

## Gene expression and characterization of 2-keto-3-deoxy-gluconate kinase, a key enzyme in the modified Entner-Doudoroff pathway of *Serratia marcescens* KCTC 2172

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**Abbreviations:** GST: Glutathione S-transferase  
KDG: 2-keto-3-deoxygluconate  
KDGK: 2-keto-3-deoxy-gluconate kinase  
KDPG: 2-keto-3-deoxy-6-phosphogluconate  
KG: 2-ketogluconate  
mms: maltose metabolism stimulation  
ORF: open reading frames  
SDS-PAGE: sodium dodecyl sulphate-spolyacrylamide gel electrophoresis

**We cloned 2-keto-3-deoxy-gluconate kinase (KDGK), which catalyzes the phosphorylation of 2-keto-3-**

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**deoxygluconate (KDG) to 2-keto-3-deoxy-6-phosphogluconate (KDPG) from *Serratia marcescens* KCTC 2172. The nucleotide sequence revealed a single open reading frame containing 1,208 bp and encoding for 309 amino acids, with a molecular weight of 33,993 Da. The enzyme was purified via GST affinity chromatography. The putative KdgT binding site was detected upstream of the initial codon. The KDG kinase utilized 2-ketogluconate (KG) and KDG as substrates. The optimal temperature and pH for KDGK activity were 50°C and 8.0, respectively.**

*Serratia* is gram-negative bacteria, classified in the large family of *Enterobacteriaceae*. *Serratia* can be distinguished from other genera by its production of three special enzymes DNase, lipase and gelatinase. Another characteristic feature of the *Serratia* among the family *Enterobacteriaceae*, in the tribe *Klebsiella* has been widely considered a red-pigmented organism. *Serratia*, like other Enterobacteria, grow well on ordinary media under anaerobic and aerobic conditions. They grow well on synthetic media using various carbon sources (Giri et al. 2004). *Serratia marcescens* is an enteric bacterium closely related to *Escherichia coli* and secretes several enzymes through the membrane into culture broth, including nucleases, lipases, proteases, chitinases (Gal et al. 1998), coenzyme A transferase, cyclic AMP receptor protein, acetoacetyl coenzyme A transferase (Yoo et al. 2000; Yoo et al. 2001), maltose metabolism stimulation, polyphosphate kinase and exopolyphosphatase (Lee et al. 2000; Lee et al. 2006).

2-Keto-3-deoxygluconate kinase (KDGK), which catalyzes the phosphorylation of 2-keto-3-deoxy-gluconate (KDG), plays a role in the carbohydrate metabolism of *Clostridia* and some of the aerobic bacteria (Ohshima et al. 2007). KDG is an important intermediate of the degradation of extracellular pectin, which is processed and incorporated into cells, and then converted into KDG in *Erwinia chrysanthemi* (Hugouvieux-Cotte-Pattat et al. 1996). In *E. coli* (Mandrand-Berthelot et al. 1984) and *E. chrysanthemi*, extracellular KDG is also utilized by assimilation via the KDG transporter. KDG is also reported to be produced from D-glucosaminatate by D-glucosaminatate dehydratase in *Pseudomonas fluorescens* (Iwamoto and Imanaga, 1991).

The pathways of pectin and galacturonate catabolism in bacteria converge to form a common intermediate, KDG, which is phosphorylated by KDG kinase. The KDGK product is the last step of pectin catabolism that is identified at the molecular level in *E. chrysanthemi*. In this organism, breakdown of pectic polymers involves various types of pectinolytic enzymes. This cleavage gives two kinds of monomeric sugar: 5-keto-4-deoxyuronate (KDU) and D-galacturonate. These monomers are catabolized by two independent pathways converging through a common intermediate: KDG. The ketodeoxyuronate catabolic pathway is composed of successive isomerization and oxidoreduction steps catalyzed by the *kduI* and *kduD* gene

products (Condemine et al. 1986; Condemine and Robert-Baudouy, 1991). The galacturonate catabolic pathway consists of three enzymes encoded by the operon *uxaCBA*. Similarly, hexuronate, glucuronate, can be degraded by three parallel steps catalyzed by the *uxaC*, *uxuB*, and *uxuA* gene products (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1987). The common catabolic product KDG, is then phosphorylated by the KDGK, yielding 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is cleaved by the KDG aldolase gene product to give intermediates of the general cellular metabolism (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985). In order to find out whether the increased expression of KDGK under the direction of a strong promoter such as *tac* promoter is necessary and genes for the putative *S. marcescens* 2-keto-3-deoxygluconate kinase are introduced into a bacterial expression vector pKK223-4. The aim of the present study was to express pKKDGK in *Escherichia coli* and determine the enzymological properties of the purified product to obtain a better understanding of the modified ED pathway in *Serratia marcescens* KCTC 2172.

## MATERIALS AND METHODS

### Cloning and expression of the *kdgK* gene

In a previous study (Yoo et al. 2000; Yoo et al. 2001), we constructed several clones from a genome library of *Serratia marcescens* KCTC 2172. In this study, pCKB17, one of the clones from the genome library, was analyzed in more detail. The nucleotide sequence of pCKB17 was determined, and a restriction map of the cloned DNA fragment and several recombinant subclones was constructed (Figure 1). The open reading frame of the cloned *kdgK* gene was amplified via PCR with the 5' and 3' primers: 5'-AGCCCGGGCTTATTGA-CGATGACTATC-3' and 5'-CATAATCAATAAGGCGGC-GGCGAAGCTTCC-3', harboring the *SmaI* and *HindIII* restriction enzyme sites. pCKB17 was utilized as a template DNA. Following digestion with *SmaI* and *HindIII*, this PCR product was cloned into pKK223-4 and digested with the corresponding enzymes for the construction of pKKDGK. The culture was cultivated in M9 minimal medium containing 0.1% glucose and casamino acids (Le et al. 2006). *E. coli* BL21 (DE3) harboring pKKDGK was induced for over-expression with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase, then incubated for 3 hrs at 37°C.

### Purification of recombinant KDGK

Cells were harvested via centrifugation (6000 rpm for 15 min at 4°C), washed with buffer 1 x PBS buffer (Diluted 10 x PBS, 1.4 M NaCl, 27 mM KCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub> and 18 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.3) and re-suspended in 1 x PBS buffer. The cells were disrupted by sonication and the supernatant was acquired through 30 min of centrifugation at 13000 rpm at 4°C. The sonicated solution was then loaded onto a GSTrap FF column (Amersham Pharmacia

Table 1. Purification of the recombinant KDGK.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	168	203	0.82	100	1
GST affinity chromatography	1.9	24	12.6	11.8	15

Biotech), equilibrated with 1 x PBS, and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 7.5) at a flow rate of 1 ml/min. The eluted fractions were dialyzed overnight against PreScission cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1 mM dichlorodiphenyltrichloroethane at pH 7.0) and concentrated using Amicon Ultra-4 strips (Millipore, Bedford, MA, USA). The purified fusion protein was then digested with PreScission protease for 12 hrs at 5°C to remove the GST region, and then the digested fusion protein was loaded onto a GSTrap FF column chromatography as described above. The unbound fraction was collected and employed as the purified enzyme.

### Enzyme assay

The enzyme coupling assay for KDGK was conducted by the method described by Ohshima et al. (2007). The standard reaction system consisted of 50 mM Tris-HCl (pH 8.5), 10 mM KDG, 10 mM glucose, 2.5 mM ATP, 10 mM MgCl<sub>2</sub>, 6H<sub>2</sub>O, 1 U of yeast glucose-6-phosphate dehydrogenase, 1 U of ADP-glucokinase, 1 mM NADP, and the enzyme in a total volume of 1.0 ml. The reaction was initiated via the addition of ATP, and an increase of absorbance at 340 nm, which is reflective of NADPH formation, followed at 50°C. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μ mol of ADP per min under the standard assay conditions, and the specific activity was expressed in U/mg of protein. Protein concentrations were determined using the method of Bradford (1976), with bovine serum albumin as the standard. ADP-dependent glucokinase was acquired from Asahi Pharma, Tokyo.

### Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted by the method of Laemmli (1970). After SDS-PAGE, the gel was stained with 0.05% Coomassie brilliant blue R-250.

### Effect of temperature and pH on enzyme activity

The effects of temperature on enzyme activity were determined by the incubation of the reaction mixtures at

temperatures ranging from 30 to 80°C in 50 mM Tris-HCl at pH 8.5. In order to determine the optimal pH for the enzyme activity, standard assay mixtures in the following buffers were utilized: 20 mM citrate buffer (pH 3.0-6.0), 20 mM sodium phosphate buffer (pH 6.0-7.6), 20 mM Tris-HCl (pH 7.5-9.0) and glycine-NaOH buffer (pH 10.0).

## RESULTS

### Cloning and expression of the *kdgK* gene

Sequence analysis showed that the presence of two open reading frames (ORFs), in which one ORF is maltose metabolism stimulation (*mms*) (Lee et al. 2000) and the other is a 2-keto-3-deoxygluconate kinase gene. Both showed opposite transcriptional directions. The KDGK ORF began with the initiation codon ATG at position 235 bp, located 10 bases downstream of the putative ribosomal binding site (AGGG), and the ending codon with the termination codon TAA at position 1160. The ATG codon was selected as the translation initiation site because its location was close to the possible ribosome binding site, 5'-AGGG-3'. The deduced KDGK amino acid sequence corresponds to a protein of 309 amino acids with a molecular weight of 33,993 Da. The putative RNA polymerase binding site evidenced the following homology to the classical consensus: 4 of 6 nt (TTGGCC) for the -35 region at position 121 and 4 of 6 nt (TAAAAC) for the -10 region at 126, with a spacing of 19 nt. At the 3' end of the KDGK gene and surrounding the KDGK translational stop, there is a GC-rich imperfect inverted repeat (GCGGCGGCG-9nt-CGCCGCC) followed by a run of A-T residues (TAATCAATAA). This sequence is typical for *rho*-independent transcription termination sites (Figure 2) (Von Hippel et al. 1984).

### Alignment of the KDGK operator with the consensus of the KdgR binding site

Most of the KDGK genes regulated by the KdgR repressor harbor a typical KdgR-binding site (Reverchon et al. 1989). The consensus sequence of the KdgR binding site was detected at the 5'-untranslated end of KDGK. Sequence matching with the consensus 5'-AATAAAAC-xx-CGTTTCA-3' of the KdgR binding site (Hugoumiex-Cotte-

Pattat et al. 1994) showed a 12/15 bp match between the consensus KdgR binding sequence and the *S. marcescens* binding site, 5'-ATTAAAC-AC-TGTTTAA-3' (Figure 3). This putative KdgR binding region partially overlaps the -10 element of the putative promoter region (Figure 2). This demonstrates that KdgR represses RNA polymerase for overlapping binding sites, thereby inhibiting transcriptional initiation.

### Amino acid sequence similarities between KDG kinase and other proteins

Comparison of the KDG kinase with the NCBI databank revealed significant homology with an approximate 70% identity with the KDG kinase of *Yersinia pseudotuberculosis*, 69% identity with *Erwinia chrysanthemi*, and 68% identity with *Salmonella enterica*. These enzymes belong to the PfkB family among carbohydrate kinase families (Wu et al. 1991) (Figure 4).

### Purification of the recombinant enzyme

The purification procedure is summarized in Table 1. *E. coli* cells harboring pKKDGK were expressed and evidenced increased enzyme activity after induction, but not the vector plasmid. The expressed enzyme was purified approximately 15 fold with an 11.8% recovery via GST affinity chromatography (Table 1). Subjecting the purified enzyme to SDS-PAGE generated a single protein band corresponding to a molecular mass of 34 kDa (Figure 5). Approximately 1.9 mg of the purified enzyme was acquired and the specific activity of the final preparation was estimated to be 12.6 U/mg (Table 1). The enzyme reaction was significant for both the substrates, 2-keto-gluconate (KG) and KDG. However, KDG proved to be approximately nine times (8.3) higher than KG (0.95) with regard to kinase specific activity.

### Effect of temperature and pH on KDG kinase activity

When we evaluated the effects of temperature on KDGK activity, we detected maximum activity at 50°C, and the activity declined suddenly at temperatures above 50°C such that the activity at 80°C was only approximately 20% that observed at 50°C (Figure 6). When we determined the effects of pH on KDGK activity, we noted that the maximum activity was observed at pH 8.0, the activity declined slightly at pH values above 8.0, and very slight activity was noted at pH 5.0 and 6.0 (Figure 7).

### DISCUSSION

The KDG kinase is the key enzyme in the semi-phosphorylative ED pathway. This enzyme is a member of the ribokinase (PfkB) enzyme family, which is composed of prokaryotic sequences related to ribokinase, including enzymes such as fructokinases, the minor 6-phosphofructokinase of *E. coli*, 1-phosphofructokinase, and archaeal ADP-dependent glucokinases and

phosphofructokinases (Ito et al. 2001). Thus far, the KDGK gene was cloned, expressed, and characterized in *E. coli*, *Erwinia chrysanthemi*, *Sulfolobus tokodaii*, and *Sulfolobus solfataricus* in the modified Entner-Doudoroff Pathway (Cynkin and Ashwell, 1960; Hugouvieux-Cotte-Pattat et al. 1994; Kim and Lee, 2006; Ohshima et al. 2007). The PfkB kinases are generally composed of 310 to 430 amino acid residues (Hugouvieux-Cotte-Pattat et al. 1994) and are distinguished by three domains (N terminal, middle, C terminal) (Henikoff and Henikoff, 1991). There are regions corresponding to the two signature patterns of carbohydrate kinases of the PfkB family. Consensus pattern 1 is [A,G]-G-[x]-[G,P]-x-N-x-[S,T,A]-6x-[G,S]-9x-G and consensus pattern 2 is [D,N,S]-[P,T,S]-x-[S,A,G]-[S,A,G]-G-D-3x-[A,G]-[A,G]-[L,I,V,M] -[L,I,V,M,S,T,A] among the consensus patterns of the PfkB family. Consensus pattern 2 was shown to be involved in ATP binding ability (Aulkemeyer et al. 1991). KDGK evidences significant homology, and approximately 70% identity with the KDG kinase of *Yersinia pseudotuberculosis*.

The apparent molecular weight of the KDGK from *Serratia marcescens* KCTC 2172 was 34 kDa, similar to that from *Sulfolobus tokodaii* (Ohshima et al. 2007); a value of 35 kDa was detected from *Sulfolobus solfataricus* (Kim and Lee, 2006) and 160 kDa from *Thermus thermophilus* (Ohshima et al. 2004). In the hyperthermophile, *Sulfolobus tokodaii*, the maximum activity of the enzyme was observed at 80°C, the activity declined rapidly at temperatures above 80°C (Ohshima et al. 2007). The maximum enzyme activity was noted at between 7.0 and 8.0 pH and the optimal pH between 7.0 and 8.0 in *S. solfataricus* KDGK (Kim and Lee, 2006). In the present study, KDG kinase activity evidenced optimal activity at a temperature of 50°C and a pH value of 8.0. In this study, we successfully expressed the *Serratia marcescens* KCTC 2172 gene and identified a product that evidenced KDGK activity.

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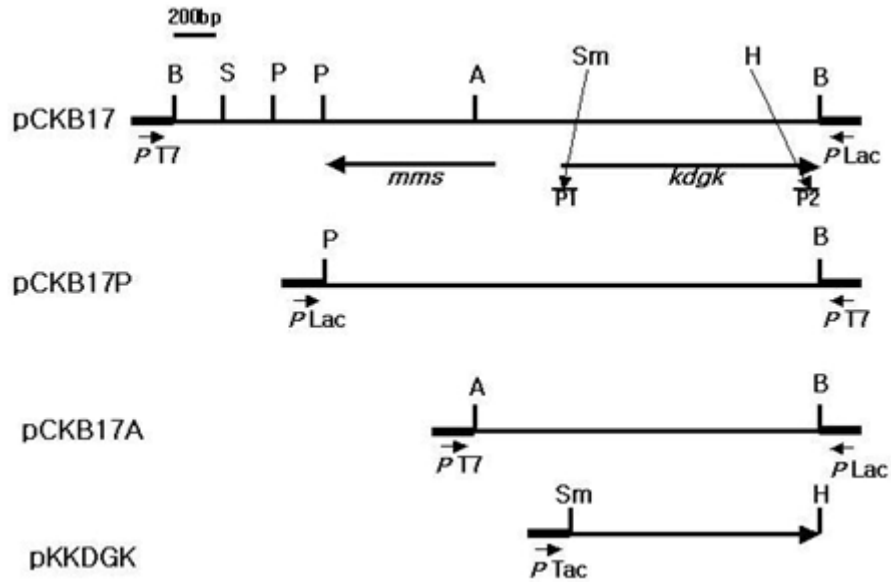
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## APPENDIX FIGURES



**Figure 1. Restriction map of the plasmid pCKB17 from *S. marcescens* and derivative plasmids.** The transcriptional directions of *mms* and *KDGK* genes are shown by the thick arrows. (A), *Ava*I; (B), *Bam*HI; (H) *Hind*II; (M), *Mlu*I; (P), *Pvu*II; (S), *Sal*I; (Sm), *Sma*I.

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1  CATATCGCCGTGGCGGGGAAGTGAGAGCGCCGCTTGGTTCTTTAC CCA ACTTATCGGCCA    60
61  ACGCCACGGTAAAACCTTTAACACGAATTGCCGGAACGCTTTCCTC TTC ACCCTGTCGGCG    120
121 ITGGCCGATCGCGATCATAAAAAATIA AAA CACTGTTTAATTTATTGACTCCCTTA CCG    180
181  CCT TCGGTGGAGACTGGTGGCTGTTGCTTCTTCTTTAGCCAGGGC TTA TTGACGATGACT    240
                                     kdgk gene      ➔ M T 2
241  ATCCGAAACCTCGCCGTGATCGGCCAATGCATGATCGAGCTGTCACAGCAGGGCGCACAG    300
      I R N L A V I G E C M I E L S Q Q G A Q    22
301  CTGACCGCGGGCTTTGGCGGGATACCTCAACACCGCCGTATACCTCGCCCGCCAAATG    360
      L T R G F G G D T L N T A V Y L A R Q M    42
361  CCGAAACAGACGTTGCAGGTGGATTACGTCACTGCGCTCGGCACCGACAGCTTCAGCGGC    420
      P K Q T L Q V H Y V T A L G T D S F S G    62
421  GAGATGGCCTGGCGGCAGGAGAAGATCGAGACCGGGTTGATTCAGCAGTTGACAACAAG    480
      E M A W R Q E K I E T G L I Q Q F D N K    82
481  CTGCCGGGCTGTACGTGATCGAAACCGACGCCCGCGGGAACGCACCTTCTACTATTGG    540
      L P G L Y V I E T D A A G E R T F Y Y W    102
541  CGCAATGACGCCCGCCCGCTATTGGCTTGC TGGACCGCAGGCCGACGCACTGTGCGCG    600
      R N D A A A R Y W L A G P Q A D A L C A    122
601  CGGCTGGCGCAGTTCGATTACCTGTACCTCAGCGGCATTAGCCTTGGGATCCTCGCGCCT    660
      R L A Q F D Y L Y L S G I S L A I L A P    142
661  GCCGACCGCACGAAGCTGCTGACGTTGCTGCGCGCTGTGCGCCAACGAAAACAGGTG    720
      A D R T K L L T L L R R C R A N G K Q V    162
721  ATCTTCGACAACAACACTACGCCCGCTCTGTGCCAAGCCGTGAGGAACTCAGCAGGCC    780
      I F D N N Y R P R L W P S R E E T Q Q A    182
781  TACCGCGAGGTGCTGGCCTGCACCGACATCGCCTTTT GACGCTGGATGACGAAGAATTG    840
      Y R E V L A C T D I A F L T L D D E E L    202
841  CTGTGGGCAAGCAGCCGGTTGAACAGATAGTGACGCGCACGCAGGCGCTCGGCGTAGGC    900
      L W G K Q P V E Q I V T R T Q A L G V G    222
901  GAGATCGTTATCAAACGCGCGCGGATGCCTGTCTGGTGTTC AATCTGGAGGGGGAGCGG    960
      E I V I K R G A D A C L V F N L E G E R    242
961  CTTGAGGTGCCGGCGATCGCGCTGCCCGCGGAGCGCGTGGTGGATACCACTGCGTGGGGC    1020
      L E V P A I A L P P E R V V D T T A W G    262
1021 GACTCTTTCAGCGCCGGCTACCTGGAGGTGCCCTGAACGGCGGGCGCCCGGCAGGCG    1080
      D S F S A G Y L A V P L N G G G A R Q A    282
1081 GCCGAGCGGGCCACCAGCTTGCTGCGACGGTGATCCAACATCGCGGCCCATCATCC    1140
      A Q R G H Q L A A T V I Q H R G A I I P    302
1141 GCAGCGGCATGCCCGCATAATCAATAAGCGGGCGGGAAGTCCGCCCGCCCGCCACGCA    1200
      A A A M P A *      ➔      ←      308
1201 ATGGATCC

```

**Figure 2. Nucleotide sequence of the *kdgK* gene from *S. marcescens*.** The predicted amino acid sequences of the coding regions are shown. The underline is indicated putative promoter sequence, bold-underline is putative ribosome binding site and boldface letters indicate KdgR binding site. The horizontal arrow indicates the regions corresponding to inverted repeat sequence. The nucleotide sequence has been assigned GenBank accession number DQ191959.





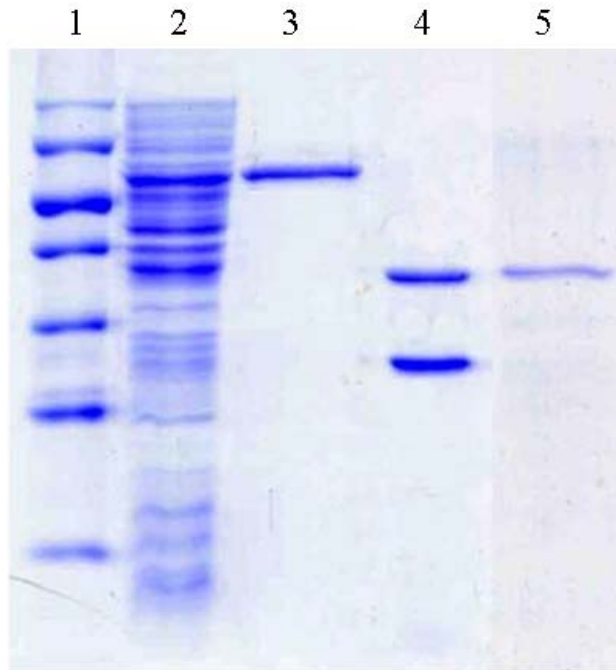


Figure 5. SDS-PAGE patterns obtained with protein preparations at each step of the purification of KDG kinase protein from *E. coli*. Lane 1, size marker; lane 2, crude extract; lane 3, GST-KDGK; lane 4, GST and KDGK digested with protease; lane 5, Purified KDGK.

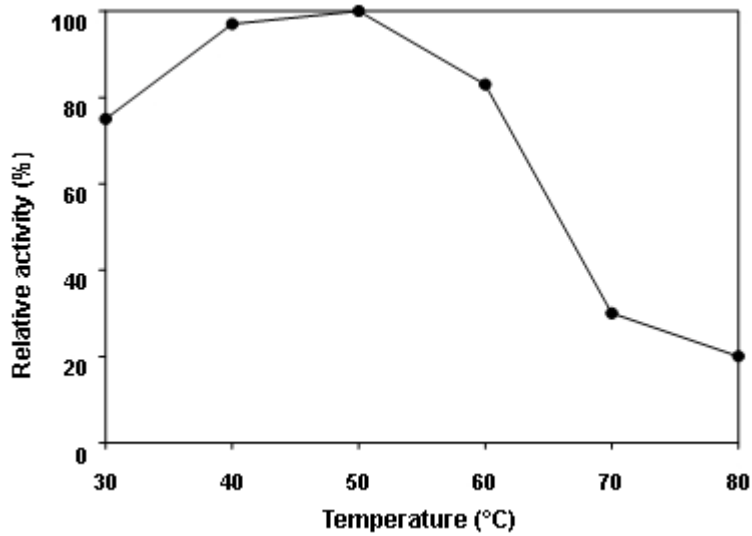


Figure 6. Effect of temperature on KDG kinase activity.

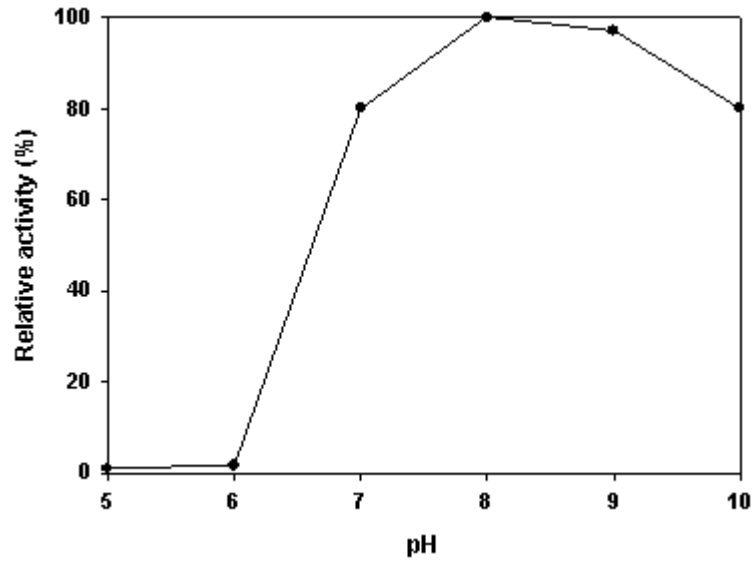


Figure 7. Effect of pH on KDG kinase activity.