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Cloning, expression and characterization of L-aspartate β-decarboxylase gene from *Alcaligenes faecalis* CCRC 11585

C-C Chen, T-L Chou and C-Y Lee

Graduate Institute of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan

L-Aspartate β-decarboxylase (Asd) is an important enzyme to produce L-alanine and D-aspartate. The genomic library of *Alcaligenes faecalis* CCRC 11585 was cloned into pBK-CMV and transformed into *Escherichia coli*. One clone, which carried the *asd* gene and expressed Asd activity, was isolated and chosen for further study. PBK-asdAE1 was subcloned and its sequence analysis revealed an open reading frame, consisting of 1599 bp, that encodes a 533-amino-acid polypeptide. The nucleotide sequence of the *asd* gene from *A. faecalis* CCRC 11585 (*asd*A) showed 84% identity with that from *Pseudomonas dacunhae* CCRC 12623, and the amino acid sequence showed 93% identity. The amino acid sequence of the AsdA showed 51–58% homology with various aminotransferases. Alignment of the AsdA with several aspartate or tyrosine aminotransferases revealed 17 conserved amino acids that appeared in most of the conserved amino acid residues within the pyridoxal-5′-phosphate (PLP) binding domains of aminotransferases. Furthermore, the *asd*A gene was cloned into expression vector pET-21a and transformed into *E. coli* BL21(DE3). A protein band sized at 61 kDa is present on the SDS-PAGE gel from the intracellular soluble form of *E. coli* BL21(DE3)/pET-asdA. The specific activities of the pET-AsdA purified by using His-Bind chromatography is 215 U/mg at 45°C and pH 5.0, which is 1000-fold higher than that of the *A. faecalis* crude extract. This is the first report of an *asd*A gene sequence from *A. faecalis* and represents the potential application of a recombinant AsdA for production of L-alanine or D-aspartic acid. *Journal of Industrial Microbiology & Biotechnology* (2000) **25**, 132–140.

Keywords: *Alcaligenes faecalis*; L-aspartate β-decarboxylase gene; sequence; expression

Introduction

L-Aspartate β-decarboxylase (Asd) catalyses the removal of the beta carboxyl group from L-aspartate with complete retention of the asymmetric centre to produce L-alanine. It is present in a number of microorganisms, including *Pseudomycobacterium, Pseudomonas* sp., *Pseudomonas dacunhae*, *Clostridium perfringens*, *Alcaligenes faecalis*, *Acetobacter* sp., *Achromobacter* sp., *Desulfovibrio desulfuricans*, *Nocardia globerula*, *Xanthomonas oryzae*, *Penicillium* and *Cunninghamella elegans* [1,3–5,7,11,14,15]. The enzyme was subsequently found in significant quantities in mammalian brain, kidney, and liver [10]. Various D-amino acids, including D-alanine, D-valine and D-phenylalanine, are found in peptide antibiotics, such as polymyxin, tyrocidin and bacitracin. It has been reported that the presence of D-amino acids increases the specificity and stability of the peptides. Recently, the application of peptides in the medical and agricultural industries has made chiral drugs an attractive subject for study. D-aspartic acid is a component of synthetic penicillin (Aspoxicillin) while L-alanine is a component of amino acid infusion and is a food additive [14]. Use of the high optical specificity of Asd for production of D-aspartic acid and L-alanine with DL-aspartic acid as substrate is the best method [14] employed in industry. DL-Aspartic acid synthesized chemically from fumaric acid and ammonia was the most suitable substrate [14].

Asd from *A. faecalis* has been purified to homogeneity and its fundamental properties have been reported [1]. However, the amino acid sequence of the enzyme is not available. Recently, Rozzell [11] cloned a gene which encodes Asd from *P. dacunhae* and is expressed in *Escherichia coli*. In this report, we describe the gene cloning and expression of Asd from *A. faecalis* CCRC 11585, and the possible evolutionary relationships between this enzyme and aminotransferases are discussed.

Correspondence: Dr C-Y Lee, University of North Carolina at Chapel Hill, Department of Microbiology and Immunology, #CB7290, 718 Mary Ellen Jones Building, Chapel Hill, NC 27599-7290
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Materials and methods

Strains, plasmids and media

Bacterial strains and plasmids are described in Table 1. *A. faecalis* was cultivated in nutrient broth medium (Difco) at 30°C overnight. *E. coli* was cultivated in LB (Lurie Bertani) medium (Difco) containing 100 μg of ampicillin/ml.

DNA manipulation

Chromosomal DNA from *A. faecalis* was isolated using the method previously reported [9]. *E. coli* XL1-Blue was transformed by means of electroporation (voltage: 2.5 kV, resistor: 200 Ω, capacitor: 25 μF, time constant: 4.8 ms, electrode gap: 0.2 cm) using the Gene Pulser apparatus (Bio-Rad Co. Ltd., Hercules, CA). Other molecular cloning methods were carried out according to standard procedures [12]. DNA sequencing was performed using the chain termination method of Sanger *et al.* [13] with appropriate synthetic oligonucleotides used as primers.

Molecular cloning

Chromosomal DNA of *A. faecalis* CCRC11585 was digested with *Eco*RI (BRL, Gaithersburg, MD), and 6 to 15 kb DNA

Table 1 Bacterial strains, phages and plasmids used in this study

Strain or phage	Characteristics	Source
P. dacunhae	Asd production strain	CCRC12623
A. faecalis	Asd production strain	CCRC11585
E. coli		
XL1-Blue MRF′	Δ(mcr)183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F′ proAB, lacI^{q}Z Δ M15, Tn10(Tet^r)]	Stratagene
XLOLR	Δ(mcr)183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, thi-1, recA1, gyrA96, relA1, lac[F′ proAB, lacI^{q}ZΔM15, Tn10(Tet^r)] Su$^-$, λ^r	Stratagene
BL21(DE3)	F$^-$ ompT hsdS$_B$(r_B^- m_B^-) gal dcm (DE3)	Novagen
ExAssist helper phage	Kan^r, used for *in vivo* excision	Stratagene
ZAP Express vector	ZAP Express vector can be excised in the form of pBK-CMV phagemid vector and used to construct a genomic library.	Stratagene
pBK-CMV	Kan^r, fl(-) origin, ColE1 replicon, *lac*Z (4.5 kb)	Stratagene
pBK-*asd*AE1	9-kb *Eco*RI fragment of *A. faecalis* CCRC 11585 carrying Asd gene cloned into pBK-CMV	This study
pBK-*asd*AAE6	5.5-kb *Apa*I–*Eco*RI fragment of pBK-*asd*AE1 cloned into the *Apa*I–*Eco*RI site of pBK-CMV	This study
pET-*asd*A	1.6 kb of Asd gene fragment cloned into the *Eco*RI–*Hin*dIII site of pET21a	This study
pET21a	Expression vector, *amp*^{r}, T7 promoter, *lac*I	Novagen

were isolated and purified using GeneGelPure Gel DNA purification kit (Watson Biotechnology Co. Ltd). The isolated fragments were ligated to *Eco*RI digested ZAP Express vector (ZAP Express undigested vector kit, Stratagene) by using DNA ligase (BRL). Litigation products were supplemented with Gigapack III gold packaging extract (Stratagene) for *in vitro* packaging, according to the directions of the manufacturer. *E. coli* XL1-Blue MRF′ and ExAssist helper phage were infected with packaged DNA at 35°C for 15 min in NZY broth (0.5% of NaCl, 0.2% of $MgSO_4 \cdot 7H_2O$, 0.5% of yeast extract, 1% of NZ amine (casein hydrolysate)), incubated at 65°C for 20 min and then centrifuged to recover the supernatant containing the recombinant phage. These supernatants were mixed with *E. coli* XLOLR and incubated at 35°C for 15 min; then, 300 μl of NZY broth were added following incubation at 35°C for 45 min in LA medium supplemented with kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). White colonies containing recombinants were selected and hybridized with a radioisotope-labeled asdA0.4 probe by means of colony blot hybridization at 65°C.

Gene probe preparation

Probe asdA was prepared by polymerase chain reaction using the primers 5′CCTGAACGAATTCACTAGGACGAA3′ (ADr1) and 5′CGTAAGCCTTGAATTCCTCGCCGT3′ (Adr2). The PCR product was a 1665-bp fragment with an *Eco*RI site at each end of the *asd*A gene. The probe asdA0.4 was amplified with the primers 5′TCCGAACTAGACAAGCTCAAGG3′ (asdAF-1) and 5′CAATGAACGCACATCGGGTAGC3′ (asdAR-1). Amplification of DNA fragments via PCR was performed using Taq DNA polymerase (Promega, Madison, WI) with the reaction buffer recommended by the supplier.

Expression of Asd in *E. coli*

The complete coding sequence of Asd was amplified by means of PCR using *pfu* polymerase (Stratagene) and the primers of 5′AAGGATTGAATTCATGAGCAAGGA3′ (asdAF-4) and 5′TCAGGCATTAAAGCTTGAGATT3′ (asdAR-3). The PCR amplicons were digested with *Eco*RI and *Hin*dIII, and ligated into the vector pET21a generating pET-asdA. *E. coli* BL21(DE3) was used as a host for transformation. The transformants were grown in LB medium containing ampicillin at 37°C, and expression was induced by IPTG (final concentration 1 mM) when the OD600 reached 0.4 to 0.6. After 4 h of induction at 28°C, cells were harvested, washed, and disrupted for enzyme assay and protein purification. Fusion protein containing six histidine residues at the C-terminal of the target protein was purified as follows.

Preparation of the intracellular fractions and purification of fusion protein

Cells were harvested and suspended in 50 mM phosphate buffer (pH 6.8). The cell suspension was sonicated in an ice-bath for 10 min, and was then centrifuged at 8050×*g* for 15 min. After this procedure, the intracellular fractions were divided into the supernatant (soluble form) and pellet (insoluble form or inclusion body). Finally, the insoluble form was resuspended in 1% Triton–50 mM phosphate buffer, pH 6.8, containing 8 M urea. The soluble protein fractions in crude extracts containing recombinant Asd were purified using an His-bind affinity column according to the directions of the supplier (Novagen) with slight modification. After unbound proteins were washed, the pET-AsdA was eluted with buffer containing 500 mM imidazole. Fractions were assayed as described below and the pooled active fractions were chosen for further characterization.

SDS-PAGE

The proteins were separated on a 7.5% SDS polyacrylamide gel as described by Laemmli [8]. Bands were detected by means of Coomassie staining.

Assay of Asd

The Asd activity was assayed using the method described by Rozzell [11]. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of L-alanine from L-aspartate in 1 min under the assay conditions. The protein concentration of the enzyme preparation was determined using the method of Bradford with serum albumin as standard [2].

Computer analysis

Sequencing data were analyzed using the software package of the Genetics Computer Group (GCG) and the NCBI GeneBank

BLAST. The nucleotide sequence and amino acid sequence of *asd*A were also analysed using PC/GENE Version 6.60.

Results and discussion

Cloning and sequencing of Asd

Southern blot of restriction enzyme digests of *A. faecalis* CCRC 11585 chromosomal DNA were hybridized with a random primed asdA probe. A unique band was observed on the chromosomal DNA digested with *Bam*HI, *Eco*RI, *Kpn*I, *Sal*I or *Sma*I (Figure 1). To clone the gene encoding Asd of *A. faecalis* CCRC 11585, a partial genomic library was constructed in ZAP Express vector pBK-CMV in *E. coli* strain XLOLR as described in Materials and Methods. The internal sequence of Asd (0.4 kb) was amplified by means of PCR and used to make random primed probe (asdA 0.4) for screening the partial genomic library. Recombinant clones were then screened by means of colony hybridization with the probe asdA 0.4, and one positive clone was found. This clone contained a 13.5-kb plasmid designated pBK-asdAE1 (Figure 2). PCR amplification, with primer pairs of ADr1/asdAR-1 and asdAF-1/asdAR-1, and restriction enzyme analysis confirmed the presence of the desired gene. Restriction analysis of the isolated plasmid revealed that pBK-asdAE1 had one *Apa*I, *Cla*I, *Nde*I, *Nhe*I and *Sma*I cutting site within a 9-kb DNA insert (Figure 2). For the determination of the nucleotide sequence, the 5.5-kb DNA insert in pBK-asdAE1 was subcloned by deletion of 3.5 kb of *Apa*I–*Eco*RI fragment, yielding plasmid pBK-asdAAE6. A partial sequence (2000 bp) of the *Apa*I–*Sal*I fragment of pBKasdAAE6 was determined in both strands using the dideoxy chain termination method [13]. The nucleotide sequence of *asd*A and its flanking regions are shown in Figure 3. Analysis of the nucleotide sequence revealed the presence of one ORF, starting at ATG at nucleotide position 1 and terminating at TAA at position 1599. A putative Shine–Dalgarno sequence (-12 to -9) was found 9 bp upstream from the ATG start codon. The ORF encodes a protein of 533

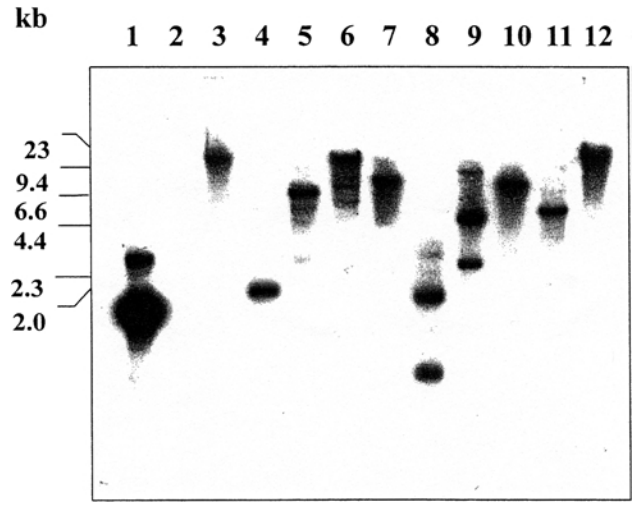

Figure 1 Southern blot of restriction-enzyme-digested chromosomal DNA of *A. faecalis* CCRC 11585 probed using the *asd*A probe. Lane 1, *asd*A probe; lane 2, λ/*Hin*dIII; lane 3, chromosomal DNA of *A. faecalis* CCRC 11585; lanes 4–12 are chromosomal DNA digested by different restriction enzymes. The restriction enzymes used are: lane 4, *Bam*HI; lane 5, *Eco*RI; lane 6, *Hin*dIII; lane 7, *Kpn*I; lane 8, *Pst*I; lane 9, *Sac*I; lane 10, *Sal*I; lane 11, *Sma*I; lane 12, *Xmn*I.

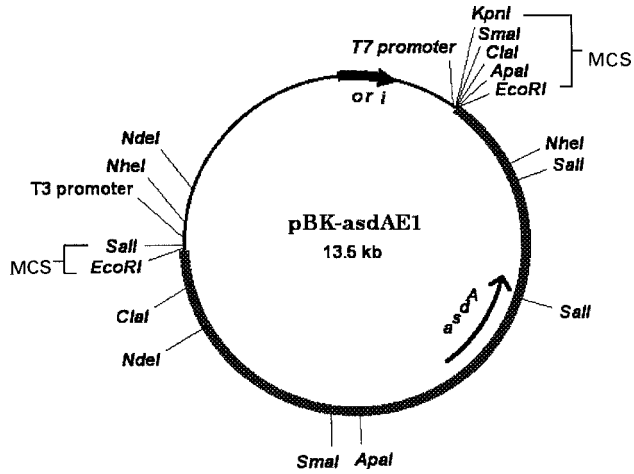

Figure 2 The restriction map of pBK-asdAE1. The cloned fragment asdAE1 is indicated by a heavy line. The black solid arrow indicates the site of Asd gene of *A. faecalis* CCRC 11585 (*asd*A). Multicloning sites (MCS) are on the pBK-CMV vector.

residues and the 3′ flanking region capable of forming a stem and loop structure. The encoded protein has a predicted molecular mass of 59.580 Da and a predicted isoelectric point of 5.0. The GC content is 55.59%. The EMBL/GenBank accession number for the asdA nucleotide sequence data presented in this paper is AF168368.

Sequence homology with other Asd and aminotransferases

A computer search of the FASTA and Swiss-Protein sequence data bank revealed a number of protein segments which are similar to various regions in the sequence of Asd from *A. faecalis*. Similarity comparisons showed that the nucleotide sequence and amino acid sequence of Asd have 84% and 93% similarity, respectively, with the sequence of that from *P. dacunhae*. The pyridoxal-5′-phosphate-depdendent enzymes (B_6 enzymes), the aspartate aminotransferase of *Methanococcus jannascii*, *Rhizobium meliloti*, *Bacillus subtilis*, *Sulfolobus solfataricus*, *Bacillus* sp. *YM-2*, *B. stearothermophilus*, the tyrosine aminotransferase of human and rat, and the elongation factor 1-alpha of *Sulfolobus acidocaldarius* also showed sequence similarity with Asd from 51% to 58%. It is interesting that the sequence near the cofactor, pyridoxal phosphate, binding lysine is highly conserved (Figure 4). Recent studies of the molecular evolution of B_6 enzymes have provided a rational approach for changing their reaction specificities. Graber *et al.* [6] created a mutant enzyme, [Y225R, R386A] aspartate aminotransferase, by site-directed mutagenesis. They found this mutant enzyme generated AsdA activity, which decarboxylates L-aspartate to L-alanine, while its transaminase activity towards dicarboxylic amino acids decreased. They concluded that the reaction specificity of B_6 enzymes may be changed by substitution of a limited number of critical residues determining the electron repartition in the coenzyme–substrate adduct [6].

Expression and Characterization of the Asd from ***A. faecalis***

The recombinant protein was highly expressed in pET-*asd*A transformed BL21(DE3) cells as compared to the Asd$^-$ host

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TCACCATCATTCCGCTGCTGATTGCCATCTTGTTTGGCAAGTATGTGCTGCGTTACGACA  -218

ACGTGGCCATGTTCGCGGGATCGCTGTCGGGCGCACGCAGTGCCAACCCCGCGTTTGGAG  -158

AGGTTCTGGACAAGGCCGGCAATTCAATCCCCACCACCCCGTTTGCAATCACTTATGCGT  -98
                                                          CCT
TGGCCAATGTGTTCCTGACTTTGTTGGGTCCGCTGGTCATTGCCTTCGCGTGAACCCCCT  -38

          ADr1 →                    asdAF-4 →
GAACGAATTCACTAGGACGAA      AAGATTGAATTCATGAGCAAGGA
GCACTGATTCACTAGGACGAATGGAAGGAGTTGCGATATGAGCAAGGATTATCAGAGTCT   23
                           RBS          M  S  K  D  Y  Q  S  L    8

GGCGAAATTGAGCCCGTTCGAGCTCAAGGATGAGTTGATCAAGATCGCCTCGAGCGACGG   83
 A  K  L  S  P  F  E  L  K  D  E  L  I  K  I  A  S  S  D  G    28

AAACCGCCTCATGCTCAATGCGGGGCGGGGCAATCCCAACTTTCTGGCCACAACGCCGAG  143
 N  R  L  M  L  N  A  G  R  G  N  P  N  F  L  A  T  T  P  R   48

AAGAGCATTTTTTCGCCTGGGCTTGTTCGCTGCAGCCGAATCGGAGCTTTCCTACTCTTA  203
 R  A  F  F  R  L  G  L  F  A  A  A  E  S  E  L  S  Y  S  Y   68

TATGACAACGGTAGGTGTTGGCGGACTGGCGAAGATCGACGGGATTGAGGGTCGCTTTGA  263
 M  T  T  V  G  V  G  G  L  A  K  I  D  G  I  E  G  R  F  E   88

ACGTTATATCGCCGAGAACCGGGATCAGGAGGGCGTGCGCTTTCTCGGTAAATCACTGAG  323
 R  Y  I  A  E  N  R  D  Q  E  G  V  R  F  L  G  K  S  L  S   108
                              •  •  •  •  •  •  •  •  •  •

CTATGTCCGTGACCAGCTGGGCTTGGACCCGGCCGCCTTCCTGCACGAAATGGTTGACGG  383
 Y  V  R  D  Q  L  G  L  D  P  A  A  F  L  H  E  M  V  D  G   128

TATTCTGGGCTGCAATTACCCCGTTCCCCCGCGGATGCTGAACATCAGTGAAAAAATCGT  443
 I  L  G  C  N  Y  P  V  P  P  R  M  L  N  I  S  E  K  I  V   148

ACGCCAGTACATCATCCGGGAAATGGGGGCCGATGCAATACCCAGTGAGTCCGTGAACCT  503
 R  Q  Y  I  I  R  E  M  G  A  D  A  I  P  S  E  S  V  N  L   168

GTTTGCAGTCGAAGGGGGAACGGCTGCCATGGCTTACATTTTCGAGAGCCTGAAGCTCAA  563
 F  A  V  E  G  G  T  A  A  M  A  Y  I  F  E  S  L  K  L  N   188

CGGTTTGCTCAAGGCCGGCGACAAGGTTGCCATTGGGATGCCGGTTTTCACGCCGTATAT  623
 G  L  L  K  A  G  D  K  V  A  I  G  M  P  V  F  T  P  Y  I   208

AGAAATTCCGGAGCTGGCCCAGTACGCACTGGAGGAAGTCGCCATCAACGCGGATCCGAG  683
 E  I  P  E  L  A  Q  Y  A  L  E  E  V  A  I  N  A  D  P  S   228

                    asdAF-1 →
                 TCCGAACTAGACAAGCTCAAGG
CCTCAATTGGCAGTATCCGGACTCTGAGCTGGACAAGCTCAAGGACCCGGCCATCAAGAT  743
 L  N  W  Q  Y  P  D  S  E  L  D  K  L  K  D  P  A  I  K  I   248

CTTTTTCTGCGTCAACCCCAGCAATCCGCCTTCGGTGAAGATGGATCAGCGAAGTCTGGA  803
 F  F  C  V  N  P  S  N  P  P  S  V  K  M  D  Q  R  S  L  E   268

ACGCGTGCGCAACATCGTTGCAGAACATCGTCCGGATCTGATGATCCTGACCGACGATGT  863
 R  V  R  N  I  V  A  E  H  R  P  D  L  M  I  L  T  D  D  V   288
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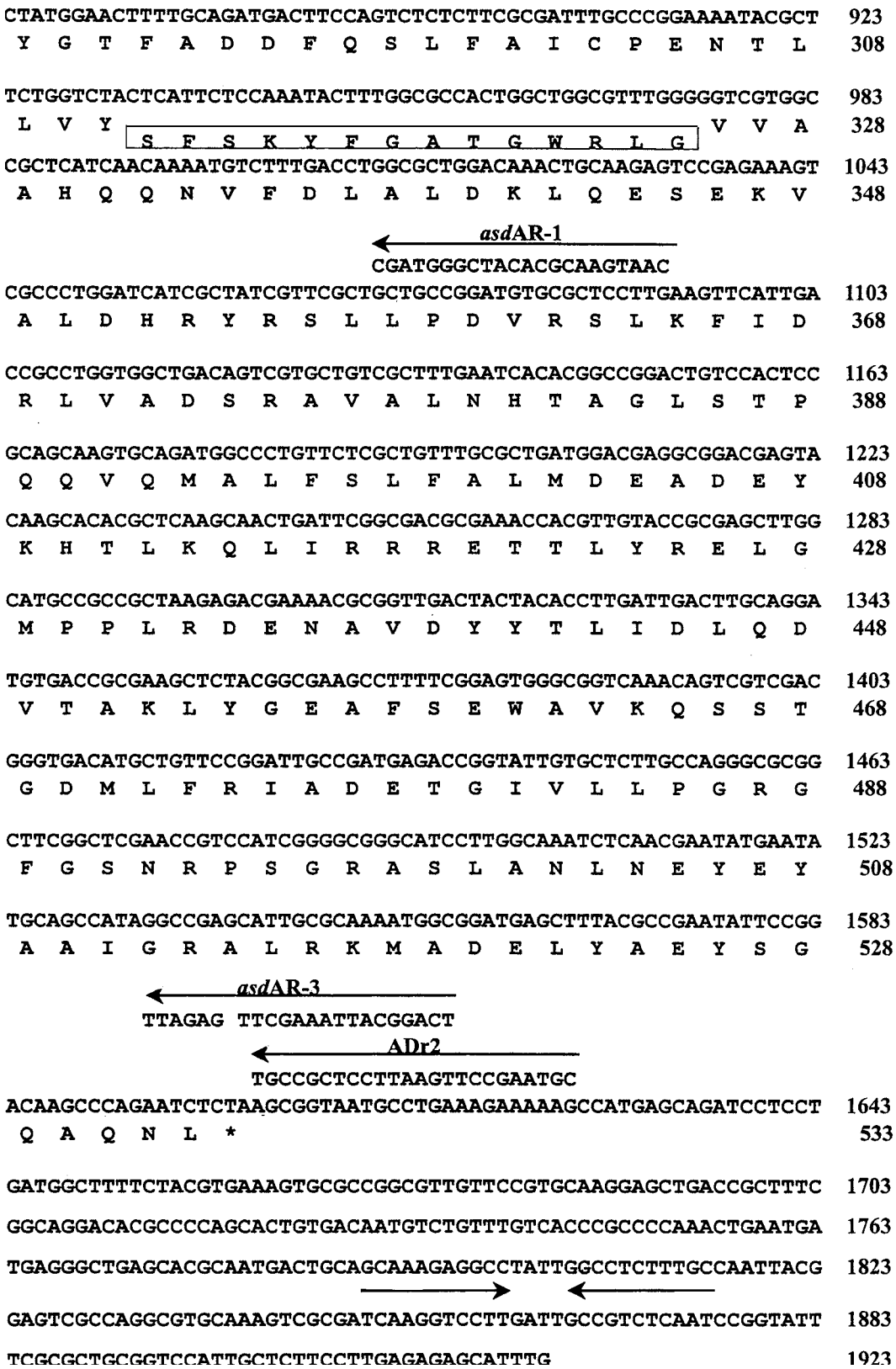

Figure 3 The nucleotide sequence and deduced amino acid sequence of the Asd gene from *A. faecalis* CCRC 11585. The putative Shine–Dalgarno sequence is underlined. The stop codon is indicated by an asterisk. The head-to-head arrow indicates the putative terminator sequence. Six regions of oligonucleotide primer are indicated by ADr1, *asd*AF-4, *asd*AF-1, *asd*AR-1, *asd*AR-3 and ADr2. The amino acid sequence is denoted below the nucleotide sequence in single letter code. ATP/GTP binding motif A (P-loop) is indicated by dots. The box represents the aminotransferases class-I pyridoxal-phosphate attachment site motif.

```
AATA RHIME  .......... .......... .......... .......... ..........
AATB RHIME  .......... .......... .......... .......... .......MTI   3
AAT1 METJA  .......... .......... .......... .......... ..........
 AAT SULSO  .......... .......... .......... .......... ..........
AAT2 BACSU  .......... .......... .......... .......... ..........
ATTY HUMAN  .......... ......MDPY MIQMSSKGNL PSILDVHVNV GGRSSVPGKM  34
  ATTY RAT  .......... ......MDSY VIQTDVDDSL SSVLDVHVNI GGRNSVQGRK  34
   AF ASDA  MSKDYQSLAK LSPFELKDEL IKIASSDGNR LMLNAGRGNP NFLATTPRRA  50
 Consensus  .......... .......... .......... .......... ..........

AATA RHIME  ...MAFLADA LSRVKPSATI AVS.QKAREL ...KAKGRDV IGLGAGEP..  41
AATB RHIME  NATVKEAGFR PASRISSIGV SEILKIGARA AAMKREGKPV IILGAGEP..  51
AAT1 METJA  .......... ..MISSRCKN IKP.SAIREI ..FNLATSDC INLGIGEP..  34
 AAT SULSO  MVSLLDFNGN MSQVTGETTL LYK.EIARNV ..EKTKKIKI IDFGIGQP..  45
AAT2 BACSU  ..MEITPSDV IKTLPRQEFS LVF.QKVKEM ..EKT.GAHI INLGQGNP..  42
ATTY HUMAN  KGRKARWSVR PSDMAKKTFN PIR.AIVDNM KVKPNPNKTM ISLSIGDPTV  83
  ATTY RAT  KGRKARWDVR PSDMSNKTFN PIR.AIVDNM KVQPNPNKTV ISLSIGDPTV  83
   AF ASDA  FFRLGLFAAA ESELSYSYMT TVGVGGLAKI DGIEGRFERY IAENRDQEGV  100
 Consensus  .......... .s........ .v........ .......... I.lg.g#p..

AATA RHIME  ..DFDTPDNI KK.......A AIDA.IDR.. GETKYTPVSG IPELREAIAK  79
AATA RHIME  ..DFDTPDNI KK.......A AIDA.IDR.. GETKYTPVSG IPELREAIAK  90
AAT1 METJA  ..DFDTPKHI IE.......A AKRA.LDE.. GKTHYSPNNG IPELREEISN  72
 AAT SULSO  ..DLPTFKRI RD.......A AKEA.LDQ.. GFTFYTSAFG IDELREKIAQ  83
AAT2 BACSU  ..DLPTPPHI VE.......A LREASLNP.. SFHGYGPFRG YPFLKEAIAA  81
ATTY HUMAN  FGNLPTDPEV TQ.......A MKDALDSG.. KYNGYAPSIG FLSSREEIAS  123
  ATTY RAT  FGNLPTDPEV TQ.......A MKDALDSG.. KYNGYAPSIG YLSSREEVAS  123
   AF ASDA  RFLGKSLSYV RDQLGLDPAA FLHEMVDGIL GCNYPVPPRM LNISEKIVRQ  150
 Consensus  ..d..t...! .........A ...a..d... g...y.p..g ...lre.!a.
                                                      *

AATA RHIME  KFKRENNLDY TAA.QTIVGT ..GGKQILFN AFMAT..... LNPGDEVVIP  121
AATB RHIME  KFQRENGLAY ELD.EITVAT ..GAKQILFN AMMAS..... LDPGDEVVIP  132
AAT1 METJA  KLKDDYNLDV DKD.NIIVTC ..GASEALML SIMTL..... IDRGDEVLIP  114
 AAT SULSO  YLNTRYGTDV KKE.EVIVTP ..GAKPALFL VFILY..... INPSDEVILP  125
AAT2 BACSU  FYKREYGVTI NPETEVALFG ..GGKAGLYV LTQCL..... LNPGDIALVP  124
ATTY HUMAN  YYHCPEAPLE AKD..VILTS ..GCSQAIDL CLAVL..... ANPGQNILVP  164
  ATTY RAT  YYHCHEAPLE AKD..VILTS ..GCSQAIEL CLAVL..... ANPGQNILIP  164
   AF ASDA  YIIREMGADA IPSESVNLFA VEGGTAAMAY IFESLKLNGL LKAGDKVAIG  200
 Consensus  y..re.g.d. .....vil.. ..Gg..al.. .f..l..... lnpg#.v.ip
                                  *

AATA RHIME  APYWVSYPEM VALCGGTPVF VPTR..QENN FKLKAEDLDR AITPKTKWFV  169
AATB RHIME  TPYWTSYSDI VQICEGKPIL IACD..ASSG FRLTAQKLEA AITPRTRWVL  180
AAT1 METJA  NPSFVSYFSL TEFAEGKIKN IDL....DEN FNIDLEKVKE SITKKTKLII  160
 AAT SULSO  DPSFYSYAEV VKLLGGKPIY ANLKWSREEG FSIDVDDLQS KISKRTKMIV  175
AAT2 BACSU  NPGYPEYLSG ITMARAELYE MPLY..EENG YLPDFEKIDP AVLEKAKLMF  172
ATTY HUMAN  RPGFSLYKTL AESMGIEVKL YNLL..PEKS WEIDLKQLEY LIDEKTACLI  212
  ATTY RAT  RPGFSLYRTL AESMGIEVKL YNLL..PEKS WEIDLKQLES LIDEKTACLV  212
   AF ASDA  MPVFTPYIEI PELAQYALEE VAINADPSLN WQYPDSELDK LKDPAIKIFF  250
 Consensus  .P.f..Y.e. .ela...... ..l....e.n ...d...ld. .i..ktk...
               *  *

AATA RHIME  FNSPSNPSGA AYSHEELKAL TDVLMKH.PH VWVLTDDMYE HLTYGDFRFA  218
AATB RHIME  LNSPSNPSGA AYSAADYRPL LDVLLKH.PH VWLLVDDMYE HIVYDAFRFV  229
AAT1 METJA  FNSPSNPTGK VYDKETIKGL AEIAEDY..N LIIVSDEVYD KIIYDKKHYS  208
 AAT SULSO  FNNPHNPTGT LFSPNDVKKI VDISRDN..K IILLSDEIYD NFVYEGKMRS  223
AAT2 BACSU  LNYPNNPTGA VADAAFYAKA AAFAKEH..N IHLIHDFAYG AFEFDQKPAS  220
ATTY HUMAN  VNNPSNPCGS VFSKRHLQKI LAVAARQ..C VPILADEIYG DMVFSDCKYE  260
  ATTY RAT  VNNPSNPCGS VFSKRHLQKI LAVAERQ..C VPILADEIYG DMVFSDCKYE  260
   AF ASDA  CVNPSNPPSV KMDQRSLERV RNIVAEHRPD LMILTDDVYG TFADDFQSLF  300
 Consensus  .n.PsNP.g. ..s....... ......h... ...l.D..Y. ...yd.....
               *   *^                          *  *
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AATA RHIME  TPVEVEPGLY ERTLTMNGVS KAYAMTGWRI GYAAGPLHLI KAMDM.....  263
AATB RHIME  TPARLEPGLK DRTLTVNGVS KAYAMTGWRI GYAGGPRALI KAMAV.....  274
AAT1 METJA  PMQ.....FT DRCILINGFS KTYAMTGWRI GYLAVSDELN KELDL.....  248
 AAT SULSO  TLEDSD..WR DFLIYVNGFS KTFSMTGWRL GYIVAKREII QKMGI.....  266
AAT2 BACSU  FLEAED..AK TVGAELYSFS KTFNMAGWRM AFAVGNEKII QAVNE.....  263
ATTY HUMAN  PLATLS..TD VPILSCGGLA KRWLVPGWRL GWILIHDRRD IFGNE.....  303
  ATTY RAT  PLANLS..TN VPILSCGGLA KRWLVPGWRL GWILIHDRRD IFGNE.....  303
   AF ASDA  AI......CP ENTLLVYSFS KYFGATGWRL GVVAAHQQNV FDLALDKLQE  344
 Consensus  .......... ...l..ngfs K...mtGWR. gy.......i ..........
                          *   *       *   *

AATA RHIME  .......... .......... ......IQGQ Q......TSG AASIAQWAAV  281
AATB RHIME  .......... .......... ......VQSQ A......TSC PSSVSQAASV  292
AAT1 METJA  .......... .......... ......INNM IKIHQYSFAC ATTFAQYGAL  272
 AAT SULSO  .......... .......... ......LAAN V......YTA PTSFVQKAAV  286
AAT2 BACSU  .......... .......... ......FQDH V......FVG MFGGLQQAAS  281
ATTY HUMAN  .......... .......... ......IRDG LVKLSQRILG PCTIVQGALK  327
  ATTY RAT  .......... .......... ......IRDG LVKLSQRILG PCTIVQGALK  327
   AF ASDA  SEKVALDHRY RSLLPDVRSL KFIDRLVADS RAVALNHTAG LSTPQQVQMA  394
 Consensus  .......... .......... .......... .........g .....Q.aa.

AATA RHIME  EAL.NGPQDF IGRNKEIFQG RRDLVVSMLN QAKGISCPTP EGA..FYVYP  328
AATB RHIME  AAL.NGPQDF LKERTESFQR RRNLVVNGLN AIEGLDCRVP EGA..FYTFS  339
AAT1 METJA  AAL.RGSQKC VEDMVREFKM RRDLIYNGLK DI..FKVNKP DGA..FYIFP  317
 AAT SULSO  KAF.DTFDE. VNQMVSLFKK RRDVMYDELT KVKGVEVSKP NGA..FYMFP  332
AAT2 BACSU  AAL.SGDPEH TESLKRIYKE RIDFFTALCE KELGWKMEKP KGT..FYVWA  328
ATTY HUMAN  SILCRTPGEF YHNTLSFLKS NADLCYGALA AIPGLRPVRP SGA..MYLMV  375
  ATTY RAT  SILQRTPQEF YHDTLSFLKS NADLCYGALA AIPGLQPVRP SGA..MYLMV  375
   AF ASDA  LFSLFALMDE ADEYKHTLKQ LIRRRETTLY RELGMPPLRD ENAVDYYTLI  444
 Consensus  .al..g.... .......fk. rrdl....l. ...g.....p .ga..fY...

AATA RHIME  SCAGLIGKTA PSG.....KV IETDEDFVSE LLETEGVAVV HGSAFGLG..  371
AATB RHIME  GCAGVARRVT PSG.....KR IESDTDFCAY LLEDSHVAVV PGSAFGLS..  382
AAT1 METJA  DVSEY..... GDG.....VE V......AKK LIENK.VLCV PGVAFGEN.G  349
 AAT SULSO  NVSKILKTSG FDV.....KS L......AIK LIEEKGVVTI PGEVFPLNIG  371
AAT2 BACSU  EIPNTFETSH Q......... ......FSDY LLEHAHVVVT PGEIFGSN.G  302
ATTY HUMAN  GIEMEHFPEF END.....VE ......FTER LVAEQSVHCL PATCFEYP..  412
  ATTY RAT  GIEMEHFPEF END.....VE ......FTER LIAEQAVHCL PATCFEYP..  412
   AF ASDA  DLQDVTAKLY GEAFSEWAVK QSSTGDMLFR IADETGIVLL PGRGFGSN..  492
 Consensus  .......... .......... ......f... l.e...!... pg..Fg.n..
                                                        ▲

AATA RHIME  .PNFRISYAT SEALLEEACR R.IQRFCAAC R.........  ...       400
AATB RHIME  .PYFRISYAT SEAELKEALE R.ISAACKRL S.........  ...       411
AAT1 METJA  ANYIRFSYAT KYEDIEKALG I.IKEIFE..  .........  ...       376
 AAT SULSO  KEFLRLSFAV NEEVIKEGIQ K.IREFAEQM MNSR......  ...       408
AAT2 BACSU  KRHVRISMVS KQEDLREFVT R.IQKLNLPF GSLQETSR..  ...       399
ATTY HUMAN  .NFIRVVITV PEVMMLEACS R.IQEFCEQH YHCAEGSQEE CDK         454
  ATTY RAT  .NFFRVVITV PEVMMLEACS R.IQEFCEQH YHCAEGSQEE CDK         454
   AF ASDA  RPSGRASLAN LNEYEYAAIG RALRKMADEL YAEYSGQAQN L..         543
 Consensus  ....R.s.a. .ee...ea.. r.i.......  .........  ...
              ▲
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Figure 4 Alignment of the deduced amino acid sequence from *A. faecalis* CCRC 11585 Asd (AF ASDA) with aminotransferases from various sources. Aspartate aminotransferase of *Rhizobium meliloti* (AATA RHIME, AATB RHIME), *Methanococcus jannaschii* (AAT1 METJA), *Sulfolobus solfataricus* (AAT SULSO), *Bacillus subtilis* (AAT2 BACSU); tyrosine aminotransferase of human (ATTY HUMAN) and rat (ATTY RAT). Identical amino acids are indicated by capital letters, and amino acids conserved at greater than 50% are indicated by the lowercase letters. The symbol "!" is any one of IV, and "#" is any one of NDQEBZ. The conserved domains of the PLP binding site and substrate binding sites are indicated by asterisks and black triangles, respectively. The aminotransferase class-I pyridoxal-phosphate attachment site motif is underlined.

and vector controls (Figure 5). This fusion AsdA was visible on SDS-PAGE with a molecular mass of 61 kDa and its specific activity was 11.27 U/mg soluble protein (Table 2). The plasmid pET-asdA encodes the AsdA tagged with an extension of six histidine residues to the carboxyl terminus, which was used to purify proteins from a His-bind column. After purification by means of His-bind column elution, 3.3 mg of purified protein was obtained from 100 ml of the

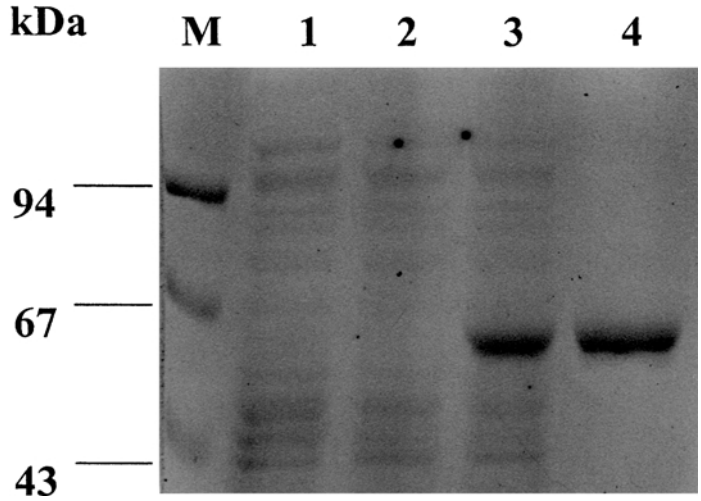

Figure 5 SDS-PAGE analysis of Asd purified from *E. coli* BL21(DE3)/pET-asdA. Lane M, protein molecular mass markers; lanes 1–3, the crude enzyme from *E. coli* BL21(DE3), *E. coli* BL21(DE3)/pET-21a + and *E. coli* BL21(DE3)/pET-*asd*A; lane 4, AsdA purified by His-Bind column (1.5 μg). The gel was stained with comassie brilliant blue R-250.

culture. The purified fusion protein appeared to be homogenous on SDS-PAGE (Figure 5), and its specific activity increased to 75.45 U/mg protein at pH 6.8 and 35°C (Table 2). The optimal pH and temperature of this enzyme are pH 5 and 45°C, respectively (Figure 6). The specific activity of the His-Bind purified AsdA is 215 U/mg at 45°C and pH 5.0 (Figure 6), which is 1000 times higher than that of the crude enzymes from *A. faecalis* and *P. dacunhae* (Table 2).

In this study we have described the cloning and functional expression in *E. coli* of Asd from *A. faecalis* CCRC 11585. A fusion recombinant AsdA was expressed in *E. coli* using the pET system. The expressed fusion protein is functionally active and could use L-aspartic acid to produce L-alanine. Rozzell [11] constructed several clones containing the *P. daunchae asd*A gene regulated by the P_L promoter, and he found in all cases examined, AsdA specific activity of 20 international units per milligram of soluble cell extract or higher was obtained. An efficient production system for L-alanine and D-aspartic acid by this recombinant AsdA enzyme will be further investigated. According to the result of computation analysis, the pyridoxal-5′-phosphate-dependent enzymes, such as the aspartate aminotransferases of several microorganisms, also share sequence similarity (51–58%) with Asd. Although the evolutionary relationship between aspartate aminotransferase and Asd is not known because no data on the primary or spatial structures of the decarboxylase are available as

Table 2 Specific activity of Asd from different sources

Enzyme source	Protein concentration (mg/ml)	Activity (unit/ml)[a]	Specific activity (unit/mg)
Crude enzyme from:			
P. dacunhae CCRC 12623	1.18	0.29±0.04	0.25
A. faecalis CCRC 11585	0.91	0.20±0.03	0.22
E. coli BL21(DE3)	2.81	0.11±0.02	0.04
E. coli BL21(DE3)/pEt-21a	3.15	0.08±0.01	0.03
E. coli BL21(DE3)/pET-*asd*A	2.05	23.12±2.10	11.27
His-Bind purified AsdA	0.16	11.77±2.21	75.45

[a]One unit is defined as the production of 1 μmol of L-alanine per minute at 35°C and pH 6.8.

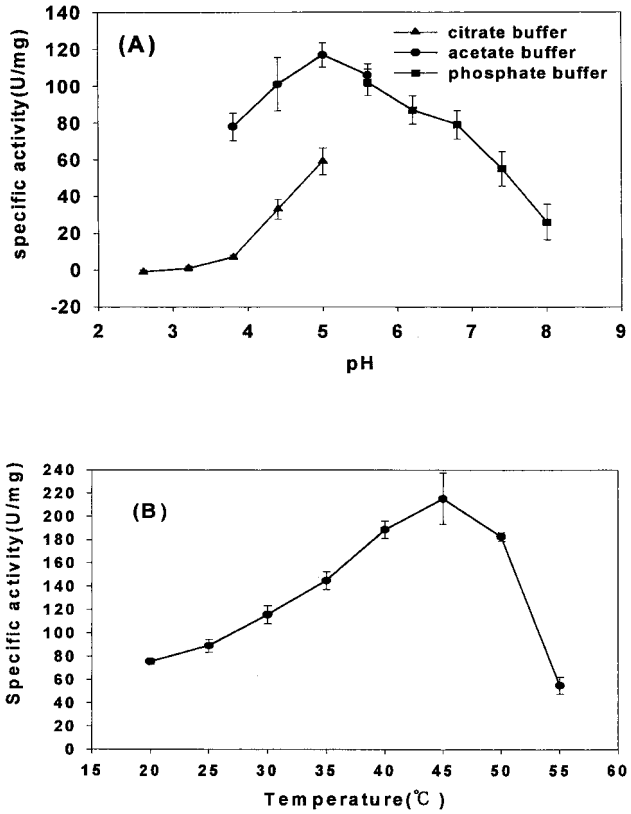

Figure 6 The optimal pH (A) and temperature (B) of Asd of *A. faecalis* CCRC 11585. The effect of pH on AsdA activity was evaluated at 35°C while the effect of temperature on AsdA activity was evaluated at pH 5.0.

yet. These enzymes might in fact constitute a family of proteins, and their structural genes may have evolved from a common ancestral gene.

Acknowledgements

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