Display of *Bombyx mori* Alcohol Dehydrogenases on the *Bacillus subtilis* Spore Surface to Enhance Enzymatic Activity under Adverse Conditions

Nan Wang, Cheng Chang, Qin Yao, Guohui Li, Lvgao Qin, Liang Chen, Keping Chen*

Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu Province, People’s Republic of China

Abstract

Alcohol dehydrogenases (ADHs) are oxidoreductases catalyzing the reversible oxidation of alcohols to corresponding aldehydes or ketones accompanied by nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) as coenzyme. ADHs attract major scientific and industrial interest for the evolutionary perspectives, afforded by their wide occurrence in nature, and for their use in industrial synthesis. However, the low activity of ADHs under extremes of pH and temperature often limits their application. To obtain ADH with high activity, in this study, we used *Bombyx mori* alcohol dehydrogenases (BmADH) as foreign gene and constructed a recombinant integrative plasmid pJS700-BmADH. This pJS700-BmADH was transformed into *Bacillus subtilis* by double cross-over and produced an amylase inactivated mutant. The fusion protein containing BmADH was expressed on the spore surface and recognized by BmADH-specific antibody. We also assayed the alcohol dehydrogenase activity of the fusion protein together with the native BmADH at different pH and temperature levels, which indicated the recombinant enzyme exhibits activity over wider ranges of temperature and pH than its native form, perhaps due to the resistance properties of *B. subtilis* spores against adverse conditions.

Citation: Wang N, Chang C, Yao Q, Li G, Qin L, et al. (2011) Display of *Bombyx mori* Alcohol Dehydrogenases on the *Bacillus subtilis* Spore Surface to Enhance Enzymatic Activity under Adverse Conditions. PLoS ONE 6(6): e21454. doi:10.1371/journal.pone.0021454

Editor: Peter Setlow, University of Connecticut, United States of America

Received March 21, 2011; Accepted May 29, 2011; Published June 29, 2011

Copyright: © 2011 Wang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from the Jiangsu Sci-Tech Support Project-Agriculture (No. BE2008379), the National Program of High-Tech Research and Development (863 High-Tech Program, No. 2008AA10Z145), and the National Natural Science Foundation (No. 30871826). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kpchen@ujjs.edu.cn

Introduction

Alcohol dehydrogenases (ADH; EC 1.1.1.1) belong to the oxidoreductase family; a class of enzymes that catalyze the reversible oxidation of alcohols to corresponding aldehydes or ketones using NAD or NADP as coenzyme. ADHs are widely distributed in nature and have been found in species throughout the three domains of life, Archaea, Bacteria and Eukarya [1–4]. ADHs play important roles in a broad range of physiological process. Based on their catalytic activities, ADHs are supposed to participate in the metabolism of steroids, retinoids, lipid peroxidation products, α-hydroxy fatty acids, xenobiotic alcohols and aldehydes [5]. However, many ADHs are generally susceptible to harsh conditions such as extremes of pH and temperature, which often hampers their industrial application [6]. Due to the ever-increasing industrial demands, microbial surface display of enzymes has been widely adopted as a promising technique for the improvement of conventional biocatalysts [7–9].

The gram-positive bacterium *Bacillus subtilis* has been extensively studied as a model prokaryotic system, which is not regarded as a pathogen but classified as a novel food currently being used as a probiotic for both human and animal consumption [10,11]. The *B. subtilis* spore offers unique resistance properties and can survive extremes of temperature, desiccation, exposure to solvents and other noxious chemicals. These attributes would make the spore an attractive vehicle for delivery of heterologous proteins that are resistant to adverse environments. Additionally, three proteins in the spore coat, CotB, CotC and CotG, have been shown to be exposed on the outside and have been used as a novel system to display passenger proteins such as different antigens [12], enzymes [13] and bioactive molecules [14].

In this study, a mutant *B. subtilis* strain, which exhibited *Bombyx mori* alcohol dehydrogenase (BmADH) on the spore surface by fusion to the spore coat protein CotC, has been successfully constructed, expressed and identified by a BmADH-specific antibody. The ethanol dehydrogenase activity of the BmADH protein expressed on the spore surface was assayed under different pHs and temperatures and this enzyme’s activity was compared with that of the native enzyme under the same conditions.

Methods

**Materials**

*B. subtilis* 168 (trp-) was obtained from Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University. The expression vector pET-30a(+) and *Escherichia coli* strains DH5α and BL21(DE3) were obtained from Novagen (CA, USA). Ex Taq polymerase, restriction enzymes, T4 DNA ligase and the subcloning vector pMD18-T were purchased from TaKaRa (Dalian, China). Chemicals are all from Sigma (MO, USA) or a domestic provider in China if not stated otherwise.
Preparation and transformation of *B. subtilis* strain 168 (trp-) competent cells were performed as previously described [15].

Expression of BmADH in *E. coli* and preparation of the specific antibody

The BmADH specific primers, *BmADH*-F1: 5′-GGGGATC-CATGGCACCGATTTCGTG-3′ (*Bam* HI site is underlined), and *BmADH*-R1: 5′-CCCTCGAGCTATGTCTTGGAGAG-TATTTGGAAG-3′ (*Xho* I site is underlined) were designed to amplify an 825 bp *BmADH* gene from a pool of silkworm cDNA. The PCR product was ligated into the pMD18-T vector and then subcloned into the pET-30a(+), expression vector and transformed into *E. coli* BL21 (DE3). *E. coli* cells containing pET30a-BmADH were grown to an optical density at about 0.6 at 600 nm and induced by addition of 0.4 mM IPTG at 16°C for 20 hours to obtain soluble protein, and cells were harvested by centrifugation at 8,000 g for 15 min at 4°C. The soluble his-tagged protein was purified by a Ni-NTA column (Qiagen, German) following manufacturer's instruction. The recombinant BmADH present in the pellet of cell lysate was mixed with SDS sample buffer, boiled for 5 min and resolved on 15% SDS-polyacrylamide gels. The recombinant BmADH was excised and extracted from gel slices. The identity of the BmADH protein was confirmed by mass spectroscopic analysis and the purity was examined by a SDS-PAGE. The antiserum was raised in New Zealand white rabbits according to the method of Sambrook et al. using Freund's adjuvant [16]. All the procedures were in line with ethical standards for treatment of animals.

**Figure 1. Cloning strategy.** (A) The construction of integration plasmid pJS700-BmADH. The fragments *amyE* 5′ and *amyE* 3′ in plasmid are homologous to the upstream and downstream of the amylase gene in *B. subtilis* 168 (trp-), respectively; *Emr*, erythromycin resistant site; *CotC*, a *B. subtilis* spore coat protein encoding gene. (B) The schematic integration of *CotC-BmADH* to *amyE* locus. Arrows indicate the positions of primer pairs used in the site-directed PCR for confirmation of the correct integration.

doi:10.1371/journal.pone.0021454.g001
Construction of plasmid pJS700-BmADH

To obtain an integration of the CotC-BmADH fusion gene at the B. subtilis amyE locus, a recombinant plasmid for double cross-over with B. subtilis chromosome was constructed. We amplified another 825 bp fragment from silkworm cDNA with primer pair BmADH-F2: 5'-GGGGTACCATGGCACCGGATTTCGTGAAGCG-3' (KpnI site is underlined), and BmADH-R2: 5'-CGACTCCTATGTCTTGGAGAGTATTTGGA-3' (SacI site is underlined). The fragment between the KpnI and SacI sites containing the BmADH gene was cloned into vector pJS700, to generate the recombinant plasmid pJS700-BmADH, in which the upstream and downstream region of erythromycin (Em)-CotC-BmADH was homologous to B. subtilis amyE, as was verified by sequencing [17].

Plasmid pJS700-BmADH was digested with BglII, and the resulted linear fragment containing Em-CotC-BmADH gene was transformed into the B. subtilis strain 168 (trp-) competent cells. The transformed cells were incubated in 2 ml LB medium and cultured at 37°C overnight with vigorous shaking, following with sprayed onto LB plate containing 0.4 mg/ml erythromycin. Plates were incubated at 37°C overnight, colonies resistant to Em were selected, and a colony with CotC-BmADH integrated at the B. subtilis amyE locus was identified by analysis of amylase activity and then confirmed by PCR.

Screening of mutants with CotC-BmADH integrated at amyE locus

The integration of CotC-BmADH at amyE locus will disrupt amyE gene causing amylase-negative phenotype on LB plates containing 1% starch [15]. After incubation at 37°C overnight, the plates were stained by iodine to examine the amylase activity. A blue

Figure 2. Identification of the mutant with CotC-BmADH integration at amyE locus. (A) Analysis of amylase activity. CotC-BmADH mutant strains and B. subtilis 168 (trp-) wide type grew on the starch-containing LB plate before (1) and after (2) being stained by iodine. The integration of CotC-BmADH might disrupt amyE and made the strain amylase deficient, while the while wide type strain showed a big white halo around colony due the secretion of amylase. (B) Site-directed PCR analysis using different primer pairs. Marker, L DNA digested by EcoT14I; W: B. subtilis 168 (trp-) wide typ; M: CotC-BmADH mutant; primer pairs used in PCR are labeled below agarose gel.

doi:10.1371/journal.pone.0021454.g002

Figure 3. SDS-PAGE analysis of CotC-BmADH and Western blotting. (A) SDS-PAGE stained by coomassie-blue. (B) CotC-BmADH detected by BmADH specific antibody. Lane 1, B. subtilis 168 (trp-); lane 2, CotC-BmADH strain.

doi:10.1371/journal.pone.0021454.g003
expression of BmADH R2 was used to detect the correct insertion of the hydrolysis of the starch in the plate. B. subtilis blue color was observed at the BmADH CGTTCC-3’ fusion in the spore surface, the proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) for western blotting using previously prepared BmADH-specific antibody at a dilution of 1:1000. Immunoreactive proteins were visualized using goat anti-rabbit IgG antibody conjugated with peroxidase as described [19].

Enzymatic activity assay
The catalytic activity of recombinant CotC-BmADH protein was assayed spectrophotometrically by measuring the increase in absorbance at 340 nm following the reduction of NAD+ to NADH in a solution containing ethanol as the substrate as described by Oudman et al. [20]. Briefly, a 3 ml reaction buffer (50 mM NaOH/Glycine buffer, pH 10.0, 0.67 M ethanol, 8 mM NAD+) was incubated at 25°C, and the reaction was initiated by adding 0.1 ml of spore suspensions. The supernatant of the reaction solution was clarified by centrifugation (12,000 g, 4°C, 30 s) for spectrophotometric analysis. The increase at A340 in the initial 10 minutes was recorded. The increase in A340 for reactions without spores was used as the blank. An extinction coefficient of 6.22 mM⁻¹ cm⁻¹ for NADH was used to calculate enzyme activity [21]. One unit of dehydrogenase activity is defined as 1 μmol NAD+ reduced per min. The enzyme specific activity calculation formula is as following: (ΔA340×V) / (6.22×b×W), where ΔA340 is the change in absorbance at 340 nm per minute, V is the final reaction volume, b is the light path, and W is the amount of protein in the reaction system. The concentration of extracted proteins was measured using the Bio-Rad DC Protein Assay kit. Spores of B. subtilis 168 (trp-) were used as negative control. The enzymatic activity was measured three times with independent samples.

Alcohol dehydrogenase specific activity at different pH levels was determined by performing standard enzyme assay at various pH levels ranging from 4.0 to 10.0. The following 0.1 M buffer systems of varying pH, but fixed ionic strength, were used: acetate buffer (NaAc-HAc) for pH 4.0; phosphate buffer (NaH₂PO₄-

Table 1. Alcohol dehydrogenase specific activities at different pHs.

| protein samples       | pH | 4    | 7    | 8    | 9    | 10   |
|-----------------------|----|------|------|------|------|------|
| BmADH (units/mg protein) |    | 3.05±0.35 | 9.14±0.82 | 80.22±4.59 | 83.77±3.27 | 12.23±1.37 |
| CotC-BmADH (units/mg protein) |    | 38.64±1.14* | 57.58±2.38* | 68.72±3.29 | 75.82±0.82 | 39.36±3.82* |

* indicates that the difference is significant (P<0.05).
Mean ± SD from three independent experiments are shown.
doi:10.1371/journal.pone.0021454.t001

Expression of CotC-BmADH on B. subtilis spore surface
Sporulation of wild type B. subtilis 168 (trp-) and recombinant strain CotC-BmADH was induced in Difco sporulation medium (DSM) by the exhaustion method as previously described [18]. Cultures were harvested 48 h after the initiation of sporulation. Spores were collected, washed several times, and purified by lysosome treatment as described by Nicholson and Setlow to break any residual sporulating cells [18]. Spore coat proteins in suspensions at high density (>1×10¹⁰ spores/ml) were extracted from B. subtilis 168 (trp-) spores and the CotC-BmADH spores using an SDS–DTT extraction buffer as previously described [15].
Table 2. Alcohol dehydrogenase specific activities at different temperatures.

| Protein samples | Temperature (°C) | BmADH (units/mg protein) | CotC-BmADH (units/mg protein) |
|-----------------|-----------------|--------------------------|-------------------------------|
|                 | 16              | 20                       | 25                            | 30                           | 37                           |
| BmADH           | 5.08±0.08       | 38.07±1.57               | 83.77±5.83                    | 60.00±3.30                   | 9.14±1.60                     |
| CotC-BmADH      | 36.35±1.15*     | 49.60±2.37               | 75.82±5.42                    | 47.32±1.78                   | 28.24±1.16*                   |

*indicates that the difference is significant (P<0.05).
Mean ± SD from three independent experiments are shown.
doi:10.1371/journal.pone.0021454.t002

Na₂HPO₄ for pH 7.0; NaOH/Glycine buffer for pH 8.0, 9.0 and 10.0.

Alcohol dehydrogenase specific activity at different temperature was determined by performing standard enzyme assays for 10 min at temperatures of 16, 20, 25, 30, and 37°C.

To verify the thermal stability of the native BmADH and spore-displayed CotC-BmADH, the two proteins were incubated respectively at 37°C for 30 min, and then assayed under optimal conditions. The relative remained activity was calculated by comparison with the activity of the untreated proteins.

Statistical analysis

The enzymatic activity difference between the native BmADH and CotC-BmADH was analyzed by t-test with a significance level of α=0.05 (Microsoft Excell in Office 97). All values are expressed as mean value ± standard deviation of three independent experiments.

Results

Construction of integrative vector containing CotC-BmADH gene

The strategy to obtain recombinant B. subtilis spores expressing BmADH on their surface was based on (i) use of the CotC gene and its promoter for the construction of translational fusions and on (ii) chromosomal integration of the CotC-BmADH gene fusions into the coding sequence of the amyE locus [15]. The CotC-BmADH gene was obtained by cloning the BmADH gene at the 3’ end of the 201-bp CotC open reading frame carried by the integrative vector pJS700 (Fig. 1A). The fusion was integrated into the B. subtilis chromosome at the amyE locus by a double cross-over event (Fig. 1B).

Identification of CotC-BmADH integrated in B. subtilis chromosome

Amylase activity assay was used to identify CotC-BmADH integration mutants. The integration of CotC-BmADH at amyE locus disrupted the production of amylase, as a result no white halo was observed around the colony on a starch-containing plate stained by iodine, but the control strain showed a big white halo around the colony due to the secretion of amylase (Fig. 2A). The disruption of amyE was further confirmed by PCR with different primer pairs (Fig. 2B). Primer pair amyE-F/amyE-R produced a 4.5 kb fragment from the CotC-BmADH integrated chromosome, in comparison with a 1098 bp fragment from the B. subtilis 168 (trp-) DNA. In addition, in the CotC-BmADH integrated chromosome, primer pairs BmADH-F/BmADH-R, BmADH-F/amyE-R, amyE-F/BmADH-R produced fragments around 825 bp, 700 bp, 2650 bp, respectively, but gave no PCR product with 168 (trp-) DNA.

Surface display of BmADH on the recombinant spores

To confirm that BmADH was expressed on the B. subtilis spore surface, approximate 5 μg coat proteins were loaded to perform SDS-PAGE analysis, which showed a distinctive protein band weights about 40 kDa (Fig. 3A). Because the CotC gene of B. subtilis encodes a 66 amino acid polypeptide (CotC) with a predicted molecular mass of 8.8 kDa [22], and the 825 bp ORF of BmADH encodes a 275 amino acid protein with a predicted molecular mass of 30 kDa, the SDS-PAGE analysis suggested that the recombinant fusion protein was expressed with expected molecular mass.

The expression of CotC-BmADH was confirmed by Western blotting with previously prepared polyclonal antibody. A 40 kDa band was detected in the extracts from recombinant spores, while no similar band in the control lane was detected, indicating the presence of the CotC-BmADH fusion protein on the spore surface (Fig. 3B).

Effect of pH and temperature on enzyme activity

By measuring the alcohol dehydrogenase specific activity of both CotC-BmADH and the native BmADH under standard enzyme assay conditions at various pH levels, we found that the spore-displayed enzyme exhibited significant higher specific activity at pH 4.0, 7.0 and 10.0 than that of the native enzyme (P<0.05), which indicated that the spore-displayed enzyme retained activity in a wider range of pH than the native protein (Fig. 4A, Table 1).

By measuring the alcohol dehydrogenase specific activity of both CotC-BmADH and the native BmADH under standard
enzyme assay under conditions at various temperatures, we found that the spore-displayed enzyme exhibited significant higher specific activity at 16°C and 37°C than that of the native enzyme (P<0.05), which indicated that the CotC-BmADH preserved activity in a wider range of temperature than the native protein (Fig. 4B, Table 2).

In addition, the protein stability of CotC-BmADH was compared with that of the native BmADH at 37°C. The catalytic activities of CotC-BmADH and BmADH were measured at optimal condition after incubation of protein at 37°C for 30 min. The measurement revealed that the spore-displayed protein retained approximate 40% of its original activity, while 10% activity was remained for the native BmADH (Fig.5), which suggesting that the fusion of BmADH to B. subtilis spore protected the protein from the irreversible destructive conformational change at 37°C.

**Discussion**

The *Bacillus subtilis* spore is encased within a complex multilayered protein structure known as the coat, whose role is to protect the spore against bactericidal enzymes and chemicals, and to influence the spore’s ability to germinate in response to appropriate germinants. The coat is composed of more than 20 polypeptides, several of which have been studied, and their structural genes (*cot* genes) have been identified [23,24]. The development of a spore surface display system was initially based on the use of CotB, a *B. subtilis* spore coat component, as a fusion partner to express a highly immunogenic tetanus toxin fragment C (TTFC) on the spore surface [25]. More recently, another coat component, CotC, was also tested as fusion partner for the expression of TTFC and of the B subunit of the heatlabile toxin of *E. coli* (LTB) [26,27]. In this study, we displayed BmADH on the *B. subtilis* Spore surface by fusing BmADH to the C- end of CotC protein, and the fusion protein exhibited catalytic activity over wider ranges of pH and temperature than its native activity was.

The measurement revealed that the spore-displayed protein retained approximate 40% of its original activity, while 10% activity was remained for the native BmADH (Fig.5), which suggesting that the fusion of BmADH to *B. subtilis* spore protected the protein from the irreversible destructive conformational change at 37°C.

**References**

1. Branden C, Jornvall H, Eklund H, Furugen B (1975) Alcohol Dehydrogenases. In: Boyer PD, Krebs EG, eds. The Enzymes, 3 ed New York: Academic Press. pp 103–190.
2. Vonschatz JP, Bethune JL, Valle BL (1964) Human Liver-Alcohol Dehydrogenase. Kinetic and Physicochemical Properties. Biochemistry 3: 1775–1782.
3. Rella R, Raia CA, Pesina FM, Gianbattista A, et al. (1987) A novel archaeabacterial NAD+-dependent alcohol dehydrogenase. Purification and properties. Eur J Biochem 167: 475–479.
4. Reid MF, Fessons CA (1994) Molecular characterization of microbial alcohol dehydrogenases. Crit Rev Microbiol 20: 13–56.
5. Dolega A (2010) Alcohol dehydrogenase and its simple inorganic models Coordination Chemistry Reviews 254: 916–937.
6. Hirakawa H, Kamiya N, Kawaiabatayashi Y, Nagamura T (2004) Properties of an alcohol dehydrogenase from the hyperthermophilic archaeon *Aeropyrum pernix K1*. J Biochem 97: 202–206.
7. Chou W, Georgous G (2002) Cell-Surface display of heterologous proteins: From high-throughput screening to environmental applications. Biotechnol Bioeng 79: 496–503.
8. Jung HC, Lebeault JM, Pan JG (1998) Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. Nature biotechnology 16: 576–580.
9. Wernersen H, Stahl S (2004) Biotechnological applications for surface-engineered bacteria. Biotechnol Appl Biochem 40: 209–229.
10. Mazza P (1994) The use of *Bacillus subtilis* as an antimicrobial microorganism. Roll Clay Farm 113: 3–18.
11. Huang JM, La Ragione RM, Cooley WA, Todryk S, Cutting SM (2008) Cytoplasmic delivery of antigens, by *Bacillus subtilis* spores. Methods 20: 95–110.
12. Omura M, Izawa D, Kamping A, Nolasco S, Cangiano A, et al. (2004) Assembly of multiple CotC forms into the *B. subtilis* spore coat. Journal of Bacteriology 186: 1129–1135.
13. Henriques AO, Moran CPJ (2000) Structure and assembly of the bacterial endospore coat. Methods 20: 95–110.
14. Cutting S, Vander-Horn P (1990) Genetic analysis. In: Harwood CR, Cutting SM, eds. Molecular biological methods for *Bacillus*. England: John Wiley & Sons, Ltd. pp 27–74.
15. Cutting S, Vander-Horn P (1990) Genetic analysis. In: Harwood CR, Cutting SM, eds. Molecular biological methods for *Bacillus*. England: John Wiley & Sons, Ltd. pp 27–74.
16. Sambrook J (2001) Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 2344 p.
17. Li Q, Ding N, Wu C (2010) Surface display of GFP using CotX as a molecular vector on *Bacillus subtilis* spores. Chinese Journal of Biotechnology 26: 264–269.
18. Nixonor W, Sellon P (1990) Sporulation, germination and outgrowth. In: Harwood C, Cutting S, eds. Molecular biological methods for *Bacillus*. Chichester: Wiley. pp 391–450.
19. Huo J, Shi H, Yao Q, Chen H, Wang L, et al. (2010) Cloning and purification of recombinant silkworm dihydrodippeptide dehydrogenase expressed in *Escherichia coli*. Protein Expr Purif 72: 95–100.
20. Oudman L, Van Delden W, Kamping A, Bija A, Ravel J (1991) Polymorphism at the *Adh* and alpha *Gpdh* loci in *Drosophila melanogaster*: effects of rearing temperature on developmental rate, body weight, and some biochemical parameters. Heredity 67: 103–113.
21. Zhang ZH, Kang YM, Yu Y, Wei SG, Schmidt TJ, et al. (2006) 11beta-hydroxysteroid dehydrogenase type 2 activity in hypothalamic paraventricular nucleus modulates sympathetic excitation. Hypertension 48: 127–133.
22. Donovan W, Zheng LB, Sanderman K, Lesiek R (1987) Genes encoding spore coat polysaccharides from *Bacillus subtilis*. J Mol Biol 196: 1–10.
23. Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, et al. (2001) Surface display of recombinant proteins on *Bacillus subtilis* spores. J Bacteriol 183: 6294–6301.
26. Mauriello EMF, Duc LH, Istitato R, Cangiano G, Hong HA, et al. (2004) Display of heterologous antigens on the Bacillus subtilis spore coat using CotC as a fusion partner. Vaccine 22: 1177–1187.

27. Barak I, Ricca E, Cutting SM (2005) From fundamental studies of sporulation to applied spore research. Mol Microbiol 55: 330–338.

28. Racker E (1955) Alcohol dehydrogenase from baker’s yeast. In: Colowich S, Kaplan N, eds. Methods in enzymology. New York: Elsevier Academic Press. pp 500–503.

29. Okuma Y, Ito Y, Endo A (1991) Purification and properties of alcohol dehydrogenase from the acid- and ethanol-tolerant yeast Candida sake. J Ferment Bioeng 71: 309–312.