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Full Length Research Paper

Cloning, expression and purification of cold adapted acetate kinase from *Shewanella* species AS-11

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A psychrotrophic bacterium, *Shewanella* sp. AS-11 was isolated from a buccinid (shell) *Neobuccinum eatoni* living in the Antarctic ice-covered sea. An open reading frame of 1203 bp, coding for acetate kinase gene, called ack, was amplified, cloned into the expression vector, pETY-16b, and the enzyme was overproduced by using T7 system in *Escherichia coli* BL21 (DE3). After extraction of crude recombinant acetate kinase, the desired enzyme was able to be purified on a Blue Sepharose CL-6B and Super-Q affinity column chromatography. The molecular mass of the enzyme is about 86 kDa, which is associated with two monomers. In respect of pH, the enzyme was stable between 6 to 8 and maximum activity was obtained at 7.5. The purified enzyme was stable at 30°C but ligand bound enzyme was stable at 40°C. The structural comparison to mesophilic and thermophilic acetate kinases demonstrates that the psychrophilic one contains lower number of salt bridges and cation-pi interaction. So, it can be suggested that the enzyme is cold adapted with thermolabile and flexible structure.

Key words: Acetate kinase, thermolabile, cold adapted, flexible, activity.

INTRODUCTION

Acetate kinase plays important role for bioconversion of organic compounds to methane and annually more than billion metric tons of methane are produced from the decomposition of organic matter by anaerobic microbial consortia (Ferry, 1992), which can help to reduce environmental pollution and energy crisis. Temperature is one of the most important factors for bioconversion of organic waste materials. In most parts of our earth that are cold as well as in all over the world, a large seasonal variation is observed. In cold environment (at low temperatures), the growth of microorganisms is reduced several times; as a result, degrading rate of the organic pollutants is decreased, which ultimate end product is methane. The bioconversion process of recalcitrant compounds can be improved by using mixed culture containing specific cold adapted microorganisms (Kumar et al., 2011). Many environment pollutant compounds such as nitrates, hydrocarbons, aromatic compounds, cellulose, chitin, lignin, protein, heavy metals, etc. are already reduced by using psychrophilic or psychrotrophic microorganisms (Timmis and Pieper, 1999; Vazquez et al., 1995). Psychrophilic or psychrotrophic organisms are colonized in cold environment and can synthesize cold adapted enzymes. These enzymes bear special character like high catalytic activity at low temperatures, a large flexibility, etc. to adapt to the organisms in cold environment (Gerday et al., 2000; Feller et al., 1999; Chiuri et al., 2009). For this specific nature, the enzymes are offered in potential economic advantages in different biochemical sectors such as bioremediation of polluted wastes, biomass conversion, detergents industry, food

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processing, etc. by catalyzing the reaction at low and moderate temperatures instead of high temperature. As a result psychrophiles have received increasing attention due to their pertinence in both basic and applied research.

Acetate kinase, (EC 2.7.2.1, ATP:acetate phosphotransferase) is a member of the ASKHA (acetate and sugar kinases/Hsc70/actin) superfamily of phosphotransferases (Buss et al., 2001), which catalyzes the phosphorylation of acetate with ATP to form acetyl phosphate. The enzymes, which belong to this group exhibit large conformational changes during catalysis and these conformational changes are easily observed by domain movement (Diao et al., 2009). Acetate kinase especially psychrophilic acetate kinase (generally, more flexible structure is formed by psychrophilic enzyme than mesophilic or thermophilic enzymes) is an ideal enzyme for effective biodegradation of organic waste materials at low temperature.

Shewanella sp. AS-11 is a Gram-negative, rod-shaped and aerobic bacterium isolated from a buccinid (shell) *Neobuccinum eatoni* living in the Antarctic ice-covered sea. *Shewanella* sp. AS-11 grows most rapidly at 20°C and can grows well at 4°C but cannot grow above 30°C, being classified to psychrotroph according to Morita (1975). The amino acid sequences of the protein encoded by acetate kinase gene have been determined from the genomic DNA of *Shewanella* sp. AS-11 bacterium by Tanoue et al. (2010).

In our present study, the acetate kinase from psychrotrophic bacterium *Shewanella* sp. AS-11 was cloned and expressed in *Escherichia coli* DH5α and BL21 (DE3), respectively. Finally, expressed enzyme was purified and partially characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzyme and reagents

Shewanella sp. AS-11 was isolated from a buccinid (shell) *N. eatoni* living in the Antarctic ice-covered sea, which was a source of cold adapted acetate kinase gene. The sources of other materials used were as follows: Deoxyribonucleic acid used as carrier DNA was obtained from Sigma-Aldrich Co. Ltd. The pET-16b vector was used from Novagen. Polymerase chain reaction (PCR) primers were purchased from Hokkaido System Science Co. Ltd. (Japan); *KOD-plus* DNA polymerase, *Bam*HI and *E. coli* from TOYOBO; *Pst*I from Wako Nippon Gene; QIAprep Miniprep kit from Qiagen; DNA ladder marker and protein marker from New England BioLabs. All other chemicals were of analytical grade for biochemical use.

PCR amplification of acetate kinase gene

Genomic DNA of *Shewanella* species AS-11 was prepared as described by Sambrook et al. (1989) and open reading frames (ORFs) of acetate kinase gene was obtained by a PCR using genomic DNA, as template with Perkin Elmer Gene PCR system-2400. The acetate kinase gene was amplified with forward (P-1) and reverse primer (P-2) 5`-**GTTTAACTTTAAGAAGGAGATATACC**ATGTCAGACAAATTAGT ACTCGT-3` and 5`-**AGCTTCCTTTCGGGCTTTGTTAGCAGCC**TCATTACTTAGCTGT GATCAGCTTAATG-3`, respectively (bold and under lines nucleotides are homologous of pETY-16b vector, starting and stop codon, respectively) under the following reaction condition: 2 min at 94°C for initial denaturation; followed by 30 cycles of 10 s at 98°C for denaturation, 30 s at 53°C for annealing, and 1 min at 68°C for extension using *KOD Plus* DNA polymerase. The primer-1 was designed to include 26-nucleotide sequences homologous to that just upstream of the starting codon of pETY-16b vector (The pETY-16b, *E. coli*-yeast shuttle vector, which was constructed by introducing a 2 micro replication origin and a selectable marker (*URA*3) of yeast to pET-16b) at the 5` end so that the recombinant enzymes is produced without His•Tag. The primer-2 was designed, to include 28-nucleotide sequence homologous to that just downstream of the *Bam*HI site of pETY-16b vector at 5` end. Amplified gene was tested on 1% agarose-S gel electrophoresis.

Transformation and isolation of recombinant plasmid

The cells of *Saccharomyces cerevisiae* were transformed according to the method of Gietz and Wood (2002). The yeast cells were suspended in 90 µl transformation mixture and cultivated. The mixture was prepared by mixing 60 µl of 50% polyethylene glycol 3500, 9 µl of 0.1 M lithium acetate, 12.5 µl of 2 mg/ml boiled SS-carried DNA, 6 µl of amplified genes (100 ng) and 2.5 µl *Bam*HI digested by pETY-16b vector (80 ng). The recombinant plasmid was isolated from the yeast cells by using QIAprep Miniprep kit (Qiagen) according to the manufacturer's instructions. The graphical representation of the amplified acetate kinase ORF into pETY-16b by homologous recombination in yeast is shown in Figure 1.

Recombinant plasmid was transferred into *E. coli* DH5α according to the method of Pope and Kent (1996) and the recombinant plasmid DNA was isolated. The plasmid DNA was digested with restriction enzyme, *Pst*I and analyzed by 1% agarose-S gel electrophoresis.

Expression and purification of acetate kinase

Recombinant plasmid DNA (isolated from *E. coli* DH5α) was transformed into *E. coli* BL21 (DE3) (Puyet A, 1987) for expression. The cells were grown at 37°C to an A_{600} of 0.6 to 0.9; at that time isopropyl β-D-thiogalactopyranoside (IPTG) was added (final concentration, 1 mM) to the culture medium and temperature of the growth medium was shifted to 20, 25, 30 and 37°C and the cells were cultivated at the shifted temperatures for an additional 2 to 24 h for determination of optimum induction temperature and time for acetate kinase expression. Cells were harvested and the total proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gel (Laemmli, 1970). Cells (25 to 30 mg/ml) were re-suspended in 20 mM Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl at 4°C and mixed with phenyl methyl sulfonyl fluoride (PMSF), freshly prepared lysozyme and sodium deoxycholate (final concentration was 1 mM, 1 mg/ml and 2.5 mg/ml, respectively). The cell suspension was incubated about 20 to 30 min with stirring at 4°C and then frozen at -80°C for 15 to 20 min. Then PMSF, DNase and RNase were added (final concentra-

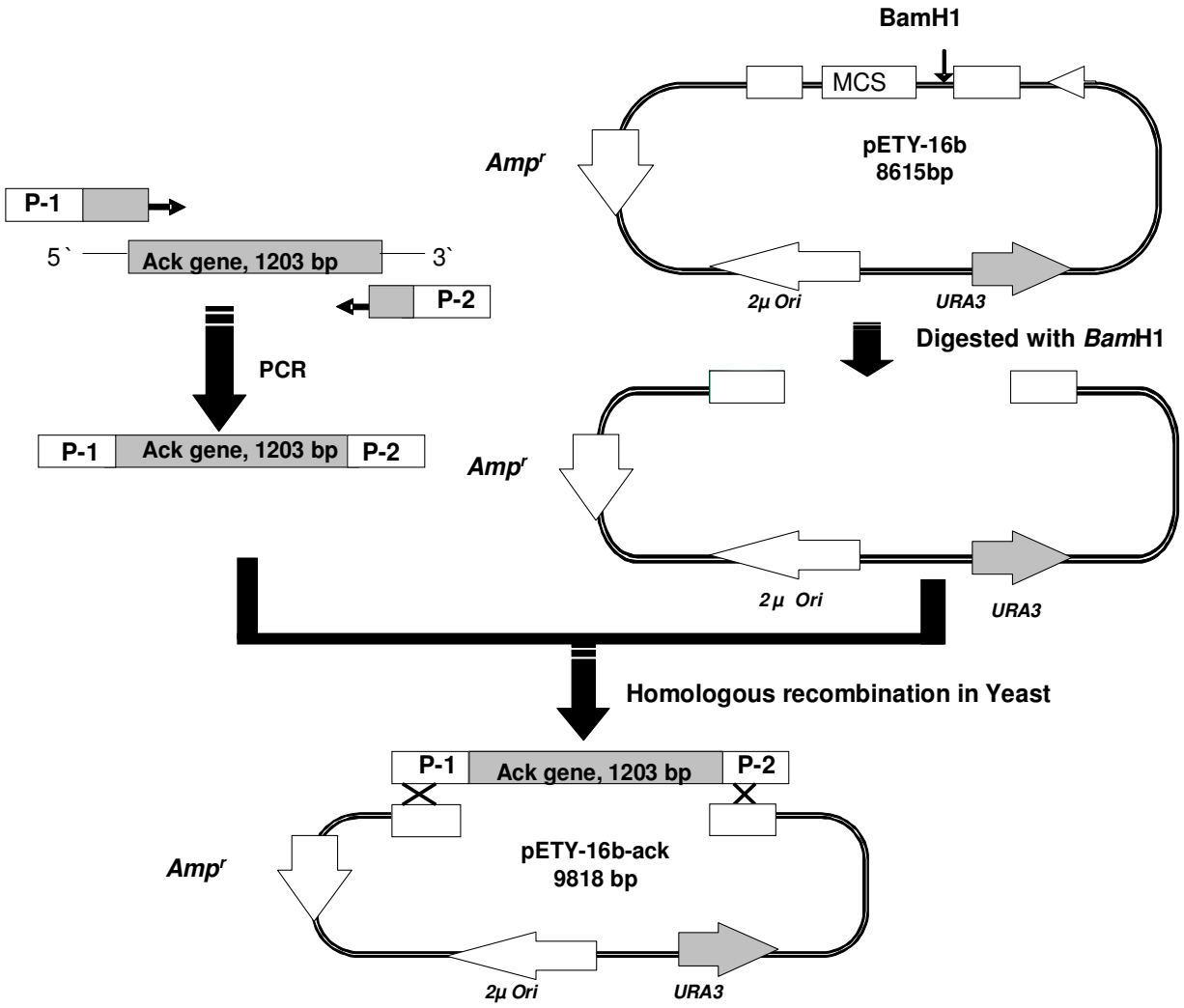

Figure 1. Graphical representation of PCR amplification and homologous recombination of acetate kinase ORF with pETY-16b. Ack is acetate kinase gene, pETY-16b-ack is pETY-16b vector contain acetate kinase gene. PCR, Polymerase chain reaction.

tion was 1 mM, 25 and 25 µg/ml, respectively) to the suspension to remove nucleic acids. The supernatant was collected and subjected to ammonium sulfate fractionation. The active fraction was collected and dialyzed against 20 mM Tris-HCl buffer containing 8 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 7.5% glycerol (v/v) at pH 7.5 and applied on a Blue Sepharose CL-6B affinity column pre-equilibrated with same buffer. The column was washed stepwise with buffer containing 50 mM KCl, 100 mM KCl and 200 mM potassium acetate at pH 7.5. Acetate kinase was eluted with same buffer containing 1 mM ATP and 200 mM potassium acetate at pH 7.5 (Fox and Roseman, 1986). The enzyme was further purified by Super-Q column chromatography and eluted by the linear gradient with same buffer containing 0.2 M NaCl, pH 7.5. The purity of the acetate kinase was assessed by SDS-PAGE using 12.5% gel. Protein concentration was determined by the Bradford method (1976) using protein dye reagent (Bio-Rad) and bovine serum albumin was used as the standard.

Superose-6 column chromatography

Superose-6 column (125 ml) was equilibrated with about 1 L of 30 mM (N-morpholino) propanesulphonic acid (MOPS)-KOH buffer pH 7.0 containing 50 mM KCl and calibration curve was prepared with Rnase A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and β-amylase (200 kDa) at a flow rate 0.5 ml/min. To determine the molecular mass of acetate kinase, 0.5 ml purified sample (1.5 mg/ml) was applied onto the same column and the retention volume was measured by same buffer and same flow rate.

Enzyme activity assays

The enzyme activity assay was measured by the hydroxamate Assay method, which detecting acetyl phosphate formation. The

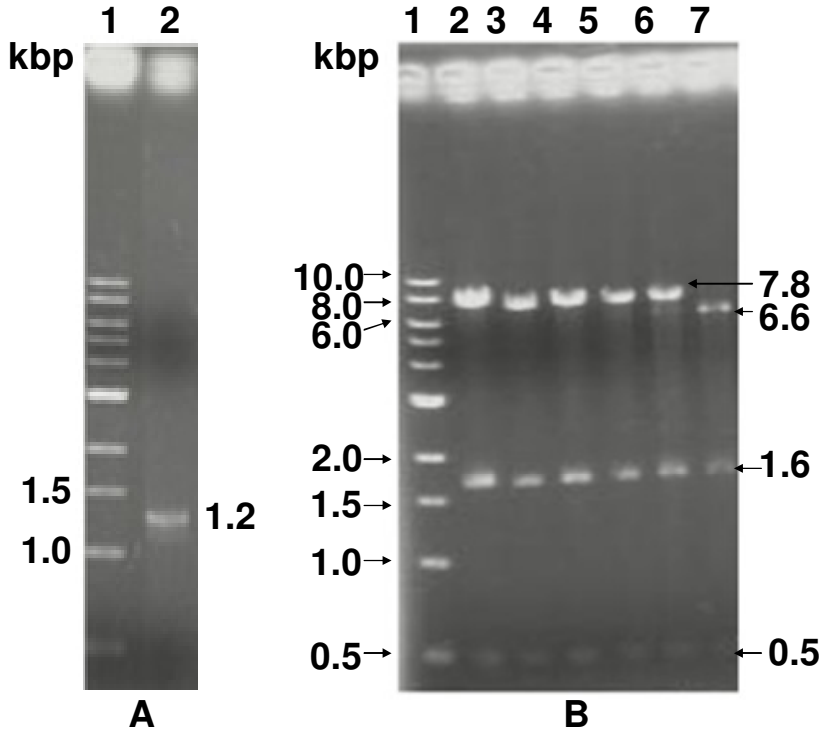

Figure 2. Agarose-S gel electrophoresis of the amplified DNA and recombinant plasmid digested with *Pst*I. (A) Lane 1, marker; lane 2, products amplified with a template of *Shewanella* sp. AS-11 chromosomal DNA; (B) lane 1, marker; lanes 2 to 6, pETY-16b-ack; lane 7, pETY-16b.

hydroxamate assay, an adaptation of the method of Rose, Lipmann and Aceti (Rose et al., 1954; Lipman, 1944; Aceti and Ferry, 1988), utilizes the reaction of acetyl phosphate with hydroxylamine to form acetyl hydroxamate, which forms a colored complex with trivalent iron. This method was slightly modified and used to measure the acetate kinase activity. 40 mM HEPES buffer, pH 7.5 was used instead of Tris-HCl buffer and the absorbance was measured at 540 nm using a micro plate reader (Bio-Rad, model 680XR). A molar absorption coefficient of 0.46 $mM^{-1}cm^{-1}$ was used for determination of acetyl phosphate concentration (Bock et al., 1999). The specific activity (Unit/mg) is reported as µmoles of product formed/min/mg of protein.

pH dependency and stability

Enzyme activity was measured in the buffer containing 20 mM sodium citrate, 20 mM sodium tetra borate and 20 mM MOPS at different (2 to 11) pH at 25°C and for pH stability measurement, acetate kinase was pre-incubated at pH values ranging from 2 to 12 in the same buffer for 30 min at 25°C and then their residual activities were measured by hydroxamate assay method at pH 7.5 at 25°C.

Thermostability

Acetate kinase was pre-incubated at different temperatures from 20 to 80°C for 15 min in 40 mM HEPES buffer, pH 7.5, with and without 10 mM ATP or 200 mM potassium acetate. The residual activities were measured by hydroxamate assay method at 25°C at pH 7.5. To determine the optimum temperature of acetate kinase, activity was measured at 5 to 80°C by above method.

Sequence alignment of psychrophilic, mesophilic and thermophilic acetate kinases

The sequence alignment, the percentages of identity of psychrophilic, mesophilic and thermophilic acetate kinases were determined by using ClustalW (1.83). The salt bridges were determined using a distance of 4.0 Å (Kumar and Nusssinov, 1999) between interacting groups with "What if web program". The cation-pi interaction was determined with CAPTURE (http://capture.caltech.edu.) (Gallivan and Dougherty, 1999).

RESULTS AND DISCUSSION

Amplification of acetate kinase gene

The ORFs of ack of psychrotrophic *Shewanella* sp. AS-11 was successfully amplified by PCR. The agarose-S gel electrophoresis showed that the amplified DNA fragment contained 1.2 kb (Figure 2A), which was

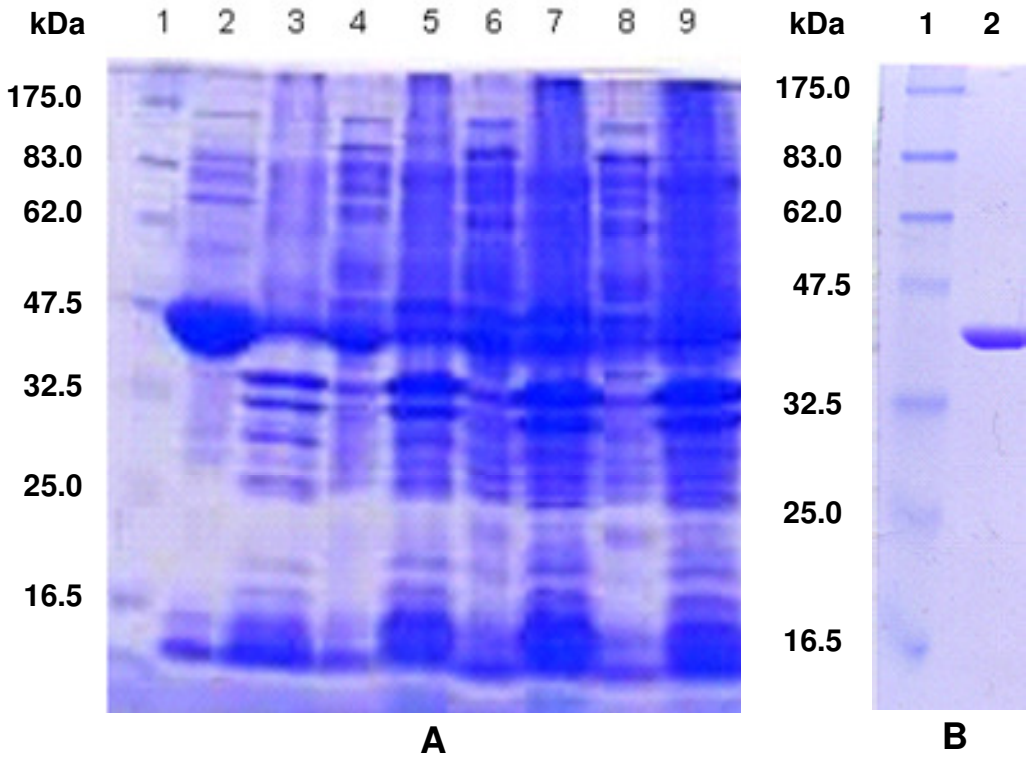

Figure 3. SDS-PAGE analyses of the soluble and insoluble proteins from *E. coli* BL21 transformants after expression at different temperatures and eluting protein from Blue Sepharose CL-6B affinity column chromatography. The gel was stained with Coomassie Blue R-250. Lane 1, molecular standard markers: MBP-ß-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), ß-lactoglobulin A (25 kDa) and lysozyme (16.5 kDa); lane 2 and 3, (soluble and insoluble, respectively) at 20° C; lane 4 and 5, (soluble and insoluble, respectively) at 25°C; lane 6 and 7, (soluble and insoluble, respectively) at 30°C; lane 8 and 9, (soluble and insoluble, respectively) at 37°C (A). Lane 1, marker; lane 2, recombinant acetate kinase (B). SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

consistent with the sizes predicted from the acetate kinase ORFs from *Shewanella* sp. AS-11.

Cloning of acetate kinase ORFs

*Bam*HI digested pETY-16b vector and PCR amplified DNA fragments were introduced into *S. cerevisiae* for *in vivo* homologous recombination. Recombinant plasmids were collected and transformed into the competent cells of *E. coli* DH5α for propagation. The recombinant plasmids were collected from *E. coli* DH5α and digested with restriction endonuclease, *Pst*I, which formed three fragments containing 7.8, 1.6 and 0.5 kb. On the other hand, the pETY-16b vector plasmid was cut by same restriction enzyme and formed three fragments containing 6.6, 1.6 and 0.5 kb (Figure 2B). The result clearly indicates that the recombinant plasmids contain the ORF of acetate kinase at a desired position and was named pETY-16b-ack.

Expression and purification of enzyme

The recombinant acetate kinase was expressed in *E. coli* BL21 (DE3) by induction with 1 mM IPTG in the culture at A_{600} of 0.6 to 0.9. The cultural growth was observed after IPTG induction and the culture growth was not inhibited. The maximum amount of soluble acetate kinase was obtained by the expression for 16 h at 20°C (Figure 3A). The results indicate that the optimum bacterial growth time is 16 h for maximum expression of acetate kinase after IPTG induction at 20°C. The cell extracts were prepared after expression and subjected to ammonium sulfate fractionation. The SDS-PAGE fractions indicate that the active enzyme was precipitated with 30 to 40%

Table 1. Purification of recombinant acetate kinase from *Shewanella* sp. AS-11.

Purification step	Volume (ml)	Protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification fold	Recovery (%)
Cell extract	175	1180.0	48	56640	1.0	100
$(NH_4)_2SO_4$ fractionation	25	486.0	105	51030	2.2	90
Blue sepharose CL-6B	30	46.8	600	28080	12.5	49
Super-Q	26	45.1	605	27285	12.6	48

ammonium sulfate saturation (figure not shown). After ammonium sulfate fractionation, partially purified acetate kinase was further purified by Blue Sepharose CL-6B affinity column chromatography. Enzyme was eluted with 20 mM Tris-HCl buffer pH 7.5 containing 1 mM ATP and 200 mM potassium acetate, which was in a pure state as judged by SDS-PAGE (Figure 3B); though, the absorbance spectra showed that the maximum absorbance was at 264 nm for the enzyme (figure not shown). These results indicate that the protein sample was contaminated by non-protein substances, such as nucleotides. In this regard, the protein was further purified by Super-Q column chromatography. Two eluting peaks were observed, first peak showed maximum absorbance at 260 nm and no protein band was observed by SDS-PAGE as well as no acetate kinase activity was observed; whereas second peak showed maximum absorbance within 278 to 280 nm (figure not shown) and was observed a single protein band by SDS-PAGE. The purification procedure is summarized in Table 1. 45 mg purified protein was obtained from 1 L induced culture (Table 1).

The molecular masses of purified enzyme have been estimated to be 86.0 ± 2.2 kDa by Superose-6 gel filtration, but the apparent molecular masses of acetate kinase was estimated 43.0 ± 1.4 kDa (Figure 3B) on SDS-PAGE, which correspond to the predicted masses (43.7 kDa) from the amino acid sequences of acetate kinase. From these results it can be suggested that acetate kinase is associated with two monomers in a native state (Fox and Roseman, 1986).

pH stability and dependency of acetate kinase

The maximum activity of recombinant acetate kinase was displayed between pH 7.0 to 7.5 (Figure 4) and the enzyme retained full activities after incubation for 30 min at pH values ranging from 6 to 8 (Figure not shown). The enzyme was not stable in a broad range of pH and both acidic and basic conditions were not good for its stability and activity. From these results it can be suggested that the neutral pH is suitable for its activity and stability.

Thermal stability and optimum temperature of acetate kinase

Thermal stability of free acetate kinase and ligand binding acetate kinase were shown in Figure 5. The free and ligand bound acetate kinase retained full activity after 15 min incubation at 30 and 40°C, respectively but completely and irreversibly inactivated at 60 and 70°C, respectively. The results indicate that the enzyme is thermolabile and ligand binding enzymes are thermally slightly more stable than free enzyme. The results are consistent with the report by Latimer and Ferry (1993). The maximum activity was expressed at 45°C and retained 27% of its maximum activity at 5°C, but, the activity was decreased abruptly at higher than the optimum temperature (Figure not shown). According to the enzyme stability curve, enzyme stability was increase when bound with substrate, which might be due to denaturation of the enzyme at higher than the optimum temperature. It can be concluded from the enzyme activity and stability curve that the enzyme is cold adapted with thermolabile.

Substrate specificity

Substrate specificity of the acetate kinase was tested with propionate and butyrate analogues of acetate. Activity was not detectable for the recombinant enzyme at 200 mM concentrations of propionate and butyrate, which concentration was saturated with acetate in hydroxamate assay method. The results of substrate specificity are in agreement with the previous reports on *E. coli* acetate kinase by Fox and Roseman (1986) and Brown and Akagi (1966).

Sequence alignment of psychrophilic, mesophilic and thermophilic acetate kinases

The sequence alignment of psychrophilic (*Shewanella* sp. AS-11), mesophilic (*E. coli* K-12) and thermophilic (*Methanosarcina thermophila*) acetate kinases is given in Figure 6. The amino acid sequence of psychrophilic

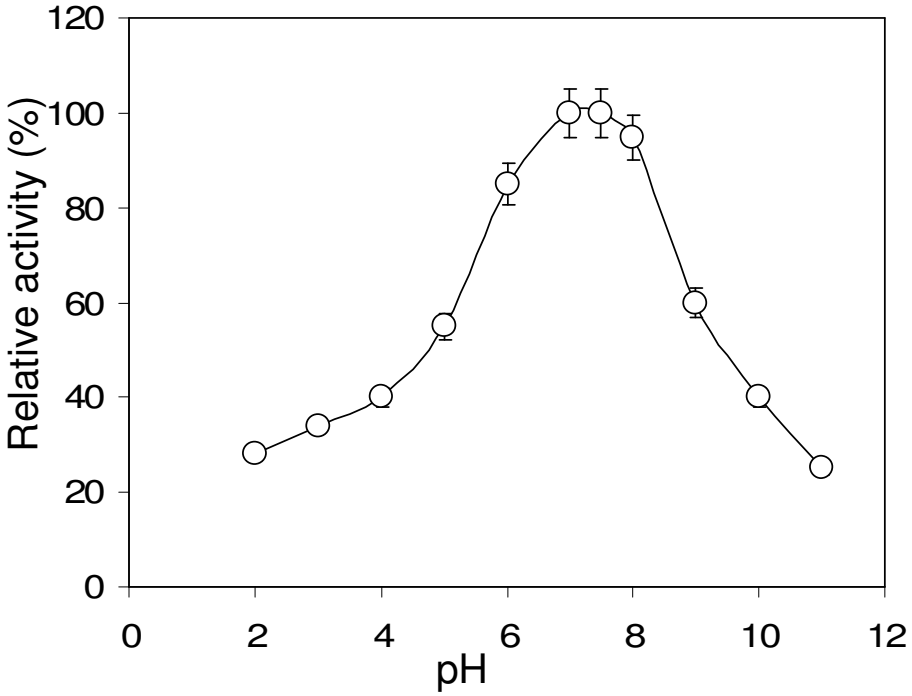

Figure 4. Effect of pH on recombinant acetate kinase activities. The activity was assayed in buffer containing 20 mM sodium citrate, 20 mM sodium tetra borate and 20 mM MOPS at different pH values (2 to 11) by hydroxamate assay method at 25°C. MOPS, (N-morpholino)propanesulphonic acid.

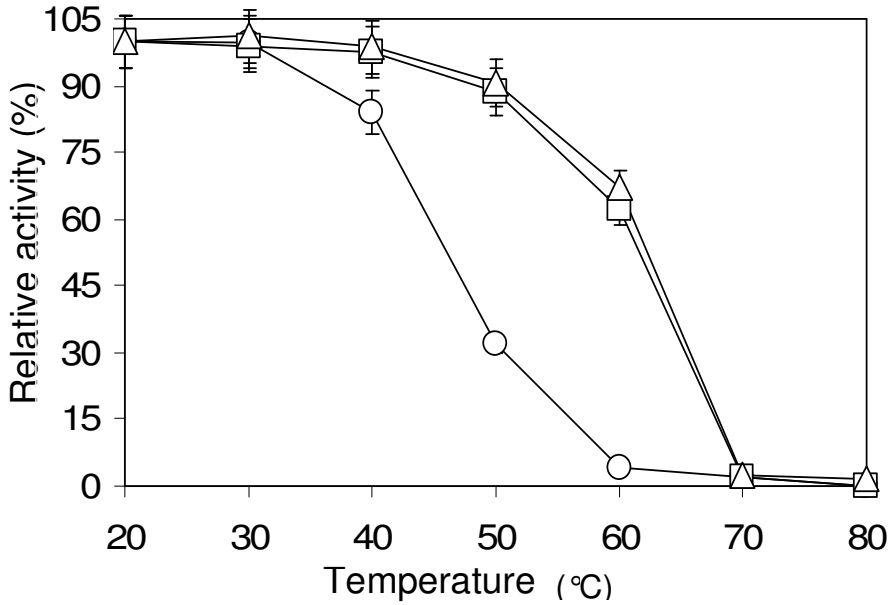

Figure 5. Thermal stability of recombinant acetate kinase. Residual activities were measured at 25°C at pH 7.5 by hydroxamate assay method after pre-incubation at different temperatures for 15 min. ○□□Free acetate kinase; Δ, acetate binding enzyme; □, ATP binding enzyme. ATP and acetate concentration was 10 mM and 200 mM, respectively.

Figure 6. Sequence alignment of acetate kinases from *Shewanella* sp. AS-11, *Escherichia coli* K-12 and *Methanosarcina thermophila*. Catalytic and substrate binding amino acid residues are indicated as bold and under line, respectively. Identical (*) and similar (: and .) residues in the sequences are indicated in the alignment.

acetate kinase shows 64 and 46% residues identity with mesophilic and thermophilic acetate kinases, respectively. The amino acid sequence identity is distributed uniformly through the sequences and mainly corresponds to catalytic residues, substrate binding residues and secondary structural elements in all acetate kinases. The catalytic residues are strictly conserved and most of the substrate binding amino acids are also conserved except acetate binding residues (Figure 6). Thermophilic acetate kinase presents Phe[179] in acetate binding pocket, which binds acetate (Buss et al., 2001; Ingram-Smith et al., 2005) but mesophilic and psychrophilic acetate kinases present Ala and Met, respectively as acetate binding residues. As listed in Table 2, the parameters derived from the primary structure (amino acid content) of psychrophilic, mesophilic and thermophilic acetate kinases failed to reveal significant differences, which could be attributed to temperature adaptation. The numbers of Gly and Pro residues, which affect the local mobility of the chain, are not significantly altered in psychrophilic and mesophilic acetate kinases. The positively charged amino acids and negatively charged amino acids have the potential to form multiple ion pairs and hydrogen bonds. The negatively charged amino acids are least abundant in psychrophilic acetate kinase, whereas the numbers of positively charged amino acids are almost similar. By contrast, the reduced number of salt bridges and cation-pi interactions of psychrophilic

Table 2. Main structural features possibly related to temperature adaptation in psychrophilic, mesophilic and thermophilic acetate kinases.

Parameter	Psychrophilic	Mesophilic	Thermophilic
Gly content	37	36	37
Pro content	13	12	16
Positive charge amino acids	54	53	57
Negative charge amino acids	44	48	58
Salt bridges	5	13	22
Cation-pi interaction	2	3	3

acetate kinase was determined from the comparison of the model structures of psychrophilic and mesophilic acetate kinases, which are consistent with the psychrophilic character.

Conclusion

Recombinant psychrophilic acetate kinase was successfully over-expressed in *E. coli* BL21 (DE3) cells and purified by two steps chromatography. Psychrophilic acetate kinase showed the activity at low and moderate temperatures and regained about one third of its maximum activity at 5°C. Thermo-labiality of this enzyme indicates that the enzyme bears unstable as well as flexible structure (Chiuri et al., 2009; D'Amico et al., 2001; Bentahir et al., 2000). The lower number of salt bridges and cation-pi interaction of psychrophilic acetate kinase than mesophilic and thermophilic acetate kinases clearly proves that the psychrophilic acetate kinase displays a more flexible structure, which is consistent with the thermolability. It has been observed that some weak intra molecular interactions, e.g. salt bridges, cation-pi interactions, hydrogen bond, etc. are missing in cold adapted enzymes (Siddiqui and Cavicchioli, 2006; Feller, 2003; Bentahir et al., 2000), as a result of a flexible structure that plays a crucial role in biological system and it is one of the main characteristics of cold-adapted enzymes (Rueda et al., 2007; Papaleo et al., 2007; Olufsen et al., 2006). These studies we can suggest that the recombinant acetate kinase from *Shewanella* sp. AS-11 is quite efficiently expressed in *E. coli* and the enzyme is cold adapted with thermolabile and flexible structure and may be this flexibility contributes to its cold adaptation.

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