene in *E. coli*, at first the gene is inserted into a suitable cloning vector, usually under the control of an inducible promoter. The product is transformed to *E. coli*. In the host, when the promoter controlling the foreign gene is recognized by the host RNA polymerase, the gene will be transcribed into mRNA molecules. The resulting mRNAs can then be translated into protein/enzyme molecules at the ribosome. The enzyme produced by this way is called recombinant enzyme.

Among a variety of *E. coli* expression systems that are commercially available, the pET system (Novagen), widely known as one of the most powerful systems for the expression of recombinant proteins, has gained increasing popularity in recent years. This system provides several vector-host combinations which enable tuning of the expression levels to optimize target gene expression. One of the most widely used vector-host combinations is pET24d in combination with the host *E. coli* BL21(DE3) strain. This combination was successfully used to express seventeen ADH genes (*adhs*) in *E. coli* BLD(DE3) strain, as reported by Machielsen, *et al* (2006). Such *adh* expression was done by two following steps: (i) inserting PCR-amplified *adh* genes into pET24d vector molecules, thereby generating recombinant plasmids designed as pWUR vectors (Fig. 4a) and (ii) transforming pWUR vectors into *E. coli* BL21(DE3) via electroporation.

The resulting desired transformants containing harboring target genes were screened by taking advantage of the antibiotic-resistance marker present in the vector. By supplementing the growth medium with a certain antibiotic, vector-free cells are killed, allowing only growth of the transformed cells. Then the presence of β -galactose in the agar plates enables to detect and isolate the desired transformants harboring target genes, which appear as white colonies. In the desired transformants, the ADH proteins were mostly produced in the soluble form (Fig. 4B). One of the generated products, designated as ADH 17 (or AdhD), showed relatively high activities towards a wide range of carbonyl substrates and relied on NAD(P) for their activity. It was purified and characterized (Uria, 2004, Machielsen, *et al.*, 2006).

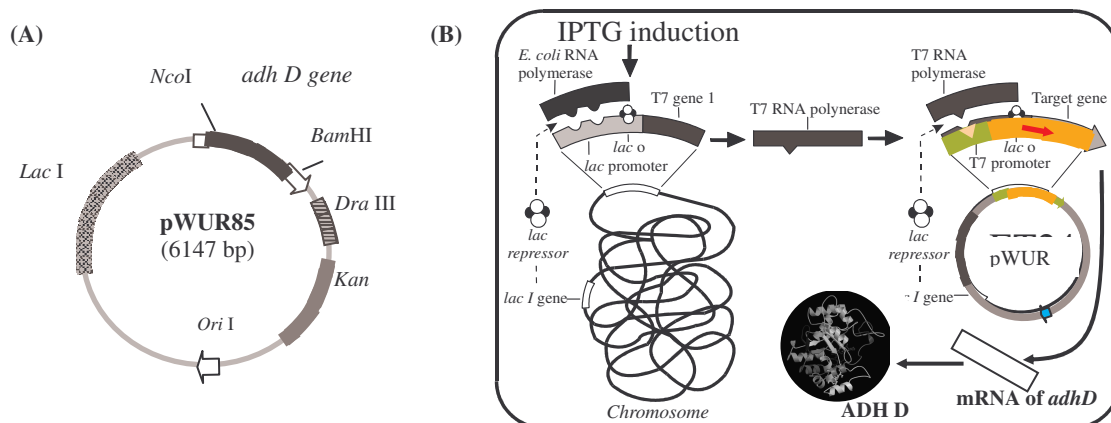


Fig. 4. pET system used for ADH expression (Novagen 2002-2003 Catalog, www.novagen.com). (A) Expression vector, pWUR85 harbouring *adhD* gene. (B) Molecular mechanisms of ADH expression in *E. coli* BLD21 (DE3) harbouring pSJS1244 after being induced with IPTG (Modified from Novagen). The T7 RNA polymerase gene was under the control of *lac* promoter located on the host chromosome. Binding the *lac* repressor molecules at the *lac* operator effectively reduces the transcription of the T7 RNA polymerase gene, leading to the inhibition of target gene expression. However, when the inducer, isopropyl- β -D-1-thiogalactopyranoside (IPTG) was supplemented into the bacterial growth medium, it inactivated the repressor binding, leading to the expression of the target gene, *adh* gene. Abbreviations: *Kan*, kanamycin resistance gene; *adh D*, alcohol dehydrogenase D gene; *Dra III*; *Lac I*, lactose gene; *Ori I*, origin of replication I.

4.2. Archaeal Systems, *Pyrococcus* and *Thermococcus*

At present, the exploitation of hyperthermophilic archaeobacteria as cell factories for overexpressing interesting heterologous ADHs is still in the early stages of development. There are some benefits of using thermophilic microorganisms in biotechnological and industrial applications, including lower fermentation/ feedstock cost, decreased viscosity of culture broth, sterility requirements are not as stringent as for mesophiles, the possibility that low biomass leads to high product yield (Bergquist, *et al.*, 1987). So far, among the extensively studied hyperthermophiles, certain species of *Sulfolobus*, *Pyrococcus* and *Thermococcus* are good candidates as the production hosts. This is due to the availability of their genome sequences in the public databases, which enables the full development of metabolic engineering. Other reasons are that they are relatively easy to handle and can be grown to high cell density (Lucas, *et al.*, 2002).

Recently, Contursi, *et al.* (2003) reported the successful expression of thermostable ADH in *S. solfataricus* by using the vector, pEXSs. In addition, a pyrococcal shuttle vector designated as pYS3 (Fig. 6) was successfully established by Lucas, *et al.* (2002). The vector was equipped with replication elements and selectable markers which allows it to work well both in *E. coli* and pyrococcal systems. The replication elements and selectable markers for *E. coli* are CoIE1 origin of replication (*Ori*) and ampicillin resistant-coffering gene (*Ap* gene); whereas those for *Pyrococcal* host are *rep75*, *dso* & *ssu* and orotidine-5'-monophosphate decarboxylase (OMPdecase) gene (*PyrF*) & phosphoribosyltransferase (OPRTase) gene (*PyrE*), respectively. Both *pyrE* and *pyrF* genes are known to be involved in the pyrimidine (uracil) biosynthesis; and microbial strains deficient in one of these genes are uracil auxotrophs and resistant to 5-fluoroorotic acid (5-FOA). Thus 5-FAO inhibits growth of the wild-type strains and can be used for positive isolation of uracil auxotrophs in many microorganisms (Lucas, *et al.*, 2002).

Strong promoters are critical elements for gene expression and they have been isolated from various microorganisms. A promising promoter for the future is the *Psip* promoter, of which the function was strongly controlled by elemental sulfur (Schut, *et al.*, 2003). This promoter has successfully been isolated and amplified from *P. furiosus* genome by using PCR and then combined with a pyrococcal *adh* gene (Uria, 2004). This *adh-Psip* hybrid then needs to be incorporated into a shuttle vector, such as pYS3, and the resulting recombinant plasmid can be transformed into *E. coli* BL21(DE3) to obtain large amounts of the plasmid. The recombinant vector containing the *adh* gene is subsequently cloned into a *pyrF*-deficient mutant of the deep-sea hyperthermophilic host, *P. abyssii*, which is resistant to 5'-FAO. The resulting *P. abyssii* transformants containing such recombinant vector are expected not to grow on the plate medium supplemented with 5'-FAO, allowing their detection on the master plate (Fig. 5). By supplementing S^0 into the growth medium, the promoter could be activated, leading to the overexpression of the *adh* gene.

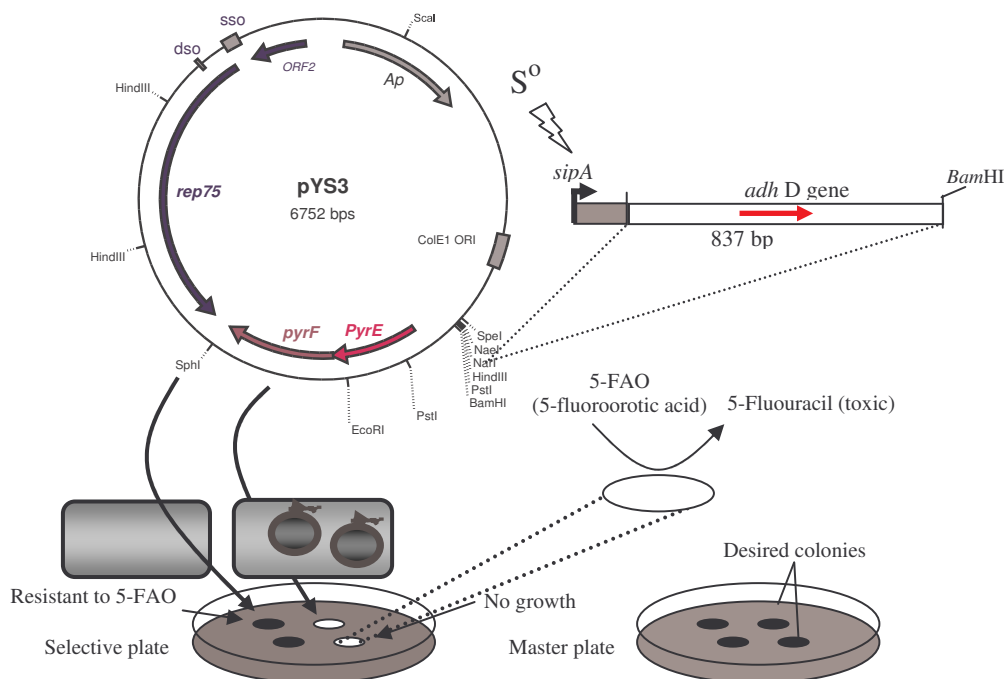


Fig. 5. A negative selection-based strategy for detecting the desired transformants harboring a recombinant shuttle vector.

However, recent reports by Sato, *et al.* (2003) showed that *pyrF*-deficient mutants (uracil auxotrophs) could grow on uracil-free plate medium despite its uracil auxotrophy in the liquid medium, as shown for KU25 (with a point mutation in *pyrF*), probably due to the presence of some pyrimidine-related compounds in the solid media. This phenomenon suggests the limitation of using *pyrF* mutant as a host and *pyrF* gene as a selectable marker. To solve such limitation, Sato, *et al.* (2003,) then developed an alternative archaeal mutant system based on tryptophan auxotrophy (*trpE*), an easily detectable phenotype, with the *trpE* as a selectable marker. This was initiated by generating uracil-auxotrophic mutants of *T. kodakaraensis* KOD1, which resistant to 5'-FAO, by cell exposure with and without UV irradiation. The two mutated strains were found to be deficient in *PyrF* gene

(*PyrF*⁻) lacking OMPdecase activity, whereas other four strains were *PyrE*⁻ mutants showing no OPRase activity. One of the six mutants, designated as KU25, was further chosen for transformation studies, because its *pyrF* gene was completely inactivated by the 1-bp deletion. In the transformation studies, a plasmid vector harboring the wild-type *pyrF* and two *trpE* genes were constructed and then transformed into the KU25 lacking *pyrF* gene, thereby causing a homologous recombination by double crossover. In such crossover event, the *pyrF* gene on the vector was integrated into the *trpE* locus on the KU25 chromosome through the disruption of the internal part of the host *trpE* and substitution with *pyrF*, leading to a phenotypic change of auxotrophy from uracil to tryptophan.

However, such tryptophan-auxotrophic thermococcal system (KU25) was considered to have following drawbacks: (i) various mutations induced by UV irradiation might occur in other genes on the KU25 chromosome, (ii) the *pyrF* mutation in KU25 was one nucleotide deletion only, suggesting unintended recombination between the selectable marker, *pyrF* gene and the mutated allele on the chromosome could always occurs. To solve the drawbacks, Sato, *et al.* (2005) at first constructed a new thermococcal system whose chromosomal *pyrF* gene was specifically and completely deleted by homologous recombination and uracil auxotrophy was solely caused by *pyrF* deficiency. Such *pyrF*-deficient system, designated as KU216, was used for constructing a *trpE* deletion mutant with the *trpE* gene as a selectable marker. One of the resulting mutants, designated as KW128, was found to be *trpE* deletion mutant in which almost the entire coding region of *trpE* on the KU216 chromosome was replaced by the *pyrF* marker with the CaCl₂ method. Therefore, this system overcomes the practical disadvantages in the previous system using *pyrF* as a selectable marker.

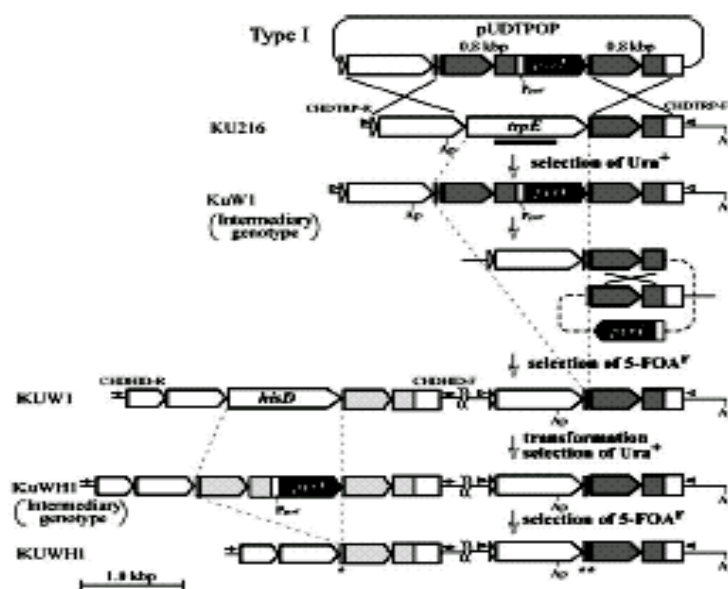


Fig. 6. Development of an archaeal system enabling multiple cycles of transformation based on pop-out recombination strategy (Sato, *et al.*, 2005). This was initiated by construction of a vector for disruption of *trpE* gene and excision of *pyrF* marker (pUDIPOP), which was followed by the transformation of such vector into the KU216. A resulting *pyrF*⁺ strain, designated as KuW1, showed the replacement of its *trpE* by *pyrF* based on the PCR analysis. This strain was subsequently cultivated on the plate medium containing 5-FAO, generating FAO-resistant strains. One of the resistant strains with the genotype of $\Delta pyrF \Delta trpE$, designated as KUW1, was transformed with pUDHPOP (a vector designed for disruption of *hisD* and subsequent pop-out excision of *pyrF*), thereby generating a triple mutant with the genotype of $\Delta pyrF \Delta trpE \Delta hisD$, designated as KUW1H1. By using such pop-out vector, the *pyrF* marker can be repeatedly used for multiple gene disruptions. The figure was copied from Sato, Fukui, Atomi, and Imanaka (2005) in *Appl. Environ. Microbiol.* 71(7):3889-3899.

The *trpE* deficient KW128, however, only enables to be used in a single cycle of transformation, since the marker used (*pyrF*) remains in the KW128 after one transformation trial. Therefore, a more versatile system enabling multiple cycles of transformation was subsequently developed through pop-out recombination, as described in Fig. 6 (Sato, *et al.*, 2005). Such recombination involved a *trpE* gene disruption and a *pyrF* marker excision, allowing the repeat use of *pyrF* marker for multiple gene disruptions.

5. PHYSIOCHEMICAL PROPERTIES

Information on the structural and functional properties of an ADH is important to know for its optimal application, as mentioned before. Many ADHs have been isolated from hyperthermophiles and investigated in term of their structural and functional properties. In our experiment, A NAD(P)-dependent alcohol dehydrogenase of *P. furiosus*, designated as AdhD, was overproduced by cloning of the expression vector harbouring *adhD* gene, pWUR85, into *Escherichia coli* BL21(DE3). The resulting AdhD was purified to homogeneity and then characterized (Uria, 2004; Machielsen *et al.*, 2006). The resulting properties are shortly described in this section.

5.1. Structural Properties

The cofactor- and substrate-binding sites for the NAD(P)-dependent ADH, from the marine hyperthermophile *P. furiosus*, were predicted using bioinformatic tools (Uria, *et al.*, 2004; Machielsen *et al.*, 2006). BLAST-P analysis, in which a sequence database is searched for sequences which are highly similar to the AdhD sequence, revealed that the amino acid sequence of AdhD showed a significant similarity to that of the enzymes belonging to aldo-keto superfamily. The AdhD sequence was very similar to enzymes from hyperthermophiles, such as *P. abyssii* morphine 6-dehydrogenase-like (M-6-DH, 80%), *S. solfataricus* aldehyde reductase (ARD, 53%), and *T. maritima* aldo-keto reductase (AKR, 69%).

Those three sequences mentioned above were aligned together with the AdhD sequence as well as the *S. scrofa* aldose reductase (ADR, 48%). The results showed that among the strict conserved residues, six of them (Asp₅₈-Ser₁₅₂-Asn₁₅₃-Gln₁₇₅-Glu₂₇₅-Asn₂₅₈) seemed to be the residues involved in the typical cofactor binding of the AKR superfamily. In addition, four of them were identified as the catalytic residues (Asp₅₈-Tyr₆₃-Lys₈₉-His₁₂₁), which form parts of the active site (Uria, 2004). The typical fingerprint of these catalytic residues is Asp₅₀-Tyr₅₅-Lys₈₄-His₁₁₇ (Jez *et al.*, 1997).

The role of such residues in catalytic reaction is shown in figure 7. The residue, Tyr₅₅ (Tyr₆₃ in *adhD*) seems to function as the catalytic acid, and His₁₁₇ (His₁₂₁ in AdhD) is responsible for orienting the carbonyl of the substrate in the active site (Sanli and Blaber 2001). Therefore, the catalytic mechanisms proposed for this enzyme is initiated by transferring a hydride ion (H⁻) from NAD(P)H to the substrate with the aid of AdhD, followed by proton transfer to the carbonium-ion intermediate. It is clear here that the NAD(P)H cofactor provides a hydride ion for reduction.

Another structural feature typical for members of the AKR superfamily that was observed for AdhD is the monomeric state of the active form. The molecular weight (MW) of an enzyme under

denatured-reduced condition can be calculated based on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. It can also be directly estimated on the basis of the amino acid sequence. Whereas, the MW in the active form is normally estimated by gel filtration chromatography. In our experiment with AdhD, the purified ADH obtained from Q-sepharose fractionation was run in a superdex 200 chromatography. The volume of the active fractions eluted was used to calculate the MW on the basis of its comparison with marker proteins. The result confirmed that the native MW of AdhD was approximately 30 kDa. This MW value was similar to the MW determined under denatured conditions by SDS-PAGE analysis, suggesting that the active form of the *P. furiosus* ADH is a monomer of approximately 32 kDa. The lower value observed from superdex chromatography in comparison with that from SDS-PAGE was due to the shape and charge of the native AdhD.

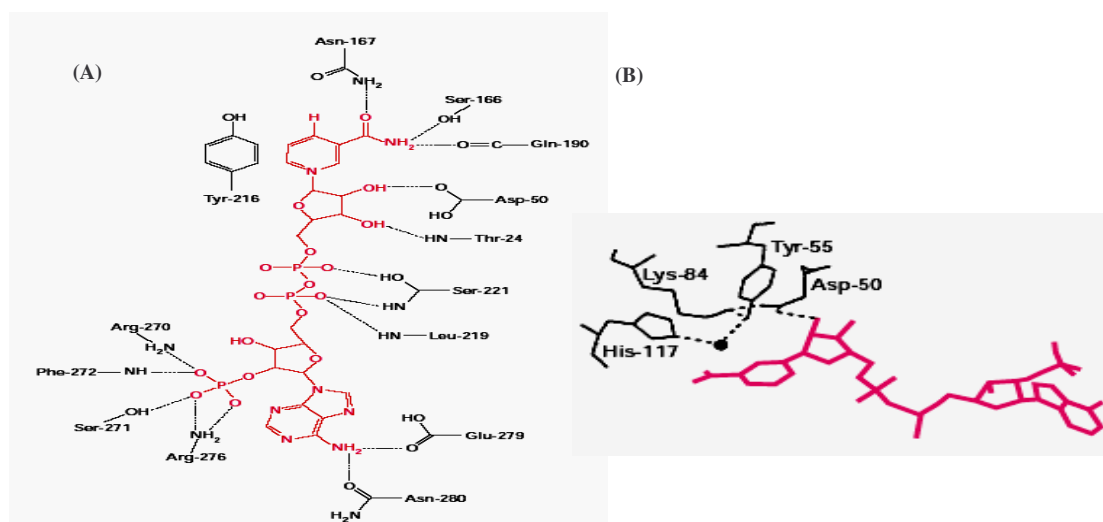


Fig. 7. (A) the active site of the AKRS (Asp₅₀-Tyr₅₅-Lys₈₄-His₁₁₇). (B) NADP⁺-binding site of the AKRs (Asp₅₀-Tyr₅₅-Lys₈₄-His₁₁₇). Copied from Jez., Bennett, Schlegel, Lewis, and Penning (1997) in *Biochem. J.* 326:625-636.

As described above, the cofactor- and substrate-binding sites of this enzyme resembled those from the members of the aldo-keto reductase (AKR) superfamily. Subsequently, this enzyme existed in a monomeric form with the molecular size of 32 kDa. Based on these properties, the conclusion can be drawn that AdhD belongs to the AKR superfamily. This conclusion was also confirmed by (i) its independency on metal for the activity, and (ii) the more efficient catalyzing of ketone reduction than alcohol oxidation, which are also features determined for the majority of the AKR superfamily. The general structure of members of the AKR superfamily is indicated in Figure 8.

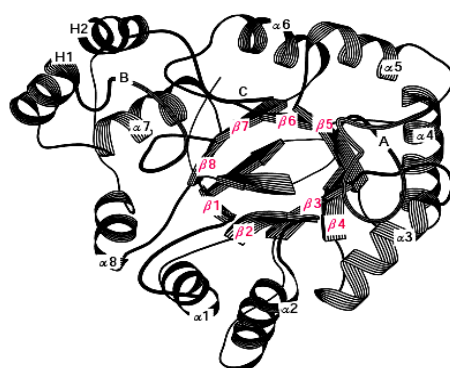


Fig. 8. A general structure of the AKRs, showing the (α:β)₈-barrel motif. The A-loop, B-loop and C-terminal are indicated. Two helices (H1 and H2) are not located on the barrel. Copied from Jez, Bennett, Schlegel, Lewis, and Penning (1997) in *Biochem. J.* 326:625-636.

5.2. Functional Properties

AdhD showed an optimal pH of around 8.8 for its oxidative activity, which was different from that for its reductive activity, pH of 6.12 (Uria, 2004; Machielsen, *et al.*, 2006). The effect of pH on the ADH activity was proposed by Koumanov, *et al.* (2003) based on the calculation of the electrostatic interactions. It was assumed that Tyr₁₅₁ and Lys₁₅₅ are involved in the pH regulation of proton abstraction via a specific mechanism called proton relay chain. In particular, at higher pH (pH >8), Tyr₁₅₁ is half protonated and will serve as a base to attract a proton from the hydroxyl group of the substrate, alcohol. Then the Tyr₁₅₁ in the protonated form donates a proton to the Lys₁₅₅ via the 2'-OH group of the ribose part of NAD⁺. When the Lys₁₅₅ is half protonated, the proton donated by the NAD⁺ is taken out from the active site by the help of an eight-member water chain. In this situation, the water chain is stabilized by a hydrogen bond connected to the carbonyl group of Asn₁₀₇ (or Asn₁₁₁). Whereas the Ser₁₃₈ itself seems to function in the correct orientation and binding of the substrate. When the pH decreases, the tendency of the 2'-OH group of the ribose part of NAD⁺ to transfer a proton to Lys₁₅₅ decreases, resulting in a discontinued proton relay.

AdhD showed a preference for mediate-chain aliphatic secondary alcohols (C₄–C₇) when compared to the primary alcohols. The activity of AdhD towards long-chain secondary alcohols (2-octanol to 2-decanol) was significantly lower, which is probably caused by the longer hydrophobic carbon chain. The highest specific activity of AdhD in the oxidative reaction was found with 2,3-butanediol (V_{\max} 108.3 U·mg⁻¹).

The thermostability and activity of AdhD at different temperatures was also determined. This enzyme showed optimal activity at 100°C (approximately 38 U/mg) with an half-life value ($T_{1/2}$) of 130 minutes at 100°C. This half-life was much higher than that of ADH from *Thermococcus* sp. strain ES-1 ($T_{1/2}$ = 15 min at 80°C) (Ma & Adams, 2001). Several factors have been suggested to contribute to the thermostability of hyperthermophilic enzymes, in general including amino acid composition and intrinsic properties and/or structural organisation (Radianingtyas & Wright, 2003).

6. PHARMACEUTICAL POTENCY OF ADHs

ADHs undoubtedly have an important future in the pharmaceutical industry since they can be applied for the preparation of single enantiomers, which are chiral intermediates required in drug synthesis. This part will provide some examples of the great potential of hyperthermophilic ADHs in preparative applications. Since the ADHs require expensive cofactors in the reactions, some strategies in cofactor regeneration for the industrial-scale application will also be discussed in this section.

6.1. Enzymatic Preparation of Chiral Compounds

A variety of ADHs have shown promising features and are future catalysts to be used in the biocatalytic synthesis of various pharmaceutical valuable chiral intermediates. Two examples of commercially available ADHs which are often used by organic chemists for laboratory-scale synthesis

are ADHs from yeast (YADH) and from horse liver (LADH). HLADH, for instance, can be applied in the preparation of (1*S*,2*R*)-cis-2-carboxymethyl-3-cyclopenten-1-ol lactone, an interesting starting material for prostaglandin synthesis. However, preparative applications using these two mesophilic enzymes are limited by their instability at temperatures above 30°C, their rather narrow substrate specificity, their quite sensitivity to organic solvents, and their tendency to loose activity during immobilization (Hummel and Kula, 1989). Some other mesophilic ADHs, such as those from *Candida parapsilosis*, *Pseudomonas* sp., and *Lactobacillus kefir*, have also been used in the preparative applications, but they also suffer the disadvantages of a narrow substrate specificity, insufficient stereospecificity or sensitivity to organic solvents (Inoue, *et al.*, 2005). For such reasons, searching and testing other ADHs with desired properties, such those from hyperthermophilic sources, seem to offer promising solution for such limitations.

NADPH-dependent ADH from the hyperthermophile, *Thermoanaerobium brockii* (*Tbr*ADH) is a good example of thermostable ADH showing industrially valuable properties. It has been widely used to prepare the pharmaceutically valuable building blocks, chiral aliphatic chloroalcohols from the corresponding chloroketones (Hummel and Kula, 1989). The great potency of hyperthermophilic ADHs is also shown in the preparation of (*S*)-2-pentanol, a key intermediate required in the organic synthesis of some potential anti-Alzheimer's drugs (Patel, 2004). So far production of (*S*)-2-pentanol has been demonstrated with *Gluconobacter oxydans* using 2-pentanone as the substrate (Patel, 2004). However, a recent report has shown that the thermostable AdhD from the hyperthermophile, *P. furiosus* is also capable of reducing 2-pentanone to the corresponding (*S*)-2-pentanol (Fig. 9A), which was determined by gas chromatography analysis (Machielsen, *et al.*, 2006). In particular, its enantioselectivity was observed when NAD⁺ was used as the cofactor. Then the enantiomeric excess (ee) value of 89.4% was obtained, while the ee value was 84.8% when NADP⁺ was used (Machielsen, *et al.*, 2006). Properties such as high thermal stability, enantio-selectivity, and a broad substrate specificity (Uria, 2004; Machielsen *et al.*, 2006) make this enzyme an interesting candidate for biocatalysts.

Another example of the potential of hyperthermophilic ADHs to the pharmaceutical industry is in the biocatalytic preparation of *S*-(-)-4-chloro-3-hydroxybutanoic acid or (*S*)-4-chloro-3-hydroxybutanoate, a key intermediate required for the total chemical synthesis of the cholesterol lowering agent, HMG-CoA reductase inhibitor (Fig 9B). So far, preparation of such key intermediate has been achieved by a secondary ADH from *Pichia finlandica* through the asymmetric reduction of ethyl 4-chloroacetoacetate to (*S*)-4-chloro-3-hydroxybutanoate. The gene encoding the ADH was cloned from *P. finlandica* and over-expressed in *Escherichia coli*. As the alternative, it had also been achieved by using cell suspensions of *Geotrichum candidum* SC 5469 (Patel, 2004).

The enzymatic preparation of the alcohol, (1*S*,2*R*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid,1,1-dimethyl-ethyl ester, a key intermediate required for the synthesis of the potent HIV inhibitor Atazanavir is also an example of the potential of alcohol dehydrogenases in the pharmaceutical industry. The diastereoselective reduction of the (*S*)-ketone,

(1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid,1,1-dimethyl-ethyl ester to such alcohol by using specific hyperthermophilic ADHs seems to be an attractive alternative way when considering the general advantages offered by the ADHs (Fig. 9C). So far preparation of this chiral alcohol has already been demonstrated with *Rhodococcus*, *Brevibacterium* and *Hansenula* strains through the enantiomeric reduction of (*S*)-2 ketone (Patel, 2004).

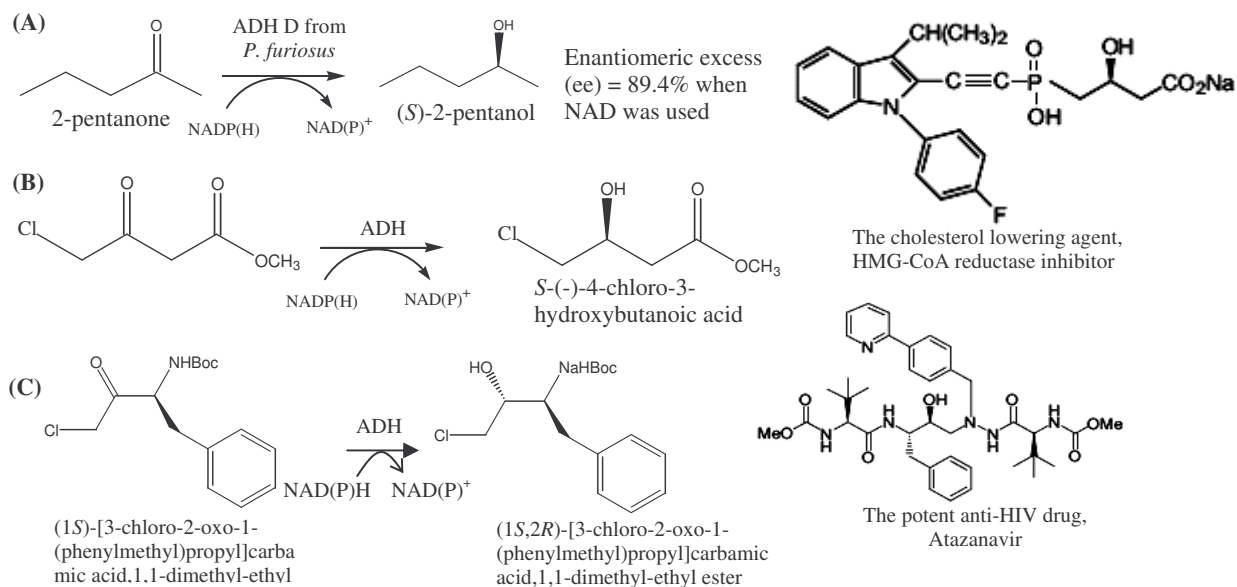


Fig. 9. Examples of the biocatalytic synthesis of chiral pharmaceutical intermediates. (A) The *P. furiosus* thermostable ADH, designated AdhD, is able to catalyse the reduction of 2-pentanone into (*S*)-2-pentanol, a key intermediate in the synthesis of several potential anti-Alzheimer's drugs. (B) ADH potential for the reduction of 4-chloro-3-oxobutanoic acid methyl ester to *S*-(-)-4-chloro-3-hydroxybutanoic acid methyl ester, a key intermediate in the total chemical synthesis of the cholesterol lowering agent, HMG-CoA reductase inhibitor. (C) Enzymatic preparation of a (1*S,2R*)-alcohol, a key intermediate in the synthesis of the potential HIV inhibitor, Atazanavir drug through the reduction of (*S*)-ketone.

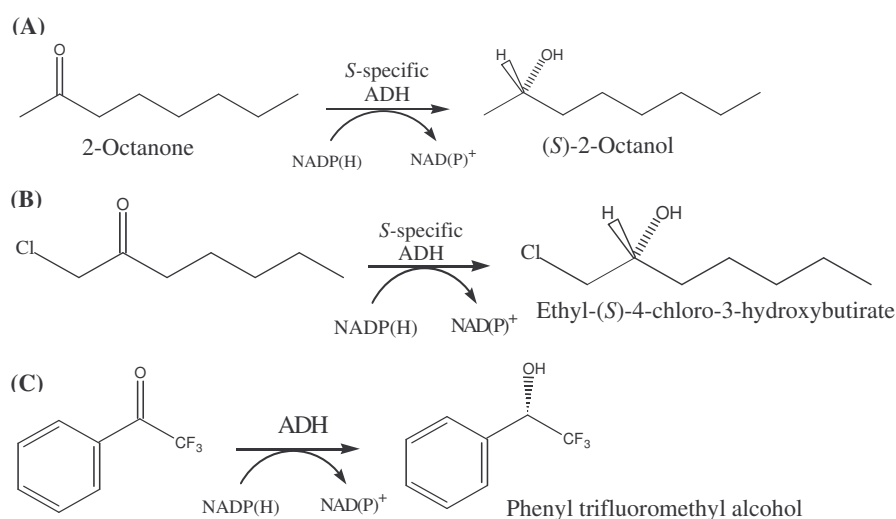


Fig. 10. Some examples of the potential of hyperthermophilic ADHs in the organic synthesis of chiral building blocks in the pharmaceutical industry. (A) Enzymatic preparation of (*S*)-2-Octanol. (B) Enzymatic preparation of Ethyl-*S*-4-chloro-3-hydroxybutyrate. (C) Enzymatic preparation of phenyl trifluoromethyl alcohol.

Two other examples of pharmaceutically important chiral compounds are (*S*)-2-Octanol and Ethyl-(*S*)-4-chloro-3-hydroxybutyrate. Here there also seems to be an interesting possibility for their production by using hyperthermophilic ADHs (Fig. 10A-B). Preparation of these two compounds have successfully been demonstrated with ADH from *Candida parapsilosis* (Zelinski, *et al.*, 1999) and ADH from *Rhodococcus erythropolis* (Wolberg, *et al.*, 2000). Hyperthermophilic alcohol dehydrogenases could also play a prominent role in the enantioselective reduction of phenyl trifluoromethyl ketone (Fig. 10C), acetophenone, and 2-heptanone into their corresponding alcohols. So far this was reported by Inoue, *et al.* (2005) for ADH from *Leifsonia* sp.

6.2. Enzymatic Regeneration of the Required Cofactor

The use of ADHs as biological catalysts for large-scale synthesis of chiral products requires efficient procedures for regeneration of the required cofactors. Continuous supply of a required cofactor into the *in vitro* catalytic system or isolation of the used cofactor and separate regeneration is too expensive. A continuously operating process is desirable for industrial applications to utilize the expensive cofactors and ADHs extensively (Patel, 2004). For such reasons, it is really necessary to build appropriate regeneration systems.

Cofactor regeneration systems can involve a single biocatalyst or multiple biocatalysts. The substrates used vary depending on biocatalyst(S) employed. For example, in the cofactor regeneration system shown in Fig. 11A, the same biocatalyst (ADH) is used for both reduction a ketone into the desired secondary alcohol and cofactor regeneration by 2-propanol. Cofactor regeneration involving multiple enzymes and different substrates are shown in Fig. 11B-C.

An example of using multiple enzymes for cofactor generation is by coupling the desired conversion by an ADH with the enzyme formate dehydrogenase (FDH) (Fig. 11B). In this system, NADH-based conversion of ketone into chiral alcohols is performed with ADH, and the resulting NAD^+ is regenerated using FDH with formate as the substrate. Another promising cofactor regeneration system, if NADH needs to be converted to NAD^+ , is based on the use of a NADH oxidase (Fig. 11C). As shown in figure 10C, to regenerate NAD^+ , the resulting NADH can be oxidized by NADH oxidase using O_2 , resulting in H_2O_2 . Since H_2O_2 is toxic, it needs to be converted back into O_2 by the help of catalase. This is intended not only to remove the toxic H_2O_2 , but also to supply O_2 into the system.

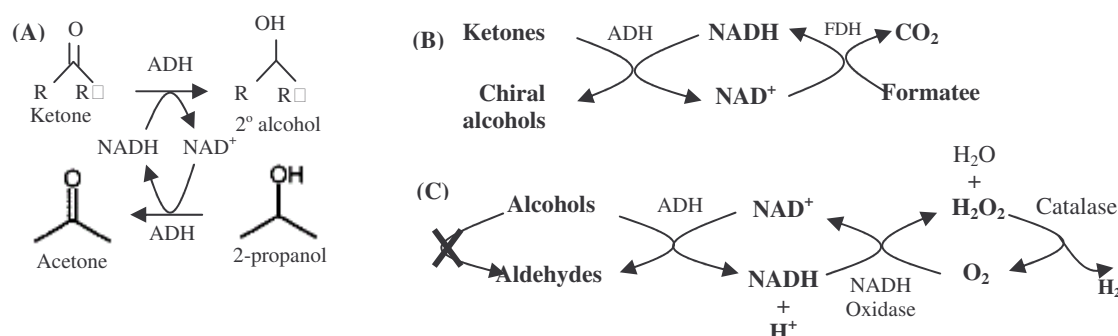


Fig. 11. Enzymatically cofactor regeneration. (A) Enantioselective reduction of a ketone into secondary alcohol, and regeneration of the required NADH with 2-propanol as the substrate. (B) Reduction of ketones into chiral alcohols using a NAD-dependent ADH, followed with the regeneration of NAD^+ using FDH. (C) Reduction of alcohols into aldehydes using NAD(P)-dependent ADH, followed with the regeneration of NAD^+ using NADH oxidase.

7. CONCLUDING REMARKS

Chiral compounds play an important role as intermediates or building blocks in the organic synthesis of pharmaceutical drugs. Production of such compounds by biocatalysis approach appears to be gaining broader acceptance, due to its efficiency, environmental friendly, and some instances, greater economic efficacy. In particular, chiral alcohols belong to the most important chiral compounds with a wide range of synthetic applications in the pharmaceutical industry. Biocatalytic synthesis of chiral alcohols has been achieved by alcohol dehydrogenases (ADHs). Although so far many chiral alcohols have successfully been generated by the use of these enzymes, but many of these enzymes originate from mesophilic organisms and have stability problems.

Over the last two decades, ADHs from marine hyperthermophilic microorganisms have attracted special attention as catalysts in several industrial processes, mainly due to some unique properties, including their relatively broad specificity towards various carbonyl substrates, their high thermal and chemical stability, as well as their suitability for cofactor regeneration. With the increased demand of chiral alcohols, there is an urgent need in exploration of the hyperthermophilic ADH potential with desired enantiomeric activities, started from their screening from various marine sources, heterologous production, characterisation until their application trials in the pharmaceutical industry.

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