CHAPTER IV

RESULTS

Result diagram

dqd gene

- Cloning of *dqd* gene into pGEM-T Easy vector
- Subcloning of *dqd* gene from pGEM-*dqd* into pET-21a vector
    - *E. coli* BL21 (DE3)/pET-*dqd* expression

skdh (GOX0859) gene

- Cloning of *skdh* (GOX0859) gene into pGEM-T Easy vector
- Subcloning of *skdh* (GOX0859) gene from pGEM-GOX0859 into pET-21a vector
    - *E. coli* BL21 (DE3)/pET-GOX0859 expression
- Subcloning of *skdh* (GOX0859) gene from pGEM-GOX0859 into pCold I vector
    - *E. coli* BL21 (DE3)/pCold I-GOX0859 expression
    - pCold I-GOX0859 and pG-KJE8 chaperone vector co-expression
        - purification

***skdh* (GOX1959) gene**

- Cloning of *skdh* (GOX1959) gene into pGEM-T Easy vector
- Subcloning of *skdh* (GOX1959) gene from pGEM-GOX1959 into pET-21a vector
    - *E. coli* BL21 (DE3)/pET-GOX1959 expression
        - purification
- Subcloning of *gdh* gene into pET-GOX1959
    - *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 expression
        - optimize expression condition
    - pET-GOX1959 and pACGD co-expression
- Subcloning of *skdh* (GOX1959) gene from pGEM-GOX1959 into pSG8 vector
    - *G. oxdans* IFO32244/pSG8-GOX1959 expression
    - IPTG effect

4.1 Cloning of *dqd* and two homologs of *skdh* genes from *Gluconobacter oxydans* 621H

Since the data of *G. oxydans* 621H genome is became available (Accession no. C000009), the nucleotide sequences of *dqd* (GOX0437) gene encoding dehydroquinate dehydratase located from 461603 to 462085 on the complementary strand of genomic DNA, *skdh* (GOX0859) gene located from 928465 to 929313 on the complementary strand of genomic DNA and *skdh* (GOX1959) gene located from 2147831 to 2148670 on the sense strand of genomic DNA were obtained. Primers positioned upstream and downstream of *dqd* (GOX0437) and *skdh* genes (GOX0859 and GOX1959) were designed base on nucleotide sequences. The preparations of the *dqd* and *skdh* genes using the PCR technique were done as described in section 3.6. Figure 4.1 shows PCR products of *dqd*, *skdh* (GOX0859) and *skdh* (GOX1959) on agarose gel electrophoresis. The major bands of *dqd*, *skdh* (GOX0859) and *skdh* (GOX1959) PCR product were observed. The PCR products were ligated into pGEM®-T easy vector and the recombinant plasmids were sequenced. The sequencing result of three genes and primer position are shown in Figure 4.2-4.4. The result showed that the insert size for *dqd* was 620 bps (83 bp upstream region, 483 bps coding region and 54 bps downstream region). Two homologs of *skdh* genes were 971 (26 bp upstream region, 849 bps *skdh* (GOX0859) coding region and 96 bps downstream region) and 863 bps (4 bp upstream region, 840 bps *skdh* (GOX1959) coding region and 19 bps downstream region), respectively.

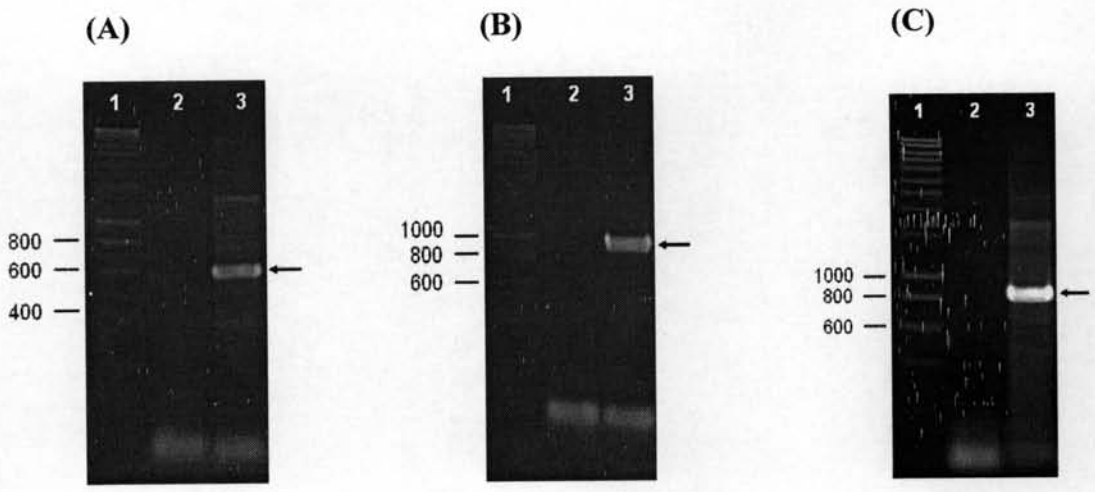


Figure 4.1 : PCR products of *dqd* and two *skdh* homologs from *G. oxydans* 621H genome on 0.8 % agarose gel **(A)** *dqd* PCR product (620 bps) **(B)** *skdh* (GOX0859) PCR product (971 bps) **(C)** *skdh* (GOX1959) PCR product (863 bps)

Lane 1 : Hyperladder 10 kb marker

Lane 2 : negative control (no DNA template)

Lane 3 : PCR product as indicated

```
                    Forward primer
dqd (GOX0437)       -----GATCGCACCAGATAGCACAAAAATGCGGCCCTGTCCCCTTTTGGACACCCTGAAG 55
dqd-F               TGATTGATCGCACCAGATAGCACAAAAATGCGGCCCTGTCCCCTTTTGGACACCCTGAAG 60
dqd-R               TGATTGATCGCACCAGATAGCACAAAAATGCGGCCCTGTCCCCTTTTGGACACCCTGAAG 60
                         ************************************************************

dqd (GOX0437)       ACGGCAGCAATAGGGCAATGACGCTTTCATGACGGCTCCGAAAGTGCTATCGCGCGGCCA 115
dqd-F               ACGGCAGCAATAGGGCAATGACGCTTTCATGACGGCTCCGAAAGTGCTATCGCGCGGCCA 120
dqd-R               ACGGCAGCAATAGGGCAATGACGCTTTCATGACGGCTCCGAAAGTGCTATCGCGCGGCCA 120
                    ************************************************************

dqd (GOX0437)       GATGAAACGCCCTCTGATCACCGTTCTCAACGGTCCGAATCTCAACATGCTGGGTCTTCG 175
dqd-F               GATGAAACGCCCTCTGATCACCGTTCTCAACGGTCCGAATCTCAACATGCTGGGTCTCCG 180
dqd-R               GATGAAACGCCCTCTGATCACCGTTCTCAACGGTCCGAATCTCAACATGCTGGGTCTCCG 180
                    ************************************************************ **

dqd (GOX0437)       CCAGCCCGGAATCTATGGTCACGCCACGCTCGATGATGTCGAGCAGGTGTGCATTCAGGC 235
dqd-F               CCAGCCCGGAATCTATGGTCACGCCACGCTCGATGATGTCGAGCAGGTGTGCATTCAGGC 240
dqd-R               CCAGCCCGGAATCTATGGTCACGCCACGCTCGATGATGTCGAGCAGGTGTGCATTCAGGC 240
                    ************************************************************

dqd (GOX0437)       TGCCGAACGGCTTGATGTCGCCATTGATTTCCGTCAGACGAACGGAGAGGGTGAACTCGT 295
dqd-F               TGCCGAACGGCTTGATGTCGCCATTGATTTCCGTCAGACGAACGGAGAGGGTGAACTCGT 300
dqd-R               TGCCGAACGGCTTGATGTCGCCATTGATTTCCGTCAGACGAACGGAGAGGGTGAACTCGT 300
                    ************************************************************

dqd (GOX0437)       GTCCTGGGTGCAGGAATGTCGCGGCCGTGCAGACGGTATCGTGATCAATCCTGCCGCTTA 355
dqd-F               GTCCTGGGTGCAGGAATGTCGCGGCCGTGCAGACGGTATCGTGATCAATCCTGCCGCTTA 360
dqd-R               GTCCTGGGTGCAGGAATGTCGCGGCCGTGCAGACGGTATCGTGATCAATCCTGCCGCTTA 360
                    ************************************************************

dqd (GOX0437)       CGGGCATACCTCGATTGCCCTGCTCGATGCGCTTCTGGCCGTCGAGCTTCCCGTGATTGA 415
dqd-F               CGGGCATACCTCGATTGCCCTGCTCGATGCGCTTCTGGCCGTCGAGCTTCCCGTGATTGA 420
dqd-R               CGGGCATACCTCGATTGCCCTGCTCGATGCGCTTCTGGCCGTCGAGCTTCCCGTGATTGA 420
                    ************************************************************

dqd (GOX0437)       GGTTCATATTTCCAATATCCATCGCAGGGAGCCGTTCCGTCATCACACCTACGTCTCGCA 475
dqd-F               GGTTCATATTTCCAATATCCATCGCAGGGAGCCGTTCCGTCATCACACCTACGTCTCGCA 480
dqd-R               GGTTCATATTTCCAATATCCATCGCAGGGAGCCGTTCCGTCATCACACCTACGTCTCGCA 480
                    ************************************************************

dqd (GOX0437)       GGCCGCCATCGGCGTGATCTGCGGCCTCGGCGTCAGGGGATACGCGCACGCGCTTCAGGC 535
dqd-F               GGCCGCCATCGGCGTGATCTGCGGCCTCGGCGTCAGGGGATACGCGCACGCGCTTCAGGC 540
dqd-R               GGCCGCCATCGGCGTGATCTGCGGCCTCGGCGTCAGGGGATACGCGCACGCGCTTCAGGC 540
                    ************************************************************

dqd (GOX0437)       AATAACCGACATGATCGAAGACGAAGGATGAGCCGTATGCTCGTGGACAAGGATGCCATT 595
dqd-F               AATAACCGACATGATCGAAGACGAAGGATGAGCCGTATGCTCGAGGACAAGGATGCCATT 600
dqd-R               AATAACCGACATGATCGAAGACGAAGGATGAGCCGTATGCTCGAGGACAAGGATGCCATT 600
                    ****************************************** ****************

                    Forward primer
dqd (GOX0437)       CGGGCATTGGCCGATATTCTGACGG----- 620
dqd-F               CGGGCATTGGCCGATATTCTGACGGAATCG 630
dqd-R               CGGGCATTGGCCGATATTCTGACGGAATCG 630
                    *************************
```

Figure 4.2 : Alignment of 620 bp *dqd* PCR product sequencing results (dqd-F and dqd-R) with *dqd* (GOX0437) gene from *G. oxdans* 621H genome sequence

```
                    Forward primer
skdh (GOX0859)  -----ACAGGCACAGATCCGAGGAGCCTCTCATGAGCCAGCAGAATTTCCGCAGCATCCT 55
skdh_F          CGATTACAGGCACAGATCCGAGGAGCCTCTCATGAGCCAGCAGAATTTCCGCAGCATCCT 60
                     ************************************************************

skdh (GOX0859)  GACCGGATCGTTCTCCACGCCATGCGATGACAACCCGACCGTCGCCATGATCGAGGCCGC 115
skdh_F          GACCGGATCGTTCTCCACGCCATGCGATGACAACCCGACCGTCGCCATGATCGAGGCCGC 120
                ************************************************************

skdh (GCX0859)  GTACCGGCACCACGACATCGATGCGCGTTACATCAACTGTGACGTCAAGGCGGACGGTCT 175
skdh_F          GTACCGGCACCACGACATCGATGCGCGTTACATCAACTGTGACGTCAAGGCGGACGGTCT 180
                ************************************************************

skdh (GOX0859)  GAAGGACGCGGTCGCGGGTGCGCGGGCCATGGAGTGGGTCGGGTTCAACTGCTCCCTGCC 235
skdh_F          GAAGGACGCGGTCGCGGGTGCGCGGGCCATGGAGTGGGTCGGGTTCAACTGCTCCCTGCC 240
                ************************************************************

skdh (GOX0859)  GCACAAGGTTGCGGTGATCGAGCATCTGGACGAACTGGCGGAGTCCGCCCGGATTATCGG 295
skdh_F          GCACAAGGTTGCGGTGATCGAGCATCTGGACGAACTGGCGGAGTCCGCCCGGATTATCGG 300
                ************************************************************

skdh (GOX0859)  TGCGGTGAACTGCGTCTCCATCCGGGACGGGCGCCTGATCGGCGACAATACGGACGGGAA 355
skdh_F          TGCGGTGAACTGCGTCTCCATCCGGGACGGGCGCCTGATCGGCGACAATACGGACGGGAA 360
                ************************************************************

skdh (GOX0859)  GGGCTTTCTGGCGTCCCTGAACAAGGTCGGGGATCCGTCCGGAAAGAAGGTCCTGCTTCT 415
skdh_F          GGGCTTTCTGGCGTCCCTGAACAAGGTCGGGGATCCGTCCGGAAAGAAGGTCCTGCTTCT 420
                ************************************************************

skdh (GOX0859)  GGGCGCGGGCGGGGCTGCGCGTGCCATCGCCGTGGAACTGGGGCTCGTTTCCGCCGCCCA 475
skdh_F          GGGCGCGGGCGGGGCTGCGCGTGCCATCGCCGTGGAACTGGGGCTCGTTTCCGCCGCCCA 480
                ************************************************************

skdh (GCX0859)  TATCATGGTCATGAACCGCGATCCCAAAAAAGCCGAAACCATTGCTGCACTGGTGCGGGA 535
skdh_F          TATCATGGTCATGAACCGCGATCCCAAAAAAGCCGAAACCATTGCTGCACTGGTGCGGGA 540
                ************************************************************

skdh (GOX0859)  CAACACCTCCGCCAAAGCCGATGTTCAGGCATGGGACGGCGAGGCCAGCGTGCCGGAAGA 595
skdh_F          CAACACCTCCGCCAAAGCCGATGTTCAGGCATGGGACGGCGAGGCCAGCGTGCCGGAAGA 600
                ************************************************************

skdh (GOX0859)  CGTGGACATCCTGATCAACGCCACGTCAATCGGTCTGGGGGATGCGGACGCCATGCCGCC 655
skdh_F          CGTGGACATCCTGATCAACGCCACGTCAATCGGTCTGGGGGATGCGGACGCCATGCCGCC 660
                ************************************************************

skdh (GOX0859)  GCTGAAGGTCGAGACCCTGCGCAAGGGCTTGATCGTCGCCGATGTCATTCCGAACCCGCC 715
skdh_F          GCTGAAGGTCGAGACCCTGCGCAAGGGCTTGATCGTCGCCGATGTCATTCCGAACCCGCC 720
                ************************************************************

skdh (GOX0859)  TGCTGCGGGAAGCAGAAAACAGGGGCTGCACCGTGCTGGACGGGCTCGGGATGCTGGTCA 775
skdh_F          TGCTGCGGGAAGCAGAAAACAGGGGCTGCACCGTGCTGGACGGGCTCGGGATGCTGGTCA 780
                ************************************************************

skdh (GOX0859)  ATCAGGGCGTGATCGGCGTGGAGCACTGGCTGGGCAGGACGTTGGACGCCGGGGTGATGG 835
skdh_F          ATCAGGGCGTGATCGGCGTGGAGCACTGGCTGGGCAGGACGTTGGACGCCGGGGTGATGG 840
                ************************************************************

skdh (GOX0859)  AGCAGACCCTGAAGGATATTTTCGGCGCGGCCTGACATGAAAAAACCCGGTGTCTCCGAA 895
skdh_F          AGCAGACCCTGAAGGATATTTTCGGCGCGGCCTGACATGAAAAAACCCGGTGTCTCCGAA 900
                ************************************************************

skdh (GOX0859)  GAGAGCACCGGGTTTTTGCAGAGCTGAAAGATCAGCGGCCTTCGAAGAAGTCGCGGACCT 955
skdh_F          GAGAGCACCGGGTTTTTGCAGAGCTGAAAGATCAGCGGCCTCCGAAGAAGTCGCGGACCT 960
                ****************************************** ******************
                 Reverse primer
skdh (GOX0859)  TGGCGAAGAAGCCGCT-------------------- 971
skdh_F          TGGCGAAGAAGCCGCTAATCACTAGTGAATTCGCGG 996
                ***************
```

Figure 4.3 : Alignment of 971 bp *skdh* PCR product sequencing result (skdh_F) with *skdh* (GOX0859) gene from *G. oxdans* 621H genome sequence

```
                                Forward primer
skdh (GOX1959)  ---------GCGCATGATTGACGGTCACACGAAACTCGCAGGCGTCATGGGTTGGCCGGT 51
skdh_F          AATTCGATTGCGCATGATTGACGGTCACACGAAACTCGCAGGCGTCATGGGTTGGCCGGT 60
                         **************************************************

skdh (GOX1959)  GGAGCATTCGCGCTCTCCGCTGATGCATAATCACTGGTGCCGGGTGAATGGCGTGAACGG 111
skdh_F          GGAGCATTCGCGCTCTCCGCTGATGCATAATCACTGGTGCCGGGTGAATGGCGTGAACGG 120
                ************************************************************

skdh (GOX1959)  GGCTTATGTGCCGCTGCCGACCCACCCCCACGGCTTTGATCAGGCCCTGCGTGGTCTGGC 171
skdh_F          GGCTTATGTGCCGCTGCCGACCCACCCCCACGGCTTTGATCAGGCCCTGCGTGGTCTGGC 180
                ************************************************************

skdh (GOX1959)  AGCGGCCGGGTTTCAGGGCGTGAATGTCACCATCCCACACAAGGAAGCGGCGATGCTGGC 231
skdh_F          AGCGGCCGGGTTTCAGGGCGTGAATGTCACCATCCCACACAAGGAAGCGGCGATGCTGGC 240
                ************************************************************

skdh (GOX1959)  CTGTGATGAACTCACCCCAACGGCTAAGCGGGCCGGCGCGGTAAACACGATTTGCTTCGT 291
skdh_F          CTGTGATGAACTCACCCCAACGGCTAAGCGGGCCGGCGCGGTAAACACGATTTGCTTCGT 300
                ************************************************************

skdh (GOX1959)  GGCTGGACGGATCATCGGTGACTGCACGGATGGTACGGGGTTCTGCGATAATCTGAGTGC 351
skdh_F          GGCTGGACGGATCATCGGTGACTGCACGGATGGTACGGGGTTCTGCGATAATCTGAGTGC 360
                ************************************************************

skdh (GOX1959)  ACATGACGTGGCGATTGCCGGTCGGGCCATGGTTCTCGGGGCGGGTGGTGCGGCGCGCGC 411
skdh_F          ACATGACGTGGCGATTGCCGGTCGGGCCATGGTTCTCGGGGCGGGTGGTGCGGCGCGCGC 420
                ************************************************************

skdh (GOX1959)  CGTGGCGGCGGCGCTTCTGGACCGGGGCTGCGAGGTCGTGATTGCCAACCGGACCCTGGA 471
skdh_F          CGTGGCGGCGGCGCTTCTGGACCGGGGCTGCGAGGTCGTGATTGCCAACCGGACCCTGGA 480
                ************************************************************

skdh (GOX1959)  ACGGGCGGAAGCACTGGTTGAGGCACTCGGTGGCGGTGAGGCTGTTGCCTGGTATGAGTG 531
skdh_F          ACGGGCGGAAGCACTGGTTGAGGCACTCGGTGGCGGTGAGGCTGTTGCCTGGTATGAGTG 540
                ************************************************************

skdh (GOX1959)  GCCGTCACTGCTATCGGGTTGTTCGCTTCTGGTGAATGCCACGTCGATGGGCATGGGCGG 591
skdh_F          GCCGTCACTGCTATCGGGTTGTTCGCTTCTGGTGAATGCCACGTCGATGGGCATGGGCGG 600
                ************************************************************

skdh (GOX1959)  CAAGGCTGGTCTGGACTGGGATGCGGCTCTTCGTGAGGCCGCGCCGGGCCTGTGCGTGAC 651
skdh_F          CAAGGCTGGTCTGGACTGGGATGCGGCTCTTCGTGAGGCCGCGCCGGGCCTGTGCGTGAC 660
                ************************************************************

skdh (GOX1959)  GGATATTGTCTACACGCCGCGCGAGACGCCGCTTCTGCTGGCCGCCCAGGCACGGGGACT 711
skdh_F          GGATATTGTCTACACGCCGCGCGAGACGCCGCTTCTGCTGGCCGCCCAGGCACGGGGACT 720
                ************************************************************

skdh (GOX1959)  GCGGACCGTGGATGGTCTGGGGATGCTGGTTCATCAGGCGCGGGCCGGGTTTCGGGCATG 771
skdh_F          GCGGACCGTGGATGGTCTGGGGATGCTGGTTCATCAGGCGCGGGCCGGGTTTCGGGCATG 780
                ************************************************************

skdh (GOX1959)  GTTCGGCGTTGATCCGCAGGCCGACCGGACGACATTCGATCTGCTGGCGGCGAGCCTGCG 831
skdh_F          GTTCGGCGTTGATCCGCAGGCCGACCGGACGACATTCGATCTGCTGGCGGCGAGCCTGCG 840
                ************************************************************
                           Reverse primer
skdh (GOX1959)  CACTGACGCGTGAAGATCATTGGTCTGACCGG---------------------- 863
skdh_F          CACTGACGCGTGAAGATCATTGGTCTGACCGGAATCACTAGTGAATTCGCGGCCGC 896
                ********************************
```

Figure 4.4 : Alignment of 971 bp *skdh* PCR product sequencing result (skdh_F) with *skdh* (GOX1959) gene from *G. oxdans* 621H genome sequence

4.2 Subcloning of *dqd* and two of *skdh* genes in to pET-21a vector

dqd and two of *skdh* genes were amplified from an open reading frame according to the method from section 3.7. The stop codon of these three genes was removed to express His-tag on C-terminal. To prepare the amplified gene product for insertion into pET-21a, the 5'-end primer comprised of *Nde*I restriction site and 5' base of each gene as shown in the method from section 3.6.1-3.6.3. The 3'-end primer comprised of *Xho*I restriction site and 3' base of each gene were designed. The PCR products were analyzed on 0.8% agarose gel (Figure 4.5). The major band of *dqd* (GOX0437), *skdh* (GOX0859) and *skdh* (GOX1959) PCR products were ligated into pET-21a vector and the recombinant plasmids were sequenced. From sequencing result in Figure 4.6-4.8, the insert size for *dqd* and two homologs of *skdh* (GOX0859 and GOX1959) genes were 489, 855 and 846 bps, respectively.

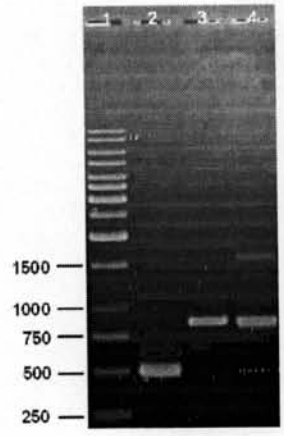


Figure 4.5 : PCR product of *dqd* and two *skdh* homologs from pGEM-T subcloning on 0.8% agarose gel

Lane 1 : GeneRuler^{TM} 1 kb DNA Ladder.

Lane 2 : *dqd* PCR product

Lane 3 : *skdh* (GOX0859) PCR product

Lane 4 : *skdh* (GOX1959) PCR product

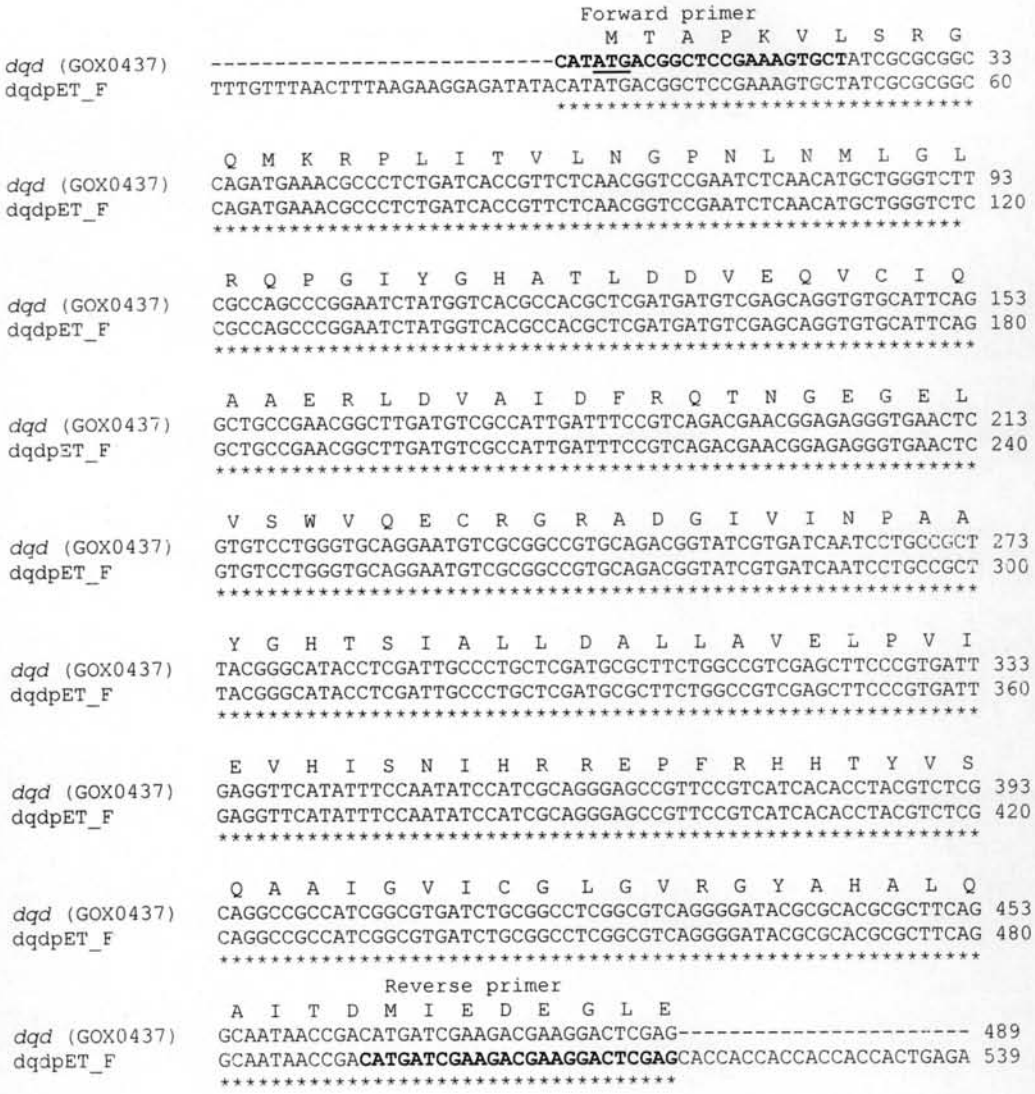


Figure 4.6 : Alignment of *dqd* PCR product for pET sequencing result (dqdpET_F) with *dqd* gene from *G. oxdans* 621H genome sequence. The deduced amino acid sequence is also shown.

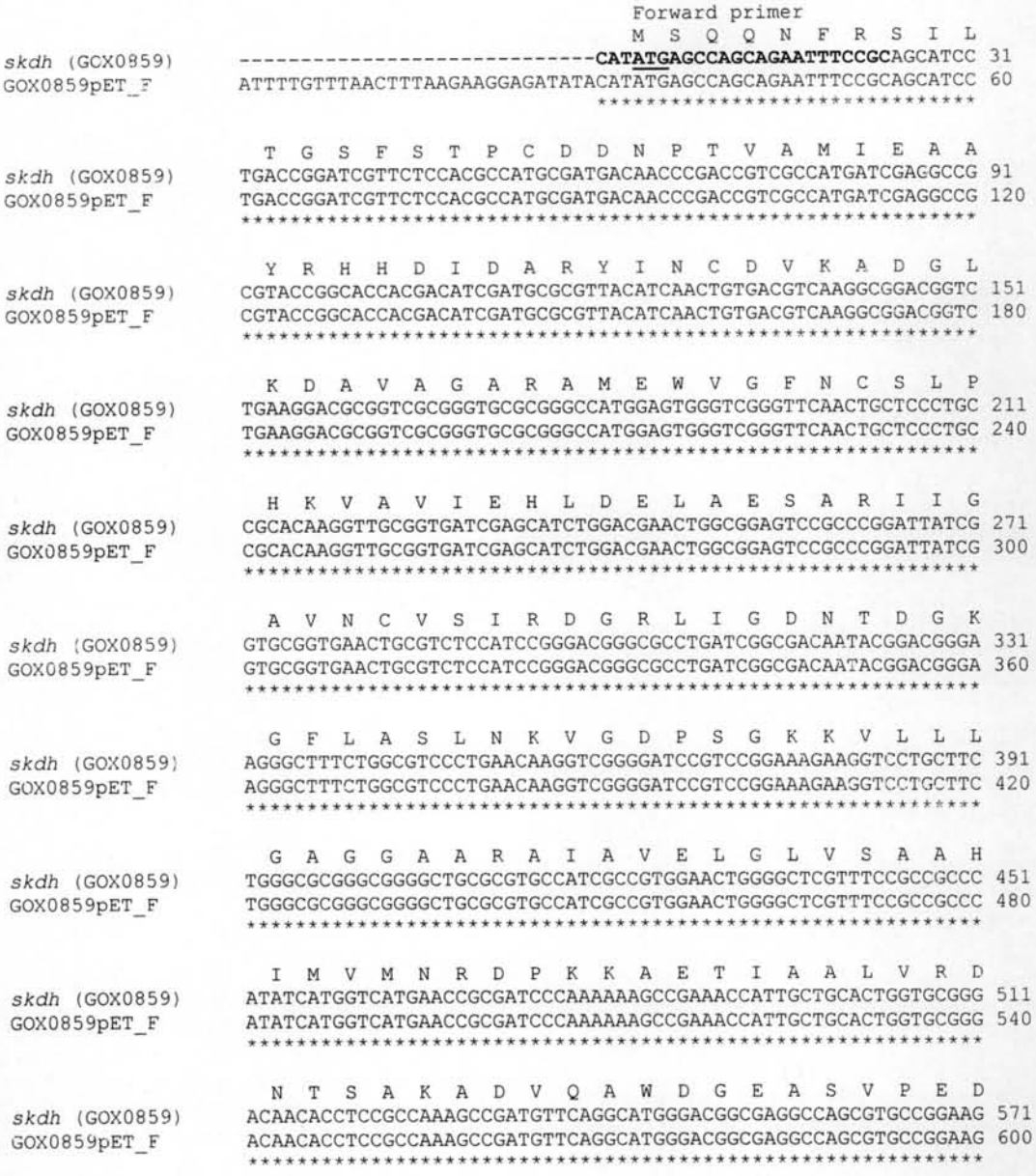


Figure 4.7 : Alignment of *skdh* (GOX0859) PCR product for pET sequencing result (GOX0859pET_F) with *skdh* (GOX0859) gene from *G. oxdans* 621H genome sequence. The deduced amino acid sequence is also shown upper.

```
                    V  D  I  L  I  N  A  T  S  I  G  L  G  D  A  D  A  M  P  P

skdh (GOX0859)      ACGTGGACATCCTGATCAACGCCACGTCAATCGGTCTGGGGGATGCGGACGCCATGCCGC 631
GOX0859pET_F        ACGTGGACATCCTGATCAACGCCACGTCAATCGGTCTGGGGGATGCGGACGCCATGCCGC 660
                    ************************************************************

                    L  K  V  E  T  L  R  K  G  L  I  V  A  D  V  I  P  N  P  P

skdh (GOX0859)      CGCTGAAGGTCGAGACCCTGCGCAAGGGCTTGATCGTCGCCGATGTCATTCCGAACCCGC 691
GOX0859pET_F        CGCTGAAGGTCGAGACCCTGCGCAAGGGCTTGATCGTCGCCGATGTCATTCCGAACCCGC 720
                    ************************************************************

                    A  A  G  S  R  K  Q  G  L  H  R  A  G  R  A  R  D  A  G  Q

skdh (GOX0859)      CTGCTGCGGGAAGCAGAAAACAGGGGCTGCACCGTGCTGGACGGGCTCGGGATGCTGGTC 751
GOX0859pET_F        CTGCTGCGGGAAGCAGAAAACAGGGGCTGCACCGTGCTGGACGGGCTCGGGATGCTGGTC 780
                    ************************************************************

                    S  G  R  D  R  R  G  A  L  A  G  Q  D  V  G  R  R  G  D  G

skdh (GOX0859)      AATCAGGGCGTGATCGGCGTGGAGCACTGGCTGGGCAGGACGTTGGACGCCGGGGTGATG 811
GOX0859pET_F        AATCAGGGCGTGATCGGCGTGGAGCACTGGCTGGGCAGGACGTTGGACGCCGGGGTGATG 840
                    ************************************************************
                                      Reverse primer
                    A  D  P  E  G  Y  F  R  R  G  L  T  L  E
skdh (GOX0859)      GAGCAGACCCTGAAGGATATTTTCGGCGCGGCCTGACACTCGAG---------------- 855
GOX0859pET_F        GAGCAGACCCTGAAGGATATTTTCGGCGCGGCCTGACACTCGAGCACCACCACCACCACG 900
                    ****************************************
```

Figure 4.7 : (continue)

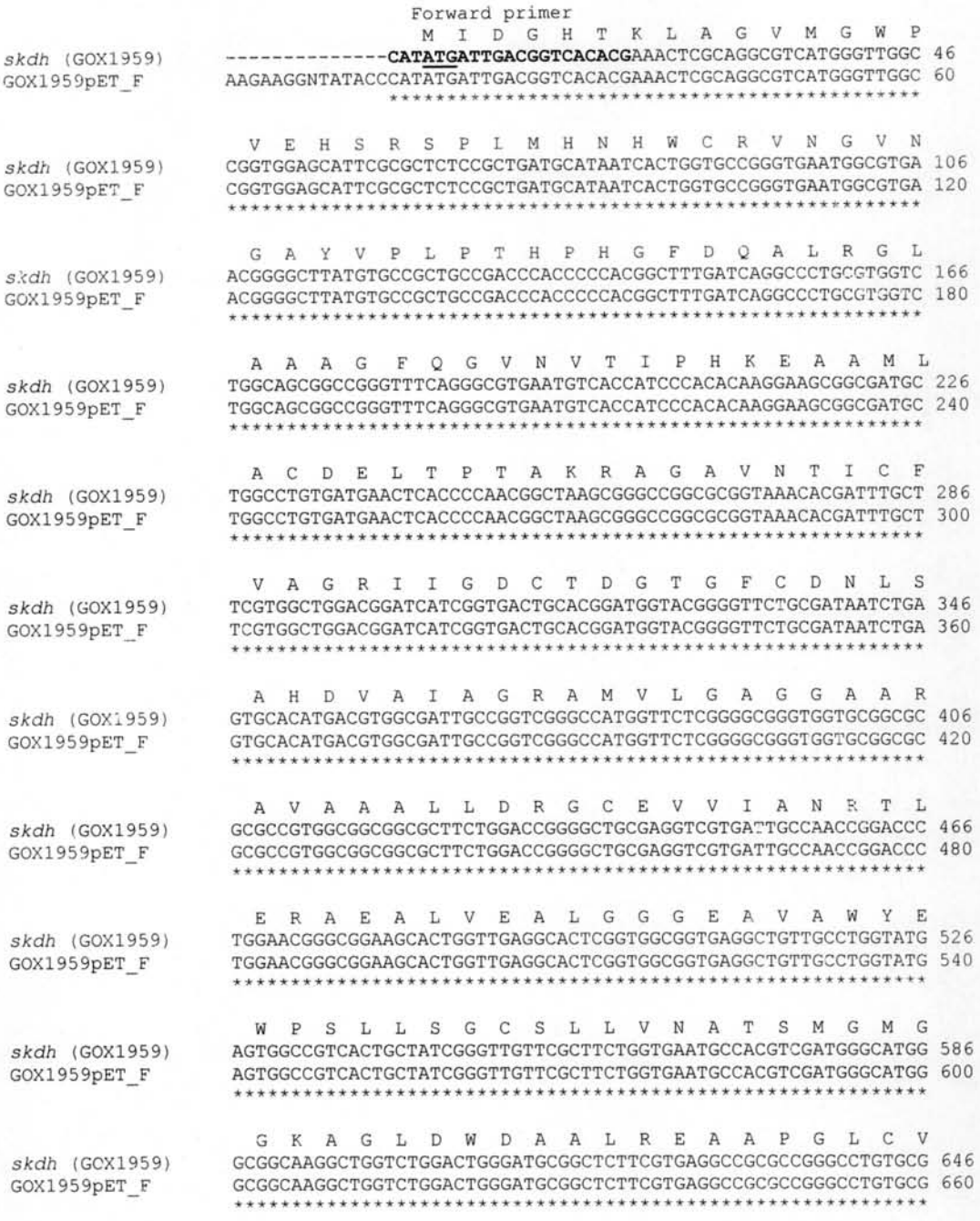


Figure 4.8 : Alignment of *skdh* (GOX1959) PCR product for pET sequencing result (GOX1959pET_F) with *skdh* (GOX1959) gene from *G. oxdans* 621H genome sequence. The deduced amino acid sequence is also shown.

```
                    T  D  I  V  Y  T  P  R  E  T  P  L  L  L  A  A  Q  A  R  G
skdh (GOX1959)      TGACGGATATTGTCTACACGCCGCGCGAGACGCCGCTTCTGCTGGCCGCCCAGGCACGGG 706
GOX1959pET_F        TGACGGATATTGTCTACACGCCGCGCGAGACGCCGCTTCTGCTGGCCGCCCAGGCACGGG 720
                    ************************************************************

                    L  R  T  V  D  G  L  G  M  L  V  H  Q  A  R  A  G  F  R  A
skdh (GOX1959)      GACTGCGGACCGTGGATGGTCTGGGGATGCTGGTTCATCAGGCGCGGGCCGGGTTTCGGG 766
GOX1959pET_F        GACTGCGGACCGTGGATGGTCTGGGGATGCTGGTTCATCAGGCGCGGGCCGGGTTTCGGG 780
                    ************************************************************

                    W  F  G  V  D  P  Q  A  D  R  T  T  F  D  L  L  A  A  S  L
skdh (GOX1959)      CATGGTTCGGCGTTGATCCGCAGGCCGACCGGACGACATTCGATCTGCTGGCGGCGAGCC 826
GOX1959pET_F        CATGGTTCGGCGTTGATCCGCAGGCCGACCGGACGACATTCGATCNGCNGGCGGCGAGCC 840
                    ********************************************** ** ***********
                     Reverse primer
                      R  T  D  A  L  E
skdh (GOX1959)      TGCGCACTGACGCGCTCGAG---------------------------------------- 846
GOX1959pET_F        TGCGCACTGACGCGCTCGAGCACCNCCACCACCANGAAAGAGATCNNNTTNTNCAAAA 898
                    ********************
```

Figure 4.8 : (continue)

4.3 Expression of *dqd* gene in *E. coli* BL21 (DE3)

pET-21a vector harboring *dqd* gene was transformed into *E. coli BL21* (DE3). The *E. coli* BL21 (DE3)/pET-*dqd* was cultured on a shaking incubator at 37°C. When OD_{600} reached 0.2, IPTG was added to final concentration 1 mM and cultivation was continued for 5 hours. Intracellular crude enzyme was prepared as described in section 3.8.3 and used to determine DQD activity (section 3.8.5). The DQD activity is shown in Table 4.1.

Intracellular crude enzyme and precipitates from wild type and transformant was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. Since the expected size of DQD is approximately 18.5 kDa, the result in Figure 4.9 showed that most of the expressed DQD was included in inclusion body (indicated by an arrow).

Table 4.1 DQD activity expressed in *E. coli* BL21 (DE3) harboring pET-*dqd* at 37°C

Samples	DQD activity (U/mg)
E. coli BL21(DE3)	0.92±0.061
E. coli BL21(DE3)/pET-21a	0.91±0.12
E. coli BL21(DE3)/pET-*dqd*	2.61±0.26

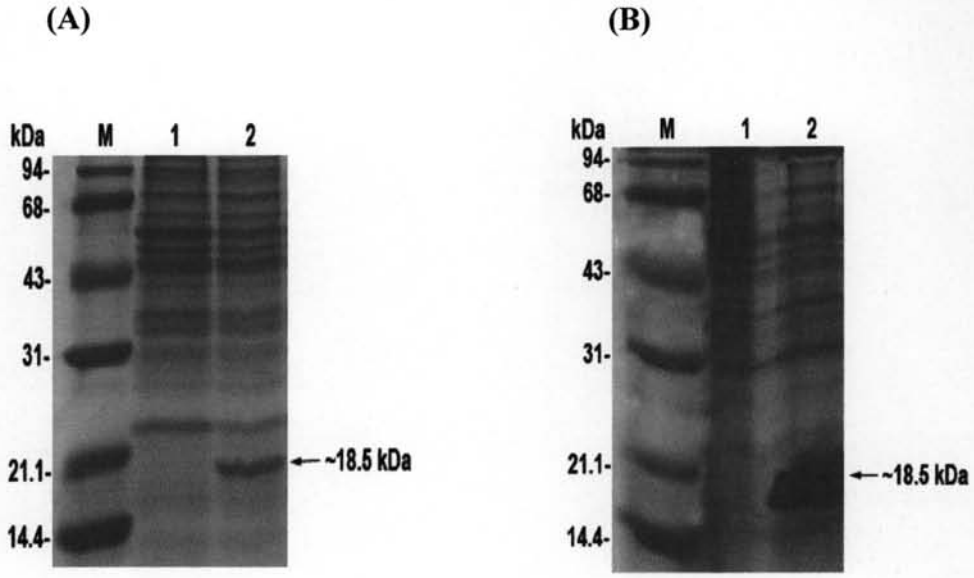


Figure 4.9 : SDS-PAGE analysis of DQD expression in *E. coli* BL21 (DE3)/pET-*dqd* at 37°C **(A)** cell free extract **(B)** cell debris

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kD
Lysozyme	14.4	kDa

Lane 1: *E. coli* BL21 (DE3)/pET-21a (30 µg protein)

Lane 2: *E. coli* BL21 (DE3)/pET-*dqd* (30 µg protein)

It has been reported that high growth temperature promotes the inclusion body formation (Strandberg and Enfors, 1991). Therefore, the transformant was expressed at 30°C instead of 37°C to decrease the inclusion bodies. The DQD activity of intracellular crude enzyme of the transformant was 10.80 U/mg as shown in Table 4.2. The DQD activity of cells expressed at 30°C was 4 fold higher than that at 37°C.

Intracellular crude enzyme and precipitates were analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. From SDS-PAGE analysis result in Figure 4.10, the protein was more soluble. The inclusion body was reduced.

Table 4.2 DQD activity expressed in *E. coli* BL21 (DE3) harboring pET-*dqd* at 30°C

Samples	DQD activity (U/mg)
E. coli BL21 (DE3)/pET-21a	0.85±0.10
E. coli BL21 (DE3)/pET-*dqd*	10.80±0.56

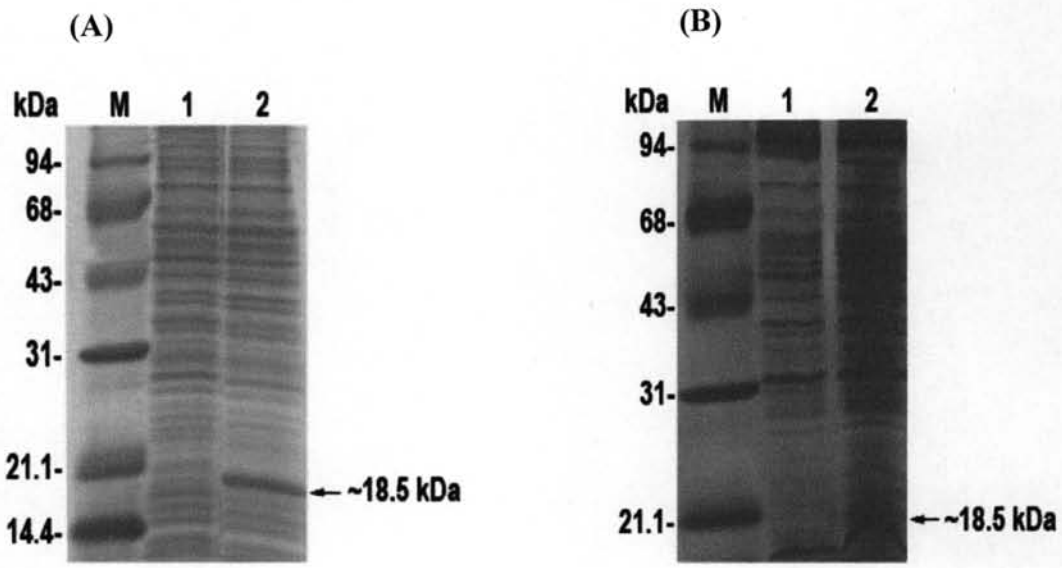


Figure 4.10 : SDS-PAGE analysis of DQD expression in E. *coli* BL21 (DE3)/pET-*dqd* at 30°C **(A)** cell free extract **(B)** precipitates

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pET21a (30 µg protein)

Lane 2 : *E. coli* BL21 (DE3)/pET-*dqd* (30 µg protein)

4.4 Expression of *skdh* (GOX0859) gene in *E. coli* BL21 (DE3)

skdh (GOX0859) gene was cloned from open reading frame into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3)/pET-GOX0859 transformant was cultured on a shaking incubator at 37°C until OD_{600} reach 0.2 and, then induced by 1mM IPTG for 5 hours. After cells were broken, the crude enzyme was used to determine SKDH activity (section 3.9.4). SKDH activity was examined using either 2 mM shikimic acid or quinic acid as a substrate and using either 0.2 mM NAD^+ or $NADP^+$ as a cofactor. The transformant has SKDH activity specificially with shikimic acid and $NADP^+$ as a cofactor. The SKDH activity is shown in Table 4.3.

Intracellular crude enzyme and precipitates were analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. Deduced amino acid from SKDH (GOX0859) is approximate 30.7 kDa, 36.3 kDa is shown in Figure 4.11. It was shown that almost expressed protein (indicated by an arrow) was in precipitates. It was concluded that GOX0859 is formed as an inclusion body.

Table 4.3 SKDH (GOX0859) activity of *E. coli* BL21 (DE3)/pET-GOX0859 assayed with shikimic acid, quinic acid and cofactor NAD^+ and $NADP^+$ at 37°C

Samples	**SKDH activity (U/mg)**			
	Shikimic acid/ NAD^+	**Shikimic acid/ $NADP^+$**	**Quinic acid/ NAD^+**	**Quinic acid/ $NADP^+$**
E. coli BL21 (DE3)	Not detected	0.044±0.002	Not detected	Not detected
E. coli BL21 (DE3)/pET-21a	Not detected	0.043±0.0031	Not detected	Not detected
E. coli BL21 (DE3)/pET-GOX0859	Not detected	0.047±0.0017	Not detected	Not detected

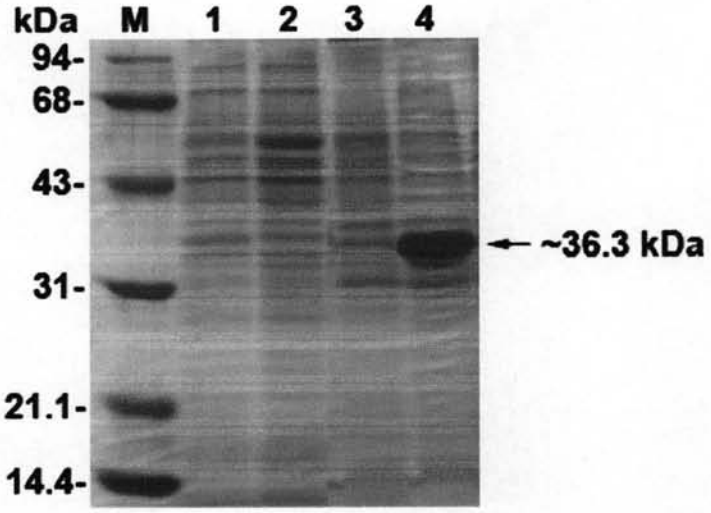


Figure 4.11 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX0859 expressed at 37°C.

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pET-21a cell free extract (30 μg protein)

Lane 2 : *E. coli* BL21 (DE3)/pET-GOX0859 cell free extract (30 μg protein)

Lane 3 : *E. coli* BL21 (DE3)/pET-21a precipitates

Lane 4 : *E. coli* BL21 (DE3)/pET-GOX0859 precipitates

From previous report, it has been suggested that high growth temperature promotes the aggregation of recombinant protein as inclusion body (Strandberg and Enfors, 1991). Therefore, the transformant was cultured at lower temperature 30°C and 20°C, respectively. After cells were grown to OD_{600} = 0.2, IPTG was added to final concentration 0.5 mM and the cultivation was continued until OD_{600} reach 1.0. Intracellular crude enzyme was prepared (section 3.9.3) and analyzed by SDS-PAGE. Intracellular crude enzyme was used to determine SKDH activity. From the result, the SKDH activity of transformant when cultured at 30°C and 20°C was not different from the *E. coli* BL21 (DE3) suggesting that *skdh* homolog might not be the gene coding for SKDH as shown in Table 4.4.

Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. From SDS-PAGE analysis in Figure 4.12 and 4.13, almost all of expressed SKDH (GOX0859) was form as an inclusion body as indicated by an arrow. In order to solve this problem, an efficient protein expression vector based on the low-temperature expression promoter gene of *E. coli*, pCold I vector (Appendix C) (Takara), was used.

Table 4.4 SKDH (GOX0859) activity of *E. coli* BL21 (DE3)/pET-GOX0859 at 30°C and 20°C

Samples	**SKDH activity (U/mg)**	
	30°C	**20°C**
E. coli BL21 (DE3)/pET-21a	0.050±0.0030	0.038±0.0015
E. coli BL21 (DE3)/pET-GOX0859	0.075±0.0052	0.037±0.0015

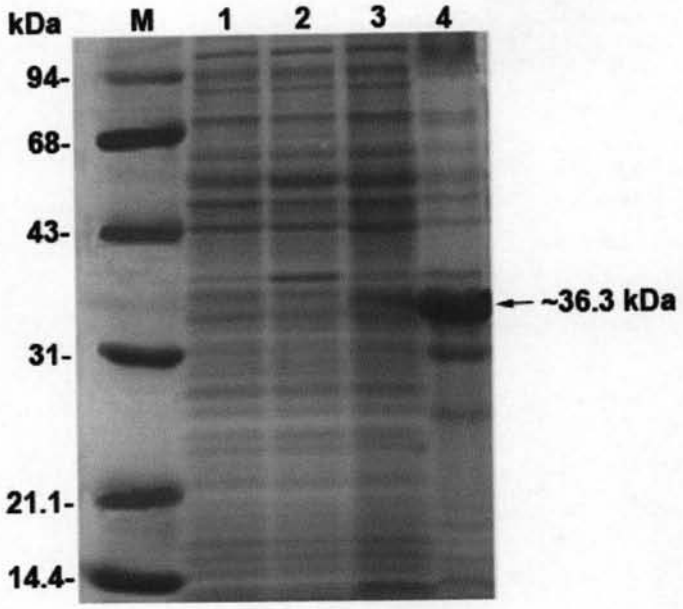


Figure 4.12: SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX0859 expressed at 30°C.

Lane M: Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1: *E. coli* BL21 (DE3)/pET-21a cell free extract (30 μg protein)

Lane 2: *E. coli* BL21 (DE3)/pET-GOX0859 cell free extract (30 μg protein)

Lane 3: *E. coli* BL21 (DE3)/pET-21a precipitates

Lane 4: *E. coli* BL21 (DE3)/pET-GOX0859 precipitates

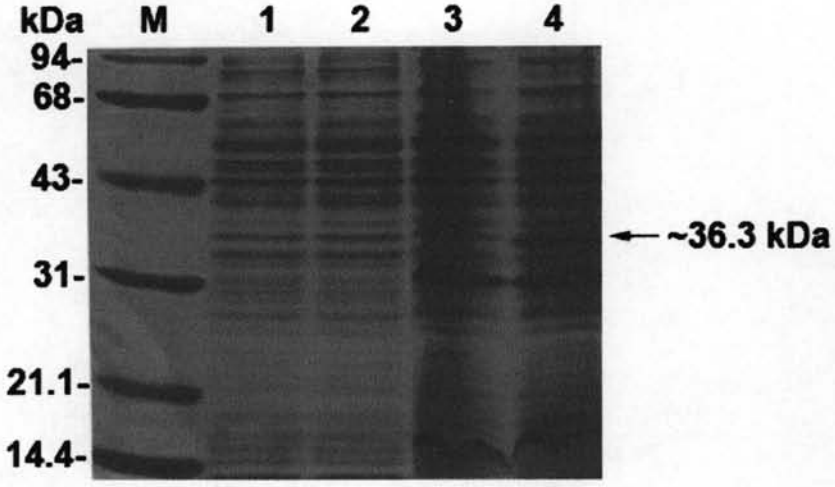


Figure 4.13 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX0859 expressed at 20°C.

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pET-21a cell free extract (30 μg protein)

Lane 2 : *E. coli* BL21 (DE3)/pET-GOX0859 cell free extract (30 μg protein)

Lane 3 : *E. coli* BL21 (DE3) precipitates

Lane 4 : *E. coli* BL21 (DE3)/pET-GOX0859 precipitates

4.5 Expression of *skdh* (GOX0859) gene in *E. coli* BL21 (DE3) by pCold I vector

Expression of gene using pCold I vector might be able to reduce inclusion body formation. Therefore, expression of *skdh* (GOX0859) in *E. coli* BL21 (DE3) using pCold I vector was carried out. The *E. coli* BL21 (DE3)/ pCold I-GOX0859 was cultured at 37°C until OD_{600} reached 0.4. The inoculum was refrigerated at 15°C for 30 minutes. IPTG was added to final concentration of 0.1 mM and cultivation was continued at 15°C for 24 hours. Intracellular crude enzyme was prepared and used to assay SKDH activity. The transformant has SKDH activity with shikimic acid and $NADP^+$. However, the activity was not different from that of the *E. coli* wild type and (Table 4.5).

Intracellular crude enzyme and precipitates were analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. According to *skdh* (GOX0859) was not inserted at start condon of the pCold I vector and stop codon of this gene was removed, protein translation was initiated at start codon of the vector and terminated by using stop codon of the vector (48 bp upstream and 45 bp downstream of the gene). Therefore, the SKDH expressed from *E. coli* BL21 (DE3)/ pCold I-GOX0859 was bigger than that of *E. coli* BL21 (DE3)/ pET-GOX0859. Deduced amino acid from SKDH (GOX0859) expressed from *E. coli* BL21 (DE3)/ pCold I-GOX0859 is approximate 33.1 kDa, 42.4 kDa is shown in Figure 4.14. It was shown that almost all of the expressed SKDH (GOX0859) was still formed as inclusion body.

Table 4.5 SKDH (GOX0859) activity of *E. coli* BL21 (DE3)/ pCold I-GOX0859 assayed with shikimic acid, quinic acid and cofactor NAD^+ and $NADP^+$

Samples	**SKDH activity (U/mg)**			
	Shikimic acid/ NAD^+	**Shikimic acid/ $NADP^+$**	**Quinic acid/ NAD^+**	**Quinic acid/ $NADP^+$**
E. coli BL21 (DE3)/pCold I	Not detected	0.054±0.0025	Not detected	Not detected
E. coli BL21 (DE3)/ pCold I-GOX0859	Not detected	0.062±0.004	Not detected	Not detected

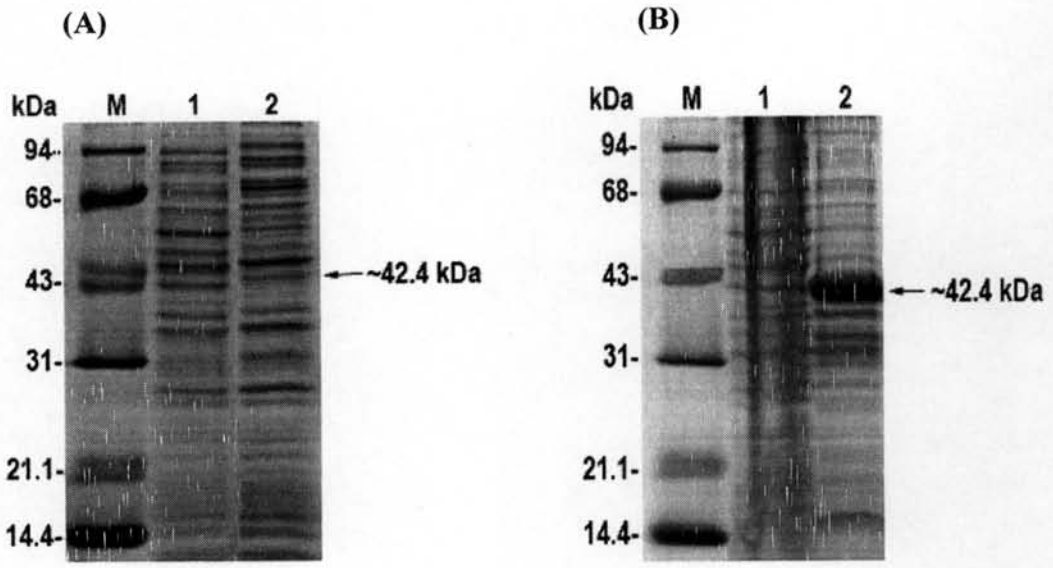


Figure 4.14 : SDS-PAGE analysis of SKDH (GOX0859) expression in *E. coli* BL21 (DE3)/pCold I-GOX0859 **(A)** cell free extract **(B)** precipitates

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pCold I (30 µg protein)

Lane 2 : *E. coli* BL21 (DE3)/pCold I-GOX0859 (30 µg protein)

Folding for many proteins is facilitated by the action of specialized proteins. Molecular chaperones are proteins that facilitating correct folding pathways or providing microenvironments in which folding can occur. It has been reported that chaperone co-expression may be minimize the non-productive side reactions during refolding (Schrodel *et al.*, 2005). Therefore, pCold I-GOX0859 was co-expressed with chaperone vector to solve inclusion body problem.

4.6 Co-expression of pCold I-GOX0859 with chaperone vector

pCold I-GOX0859 was transformed into *E. coli* BL21 (DE3) harboring chaperone vector, pG-KJE8. The transformant was cultured as described in section 3.11 and intracellular was prepared as described in section 3.11.4. According to the result shown in Table 4.6, the SKDH activities between the transformant and the wild type were not different. The cell free extracts and precipitates are analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. From the result in Figure 4.15, co-expression of pCold I-GOX0859 with chaperone vector made expressed protein more soluble.

Table 4.6 SKDH (GOX0859) activity of *E. coli* BL21 (DE3)/pCold I-GOX0859 co-expressed with chaperone vector

Samples	SKDH activity (U/mg)
E. coli BL21 (DE3)/pCold I	0.058±0.0015
E. coli BL21 (DE3)/pCold I-GOX0859/pG-KJE8	0.058±0.0006

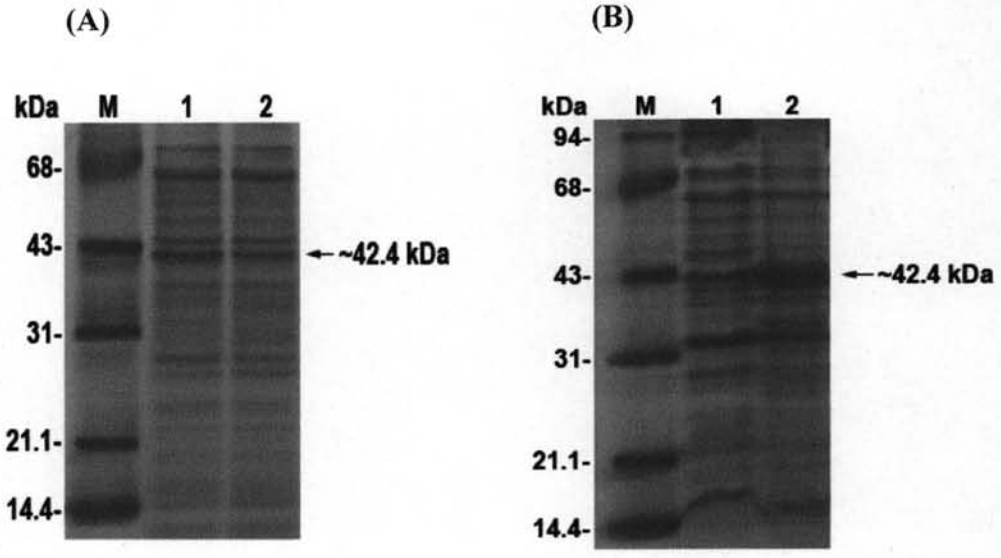


Figure 4.15 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pCold I-GOX0859 co-expressed with pG-KJE8 vector **(A)** cell free extract **(B)** precipitates

Lane M: Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pCold I (30 µg protein)

Lane 2 : *E. coli* BL21 (DE3)/pCold I-GOX0859/pG-KJE8 (30 µg protein

4.7 Purification of *skdh* (GOX0859) by Ni-NTA agarose column chromatography

The expressed SKDH (GOX0859) has six-histidine residues at C-terminal. The intracellular crude enzyme was prepared as described in section 3.11.4 and subjected to Ni-NTA Agarose column chromatography as described in section 3.13. The elution profile of SKDH (GOX0859) was shown in Figure 4.16. Unbound proteins were eluted from the column by 1 column volume native wash buffer. The bound protein was eluted with 50 mM sodium phosphate buffer pH 8.0 with 250 mM imidazole buffer containing 0.5 M NaCl. Each fraction was measured the absorbance at 280 nm and SKDH activity was assayed. However, no SKDH activity was observed in any fraction. Therefore, protein peak fraction number 4 and 5 were pooled and dialyzed in 20 mM Tris-HCl buffer pH 8.0.

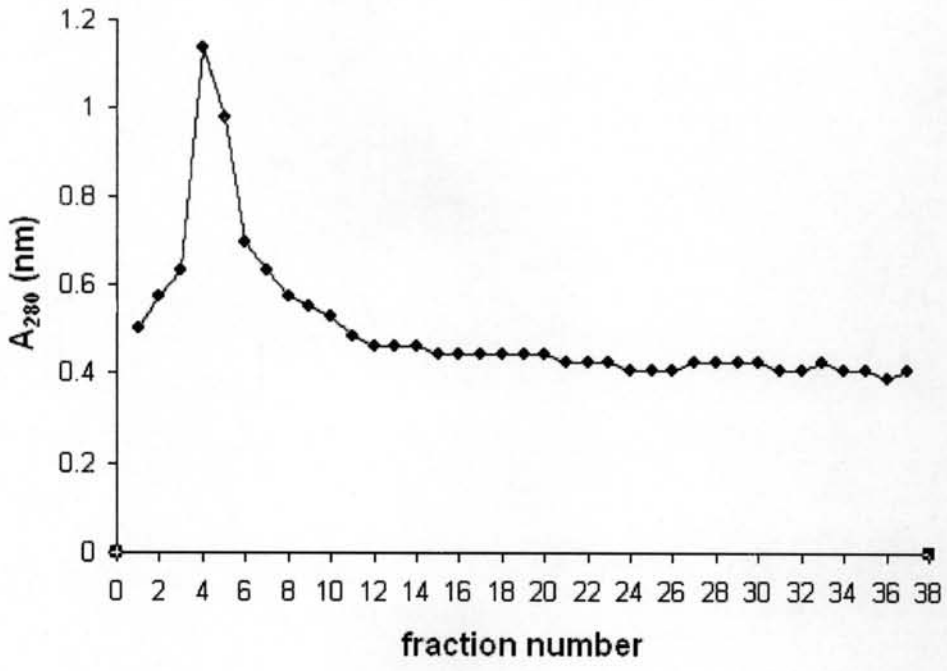


Figure 4.16 : The elution profile of SKDH (GOX0859) on Ni-NTA agarose column chromatography eluted by 50 mM sodium phosphate buffer pH 8.0 with 250 mM imidazole buffer containing 0.5 M NaCl. (1 ml fraction)

After assay SKDH activity of pooled fraction, no SKDH activity was Observed (Table 4.7). The purified protein was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme and purified enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. From Figure 4.17, Ni-NTA Agarose Column Chromatography could not remove all of protein impurity. SKDH (GOX0859) molecular weight was calculated from the standard curve in Figure 4.18 to be 42.4 kDa.

Table 4.7 SKDH (GOX0859) activity of E. coli BL21 (DE3)/pCold I-GOX0859/pG-KJE8 after purification

Samples	SKDH activity (U/mg)
Crude SKDH (GOX0859)	0.036±0.001
Pooled fraction (no. 4 and 5)	Not detected

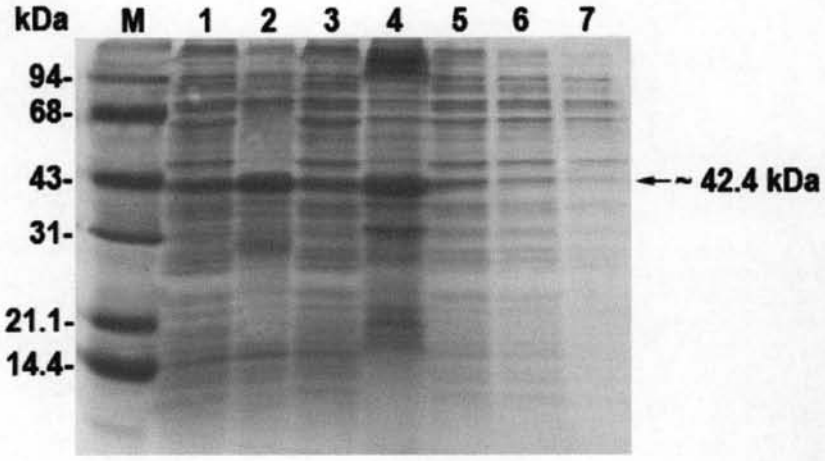


Figure 4.17 : SDS-PAGE analysis of SKDH (GOX0859) from *E. coli* BL21 (DE3)/pCold I-GOX0859 co-expressed with pG-KJE8 vector after purification

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : Crude SKDH (GOX0859) (30 μg protein)

Lane 2 : Purified SKDH (GOX0859) (30 μg protein)

Lane 3 : Cell free extract solution after binding with Ni-NTA agarose (30 μg protein)

Lane 4 : Precipitates

Lane 5 : washed solution number 1

Lane 6 : washed solution number 2

Lane 7 : washed solution number 3

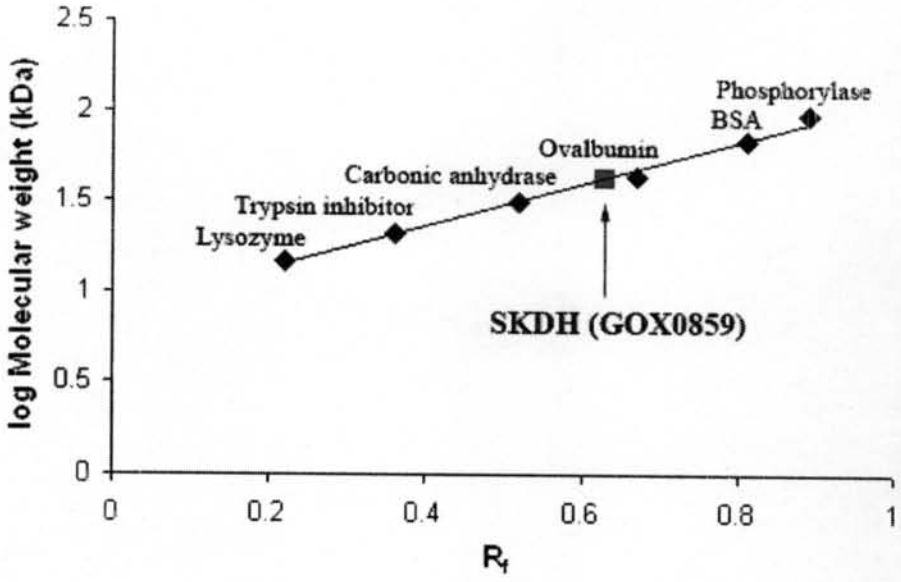


Figure 4.18 : Molecular weight calibration curve of standard proteins by 12.5% SDS-PAGE for molecular weight determination of SKDH (GOX0859) from *E. coli* BL21 (DE3)/pCold I-GOX0859 co-expressed with pG-KJE8 vector

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

4.8 Expression of *skdh* (GOX1959) gene in *E. coli* BL21 (DE3)

From *G. oxydans* 621H genome database, two *skdh* genes (GOX0859 and GOX1959) were reported to encoded shikimate dehydrogenase. After *E. coli* BL21 (DE3)/ pCold I-GOX0859 expression, the *skdh* (GOX0859) was not shown SKDH activity. Therefore, *E. coli* BL21 (DE3)/ pET-GOX1959 expression was done. The *E. coli* BL21 (DE3)/ pET-GOX1959 was cultured at 37°C until OD_{600} reach 1.2. Intracellular crude enzyme was prepared as described in section 3.12.3. After SKDH activity assay, the transformant showed activity with shikimic acid and $NADP^+$ at 92.49 U/mg (Table 4.8).

Crude enzyme and precipitates were analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. Since the expected size of SKDH (GOX1959) is approximately 30.2 kDa, the result in Figure 4.19 showed that some of the expressed SKDH (GOX1959) was formed as inclusion body.

Table 4.8 SKDH (GOX1959) activity of *E. coli* BL21 (DE3)/ pET-GOX1959 assayed with shikimic acid, quinic acid and cofactor NAD^+ and $NADP^+$

Samples	**SKDH activity (U/mg)**			
	Shikimic acid/ NAD^+	**Shikimic acid/ $NADP^+$**	**Quinic acid/ NAD^+**	**Quinic acid/ $NADP^+$**
E. coli BL21 (DE3)	Not detected	0.043±0.004	Not detected	Not detected
E. coli BL21 (DE3)/pET-21a	Not detected	0.047±0.0017	Not detected	Not detected
E. coli BL21 (DE3)/ pET-GOX1959	Not detected	92.49±0.28	Not detected	Not detected

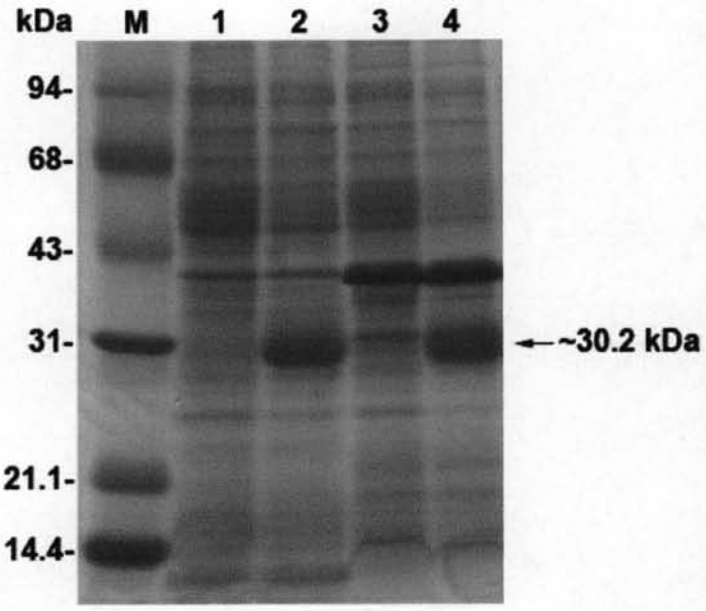


Figure 4.19 : SDS-PAGE analysis of SKDH (GOX1959) expression in *E. coli* BL21 (DE3)/pET-GOX1959

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pET-21a cell free extract (30 μg protein)

Lane 2 : *E. coli* BL21 (DE3)/pET-GOX1959 cell free extract (30 μg protein)

Lane 3 : *E. coli* BL21 (DE3)/pET-21a precipitates

Lane 4 : *E. coli* BL21 (DE3)/pET-GOX1959 precipitates

4.9 Purification of SKDH (GOX1959) by Ni-NTA agarose column chromatography

The expressed SKDH (GOX1959) has six-histidine residues at C-terminal. The intracellular crude enzyme was prepared as described in section 3.12.3 and subjected to Ni-NTA Agarose Column Chromatography as described in section 3.13. Unbound proteins were eluted from the column by 50 mM sodium phosphate buffer pH 8.0 with 250 mM imidazole buffer containing 0.5 M NaCl. The bound protein was eluted with native elution buffer. Each fraction was measured absorbance at 280 nm and assayed for SKDH activity. The elution profile of SKDH (GOX1959) was shown in Figure 4.20. Fraction number 7, 8 and 9 were pooled and dialyzed in 20 mM Tris-HCl buffer pH 8.0. The specific activity and purification fold of the enzyme was 454.03 U/mg and 7.33 folds (Table 4.9). Crude enzyme, purified enzyme and washed fraction were analyzed by SDS-PAGE. Thirty microgram protein of crude enzyme and purified enzyme were subjected to electrophoresis on 12.5 % SDS-PAGE. From the result in Figure 4.21, protein impurities were almost removed.

The molecular weight calibration curve was ploted between relative mobility (R_f) and log molecular weight. From Figure 4.22, SKDH (GOX1959) has a molecular weight at 30.9 kDa.

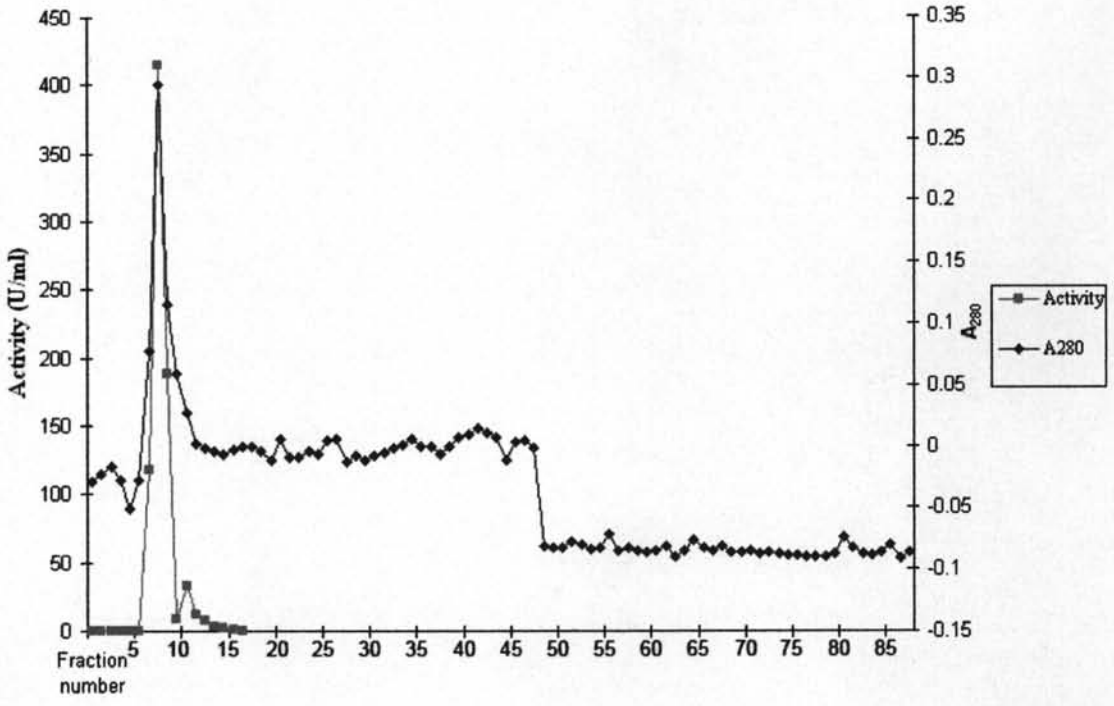


Figure 4.20 : The elution profile of SKDH (GOX1959) on Ni-NTA agarose column chromatography eluted by 50 mM sodium phosphate buffer pH 8.0 with 250 mM imidazole buffer containing 0.5 M NaCl.

Table 4.9 Purification of SKDH (GOX1959) from *E. coli* BL21 (DE3)/pET-GOX1959

Samples	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield
Crude	2	1,611.27	26.14	61.64	1.00	100
purified SKDH (GOX1959)	2	605.72	1.34	452.03	7.33	37.59

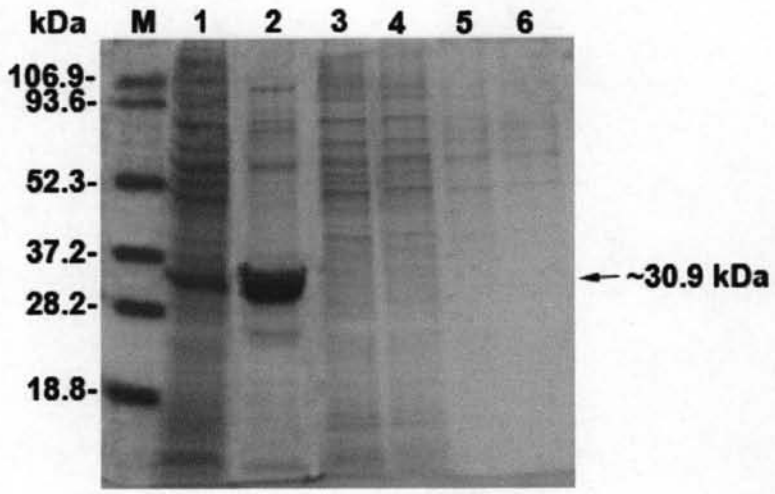


Figure 4.21 : SDS-PAGE analysis of purified SKDH (GOX1959)

Lane M : Protein molecular weight marker

Phosphorylase B	106.9	kDa
BSA	93.6	kDa
Ovalbumin	52.3	kDa
Carbonic anhydrase	37.2	kDa
Soybean trypsin inhibitor	28.2	kDa
Lysozyme	18.8	kDa

Lane 1 : crude SKDH (GOX1959) (30 μg protein)

Lane 2 : purified SKDH (GOX1959) (30 μg protein)

Lane 3 : washed column number 1

Lane 4 : washed column number 2

Lane 5 : washed column number 3

Lane 6 : washed column number 4

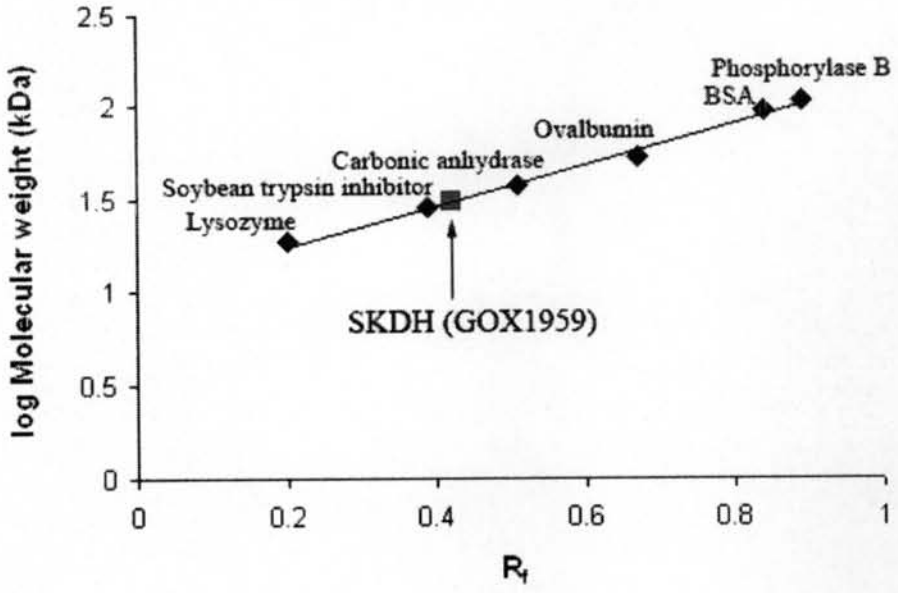


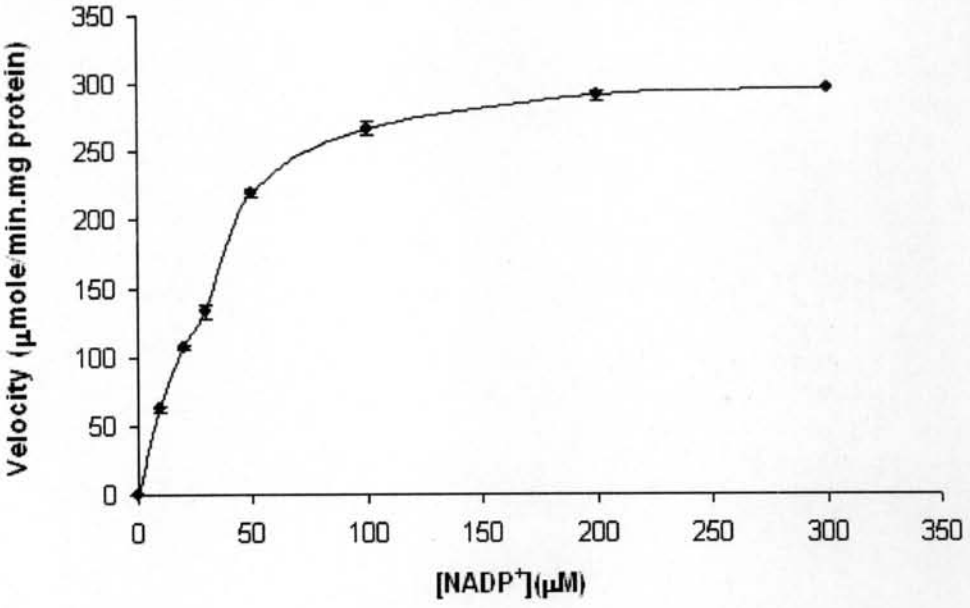
Figure 4.22 : Molecular weight calibration curve of standard proteins by 12.5% SDS-PAGE for molecular weight determination of SKDH (GOX1959) from *E. coli* BL21 (DE3)/pET -GOX1959

Phosphorylase B	106.9	kDa
BSA	93.6	kDa
Ovalbumin	52.3	kDa
Carbonic anhydrase	37.2	kDa
Soybean trypsin inhibitor	28.2	kDa
Lysozyme	18.8	kDa

4.10 Determination of kinetic parameters of SKDH (GOX1959)

Purified SKDH (GOX1959) was used to determine kinetic parameters. K_m and V_{max} values for shikimate and $NADP^+$ were determined at saturating concentration of co-substrate 50 mM as described in section 3.14. The typical Lineweaver-Burk plot was shown for $NADP^+$ and shikimic acid as the substrate of SKDH (GOX1959) (Figure 4.23 and 4.24, respectively). Kinetic parameters with various substrates were summarized in Table 4.10. The K_m and k_{cat} value of $NADP^+$ was lower than shikimic acid while V_{max} value of $NADP^+$ was higher than shikimic acid.

(A)



(B)

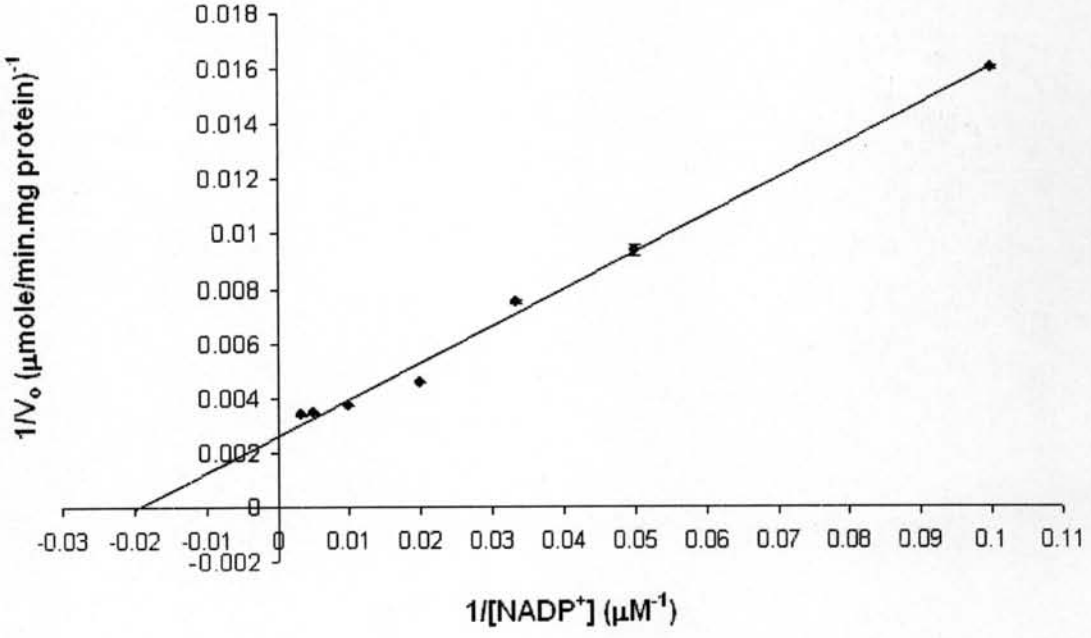
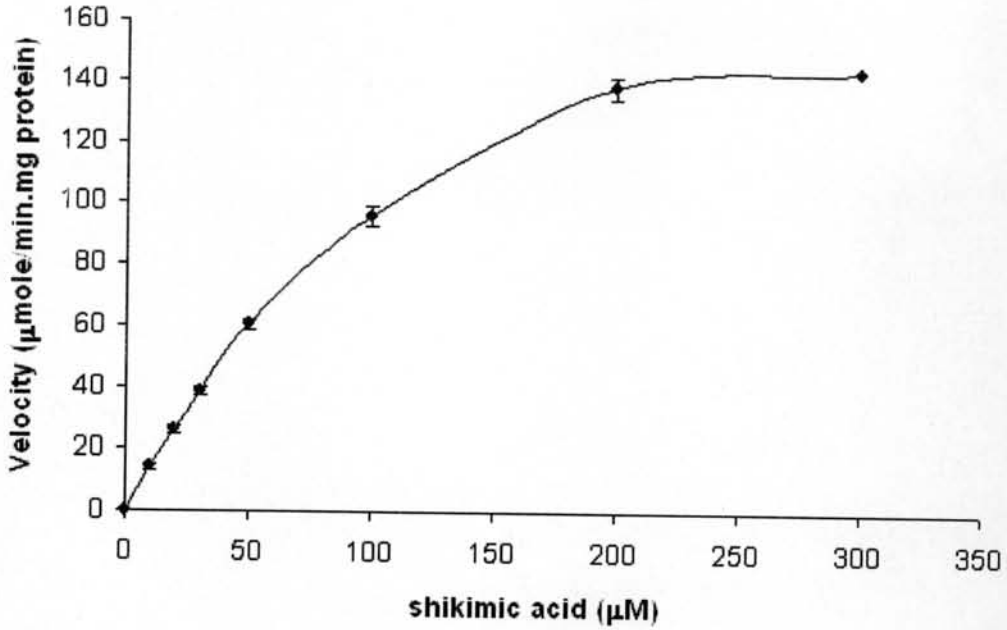


Figure 4.23 : Kinetic studies of SKDH (GOX1959) with $NADP^+$ as a substrate (n=3)

(A) Saturation curve

(B) Lineweaver-Burk plot

(A)



(B)

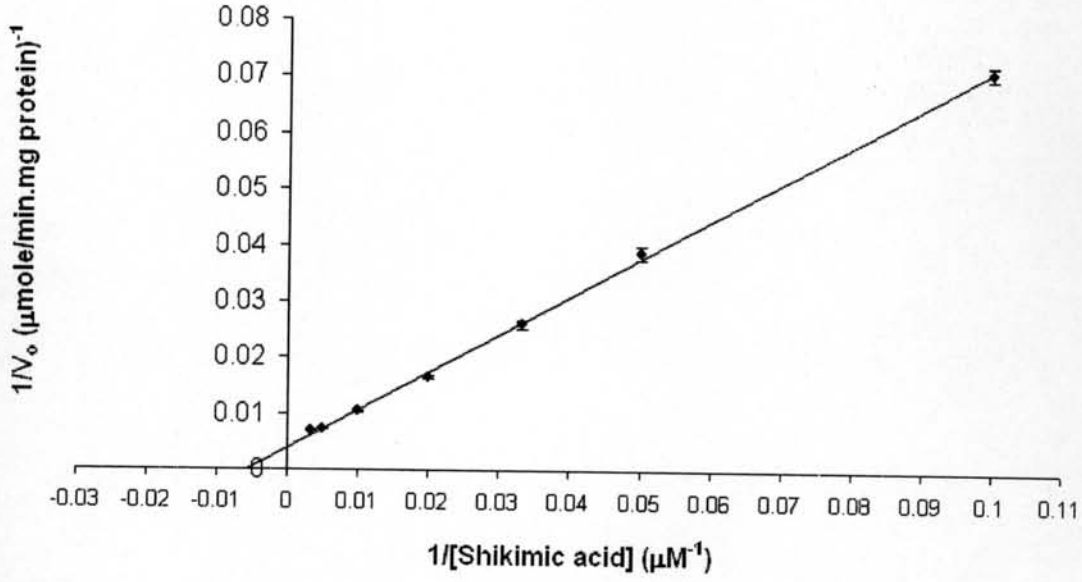


Figure 4.24 : Kinetic studies of SKDH (GOX1959) with shikimic acid as a substrate (n=3)

(A) Saturation curve

(B) Lineweaver-Burk plot

Table 4.10 Kinetic parameters of SKDH (GOX1959)

Substrate	K_m **(μM)**	V_{max} **(μmole/min.mg protein)**	k_{cat} **(min^{-1})**
$NADP^+$	51.7	384.6	1.16×10^5
Shikimic acid	250	168.4	8.74×10^5

4.11 Cloning of NAD(P)-dependent *Glucose dehydrogenase* gene from *Gluconobacter oxydans* 621H

From the genome sequence of *G. oxydans* 621H, primers positioned upstream and downstream of NADP-*gdh* gene (GOX2015) was designed. To prepare the amplified gene product for insertion into pET-GOX1959, the 5'-end primer comprised of *Xho*I restriction site and 5'-base sequence of *gdh* gene. The 3'-end primer comprised of *Xho*I restriction site and 3'-base sequence of *gdh* gene according to the method described in section 3.15.1. Figure 4.25 showed the 850 bps PCR product of *gdh* gene. After PCR amplification, the PCR products were ligated into pET-GOX1959 and the recombinant plasmid was sequenced. The sequencing result showed that the exact insert size was 875 bps (29 bp upstream region, 801 bp coding region, 45 bp downstream) (Figure 4.26).

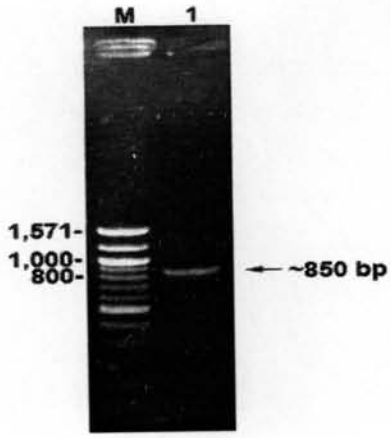


Figure 4.25 : *gdh* PCR products from *G. oxydans* 621H genome on 0.8 % agarose gel

Lane M : 100 bp DNA Ladder

Lane 1 : *gdh* PCR products

```
                                        Forward primer                M  P  A  P
gdh (GOX2015)   ---------------------TCTCGAGTAACAAGGAGAGGTGAGGCCAGATGCCTGCCC 39
gdh_F           CTATTCTCGAGCGGTTTCAAACCTCGAGTAACAAGGAGAGGTGAGGCCAGATGCCTGCCC 60
                                     ************************************

                  Y  K  D  R  F  A  G  K  K  V  L  V  T  G  A  S  Q  G  I  G
gdh (GOX2015)   CTTACAAAGACCGTTTCGCCGGCAAGAAAGTCCTCGTCACCGGGGCCTCCCAGGGAATTG 99
gdh_F           CTTACAAAGACCGTTTCGCCGGCAAGAAAGTCCTCGTCACCGGGGCCTCCCAGGGAATTG 120
                ************************************************************

                  E  A  T  A  L  R  F  A  E  E  G  A  Q  V  A  L  N  G  R  K
gdh (GOX2015)   GCGAGGCCACCGCGCTTCGTTTTGCCGAAGAAGGCGCGCAGGTCGCCCTCAACGGCCGCA 159
gdh_F           GCGAGGCCACCGCGCTTCGTTTTGCCGAAGAAGGCGCGCAGGTCGCCCTCAACGGCCGCA 180
                ************************************************************

                  E  D  K  L  I  A  V  R  E  K  L  P  K  V  S  G  G  E  H  P
gdh (GOX2015)   AGGAAGACAAGCTGATCGCCGTCCGCGAGAAGCTGCCCAAGGTTTCCGGCGGAGAGCACC 219
gdh_F           AGGAAGACAAGCTGATCGCCGTCCGCGAGAAGCTGCCCAAGGTTTCCGGCGGAGAGCACC 240
                ************************************************************

                  I  A  T  G  D  I  S  K  E  D  D  V  K  R  L  V  A  E  S  I
gdh (GOX2015)   CGATCGCCACGGGTGACATTTCCAAAGAAGACGACGTCAAACGTCTGGTTGCCGAGAGCA 279
gdh_F           CGATCGCCACGGGTGACATTTCCAAAGAAGACGACGTCAAACGTCTGGTTGCCGAGAGCA 300
                ************************************************************

                  K  A  M  G  G  L  D  V  L  V  C  N  A  G  Y  Q  I  P  S  P
gdh (GOX2015)   TCAAGGCCATGGGTGGTCTCGACGTTCTGGTCTGCAATGCGGGCTATCAGATCCCCTCCC 339
gdh_F           TCAAGGCCATGGGTGGTCTCGACGTTCTGGTCTGCAATGCGGGCTATCAGATCCCCTCCC 360
                ************************************************************

                  S  E  D  I  K  L  E  D  F  E  G  V  M  A  V  N  V  T  G  V
gdh (GOX2015)   CGTCAGAAGACATCAAGCTCGAAGATTTTGAAGGCGTGATGGCCGTCAACGTCACGGGGG 399
gdh_F           CGTCAGAAGACATCAAGCTCGAAGATTTTGAAGGCGTGATGGCCGTCAACGTCACGGGGG 420
                ************************************************************

                  M  L  P  C  R  E  V  I  R  Y  W  L  E  N  G  I  K  G  T  I
gdh (GOX2015)   TGATGCTGCCCTGTCGCGAAGTCATCCGCTACTGGCTGGAAAACGGCATCAAGGGCACGA 459
gdh_F           TGATGCTGCCCTGTCGCGAAGTCATCCGCTACTGGCTGGAAAACGGCATCAAGGGCACGA 480
                ************************************************************

                  I  V  N  S  S  V  H  Q  I  I  P  K  P  H  Y  L  G  Y  S  A
gdh (GOX2015)   TCATTGTGAACTCCTCCGTTCACCAGATCATCCCGAAACCGCATTATCTGGGCTATTCCG 519
gdh_F           TCATTGTGAACTCCTCCGTTCACCAGATCATCCCGAAACCGCATTATCTGGGCTATTCCG 540
                ************************************************************

                  S  K  G  A  V  G  N  I  V  R  T  L  A  L  E  Y  A  T  R  G
gdh (GOX2015)   CCTCCAAGGGTGCCGTCGGCAACATTGTCCGCACGCTGGCACTGGAATATGCCACCCGCG 579
gdh_F           CCTCCAAGGGTGCCGTCGGCAACATTGTCCGCACGCTGGCACTGGAATATGCCACCCGCG 600
                ************************************************************
```

Figure 4.26 : Alignment of *gdh* PCR product sequencing result (gdh_F) with *gdh* (GOX2015) gene from *G. oxdans* 621H genome sequence. The deduced amino acid sequence is also shown.

```
                I  R  V  N  A  V  A  P  G  A  I  V  T  P  I  N  M  S  W  I
gdh (GOX2015)   GCATCCGGGTGAATGCCGTGGCGCCCGGCGCCATCGTGACGCCGATCAACATGTCGTGGA 639
gdh_F           GCATCCGGGTGAATGCCGTGGCGCCCGGCGCCATCGTGACGCCGATCAACATGTCGTGGA 660
                ************************************************************

                D  D  P  E  Q  Y  K  A  V  S  S  H  I  P  M  K  R  P  G  E
gdh (GOX2015)   TCGACGATCCCGAACAGTACAAGGCCGTTTCGAGCCACATCCCGATGAAGCGCCCGGGCG 699
gdh_F           TCGACGATCCCGAACAGTACAAGGCCGTTTCGAGCCACATCCCGATGAAGCGCCCGGGCG 720
                ************************************************************

                S  R  E  I  A  D  A  I  T  F  L  A  A  E  D  S  T  Y  I  T
gdh (GOX2015)   AAAGCCGCGAAATCGCGGATGCCATCACCTTCCTCGCCGCCGAGGACAGCACCTACATCA 759
gdh_F           AAAGCCGCGAAATCGCGGATGCCATCACCTTCCTCGCCGCCGAGGACAGCACCTACATCA 780
                ************************************************************

                G  Q  T  L  Y  V  D  G  G  L  T  L  Y  G  D  F  E  N  N  W
gdh (GOX2015)   CGGGTCAGACCCTGTATGTCGATGGTGGTCTGACGCTCTACGGCGATTTCGAAAACAACT 819
gdh_F           CGGGTCAGACCCTGTATGTCGATGGTGGTCTGACGCTCTACGGCGATTTCGAAAACAACT 840
                ************************************************************

                S  S                                Reverse primer
gdh (GOX2015)   GGTCCTCGTAAACTTATATGGCCCCTTCCCTTACCGTTCTGCTGATCGACTCGAGA---- 875
gdh_F           GGTCCTCGTAAACTTATATGGCCCCTTCCCTTACCGTTCTGCTGATCGACTCGNGAGCNA 900
                ************************************************** **
```

Figure 4.26 : (continue)

4.12 Co-expression of *skdh* (GOX1959) with *gdh* gene in *E. coli* BL21 (DE3)

gdh (GOX2015) gene was cloned with 29 bp and 45 bp upstream region and downstream regions, respectively, into pET-GOX1959. The *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 was cultured at 37°C until OD_{600} reach 1.2. Intracellular crude enzyme was prepared as described in section 3.16.3. From the activity result, SKDH activity was high but GDH activity was low as shown in Table 4.11.

Crude enzyme and precipitates were analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. From deduced amino acid sequence, GDH has a molecular weight of about 30.3 kDa, which is close to that of SKDH (GOX1959). Therefore, SDS-PAGE could not separate GDH and SKDH (GOX1959) bands (Figure 4.27).

Table 4.11 SKDH and GDH activity expressed in *E. coli* BL21 (DE3) harboring pET-GOX1959-GOX2015

Samples	SKDH (GOX1959) activity (U/mg)	GDH (GOX2015) activity (U/mg)
E. coli BL21(DE3)	0.042±0.002	Not detected
E. coli BL21(DE3)/pET-21a	0.046±0.0029	Not detected
E. coli BL21(DE3)/pET-GOX1959-GOX2015	109.04±0.46	0.55±0.010

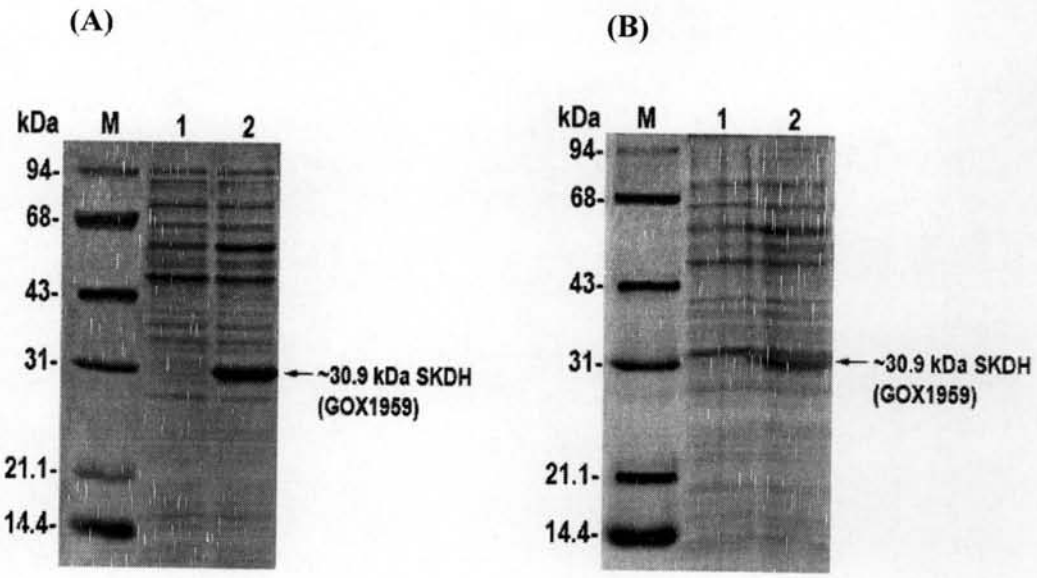


Figure 4.27 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 cell free extract **(A)** cell free extract **(B)** precipitates

Lane M: Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3)/pET-21a (30 μg protein)

Lane 2 : *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 (30 μg protein)

4.13 Optimization of co-expression conditions for SKDH and GDH activities

4.13.1 Effect of medium volume on SKDH and GDH activities

The medium volume is an important parameter that effect to the dissolved oxygen level in flask. It may be influence to the proteins expression. To study the effect of medium volume on the SKDH (GOX1959) and GDH (GOX205) expression, the *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 was cultured in LB medium using the medium volume of 25, 50, 75, 100 and 125 ml at 37°C until OD_{600} reach 1.2. Intracellular crude enzyme was prepared as described in section 3.16.3. The SKDH and GDH activity were shown in Table 4.12 and Figure 4.28. It is obvious that the specific activity of SKDH and GDH were highest when cells were grown in 100 ml medium volume. Therefore, 100-ml medium was used to culture the transformant for further study. Intracellular crude enzyme was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme was subjected to electrophoresis on 12.5 % SDS-PAGE. The result in Figure 4.29 showed that the intensity of major protein band was corresponded to the level of enzyme activity.

Table 4.12 SKDH and GDH activity of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 when grown at different volumes

Culture volume	Crude enzyme from *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015	
	SKDH activity (U/mg)	**GDH activity (U/mg)**
volume 25 ml	22.3±2.08	0.011±0.0006
volume 50 ml	52.48±1.92	0.055±0.0046
volume 75 ml	93.37±0.79	0.10±0.014
volume 100 ml	98.97±0.82	0.15±0.017
volume 125 ml	83.41±1.88	0.13±0

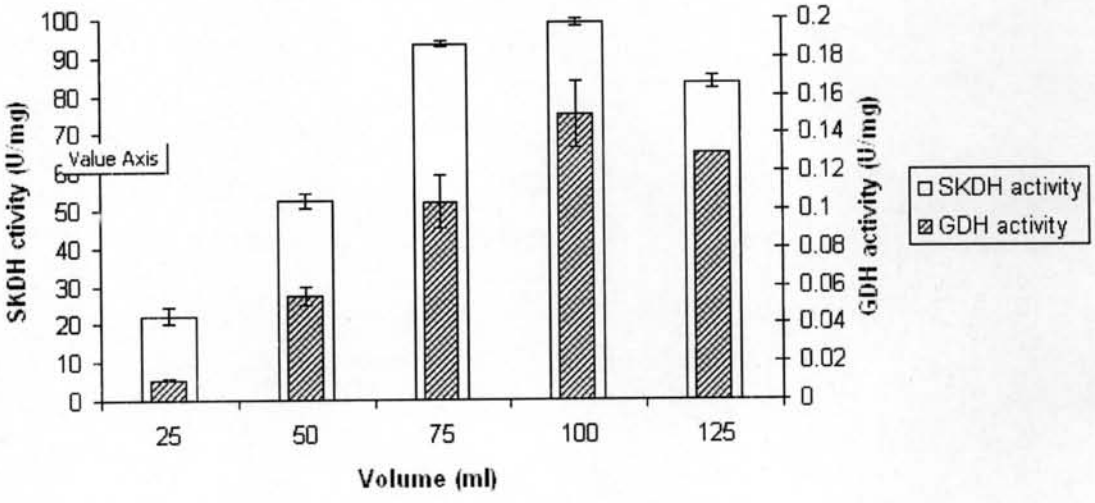


Figure 4.28 : SKDH and GDH activities of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 grown at different medium volumes

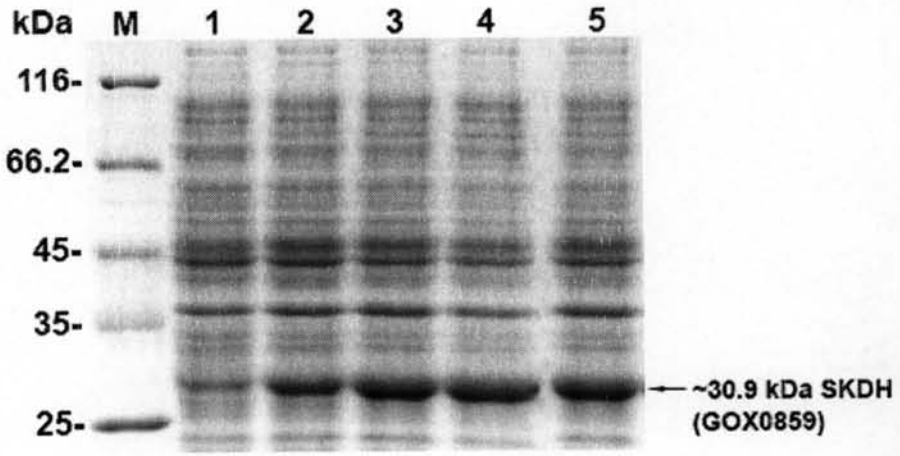


Figure 4.29 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 cell free extract when culture at different medium volumes

Lane M : Protein molecular weight marker

β-galactosidase	116 kDa
BSA	66.2 kDa
Ovalbumin	45 kDa
Lactate dehydrogenase	35 kDa
REase Bsp98I	25 kDa

Crude enzyme from cell grown at different medium volumes:

Lane 1 : volume 25 ml (30 μg protein)

Lane 2 : volume 50 ml (30 μg protein)

Lane 3 : volume 75 ml (30 μg protein)

Lane 4 : volume 100 ml (30 μg protein)

Lane 5 : volume 125 ml (30 μg protein)

4.13.2 Effect of IPTG concentration on SKDH and GDH activities

The *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 was cultured in 100 ml LB medium at 37°C. When OD_{600} reach 0.2, IPTG was added to final concentration of 0.2, 0.5, 0.8 and 1.0 mM, respectively and cultivation was continued at 37°C for 5 hours (final OD_{600} of 1.2). Intracellular crude enzyme was prepared as described in section 3.12.3 and used to assay SKDH and GDH activity. The SKDH and GDH activity were shown in Table 4.13 and Figure 4.30. The highest SKDH and GDH activities were obtained when induced with 0.5 and 0.2 mM, respectively. Intracellular cellular enzyme was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme was subjected to electrophoresis on 12.5% SDS-PAGE. The result in Figure 4.31 showed that the intensity of major protein band was corresponded to the level of SKDH activity. Because $NADP^+$ was reproduced, the GDH activity should be high enough to reproduce $NADP^+$ in a proper amount. From activity result, the GDH activity was low. Therefore, the transformant was induced with 0.2 mM IPTG to obtain the highest GDH activity.

Table 4.13 SKDH and GDH activity of E. *coli* BL21 (DE3)/pET-GOX1959-GOX2015 when induced with different IPTG concentrations

IPTG concentration	Crude enzyme from *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015	
	SKDH activity (U/mg)	GDH activity (U/mg)
0.2 mM IPTG	275.87±0.49	1.43±0.011
0.5 mM IPTG	333.17±0.74	1.21±0.095
0.8 mM IPTG	215.88±0.34	0.96±0.035
1 mM IPTG	150.40±1.00	0.94±0.012

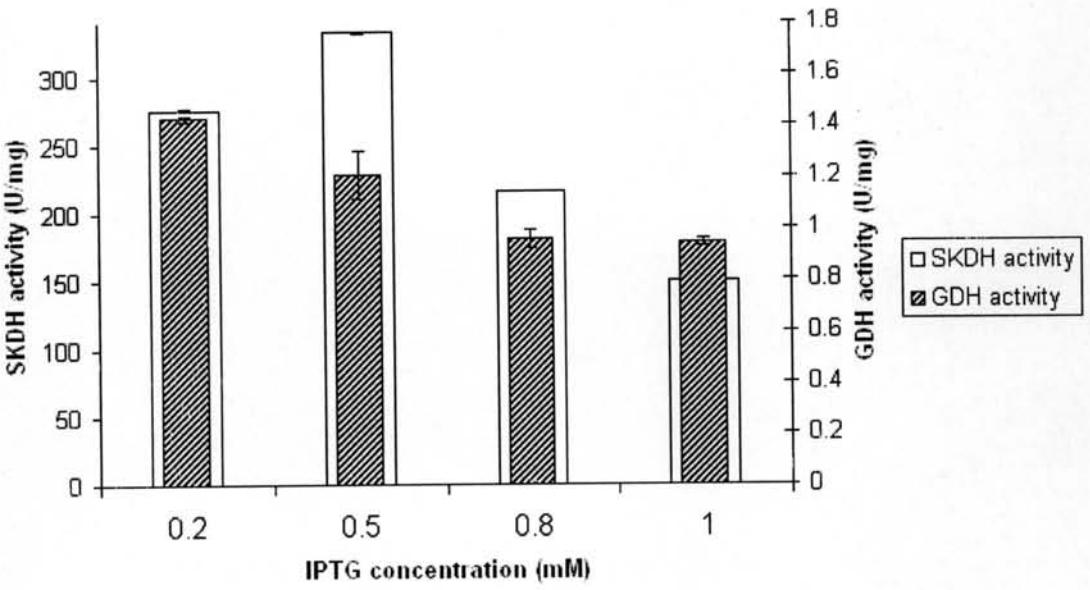


Figure 4.30 : SKDH and GDH activities of E. *coli* BL21 (DE3)/pET-GOX1959-GOX2015 induced with different IPTG concentrations

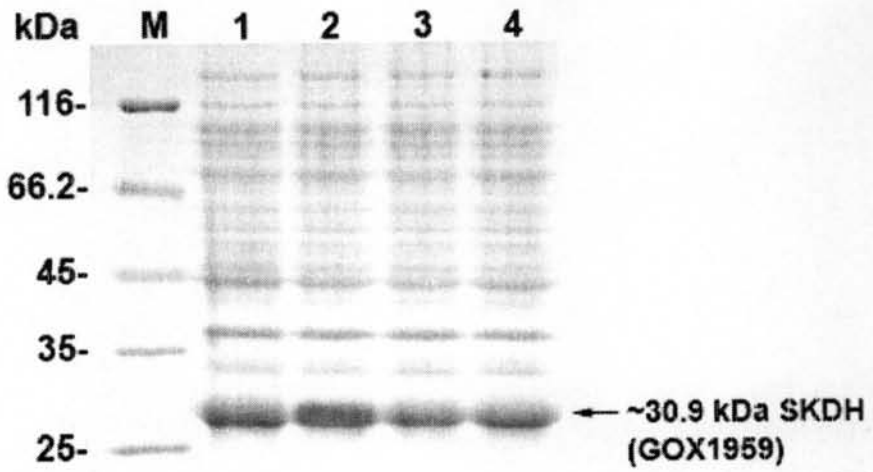


Figure 4.31 : SDS-PAGE analysis *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 cell free extract when induce with different IPTG concentrations

Lane M : Protein molecular weight marker

β-galactosidase	116 kDa
BSA	66.2 kDa
Ovalbumin	45 kDa
Lactate dehydrogenase	35 kDa
REase Bsp98I	25 kDa

Crude enzyme from a 100-ml cell culture induced with different IPTG concentrations:

Lane 1 : 0.2 mM IPTG (30 μg protein)

Lane 2 : 0.5 mM IPTG (30 μg protein)

Lane 3 : 0.8 mM IPTG (30 μg protein)

Lane 4 : 1.0 mM IPTG (30 μg protein)

4.13.3 Effect of induction temperature on SKDH and GDH activities

For the pET system, the proteins expression was induced by adding IPTG to the culture. Therefore, IPTG concentration should be optimized to obtain optimum expression condition. The *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 was cultured in an 100-ml LB medium at 28, 30, 34, 37 and 40°C, respectively. When OD_{600} reach 0.2, IPTG was added to the final concentration of 0.2 mM and cultivation was continued at each temperature for 5 hours (final OD_{600} of 1.2). Intracellular crude enzyme was prepared as described in section 3.12.3 and used to determine SKDH and GDH activity. Table 4.14 and Figure 4.32 show SKDH and GDH activity. The highest SKDH and GDH activity were obtained when cultured at 30°C and 37°C, respectively. Because $NADP^+$ was reproduced, the activity of GDH should be high enough. Therefore, the transformant was cultured at 37°C to obtain the highest GDH activity. Crude enzyme was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme was subjected to electrophoresis on 12.5% SDS-PAGE. From SDS-PAGE result in Figure 4.33, the intensity of major protein band was corresponded to the level of SKDH activity.

Table 4.14 SKDH and GDH activity of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 when grown at different temperatures

Temperature	Crude enzyme from *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015	
	SKDH activity (U/mg)	**GDH activity (U/mg)**
28°C	163.87±0.74	0.78±0.006
30°C	412.95±0.74	0.85±0.01
34°C	375.13±0.60	0.95±0.006
37°C	279.24±0.59	1.03±0.012
40°C	76.09±0.61	0.53±0.012

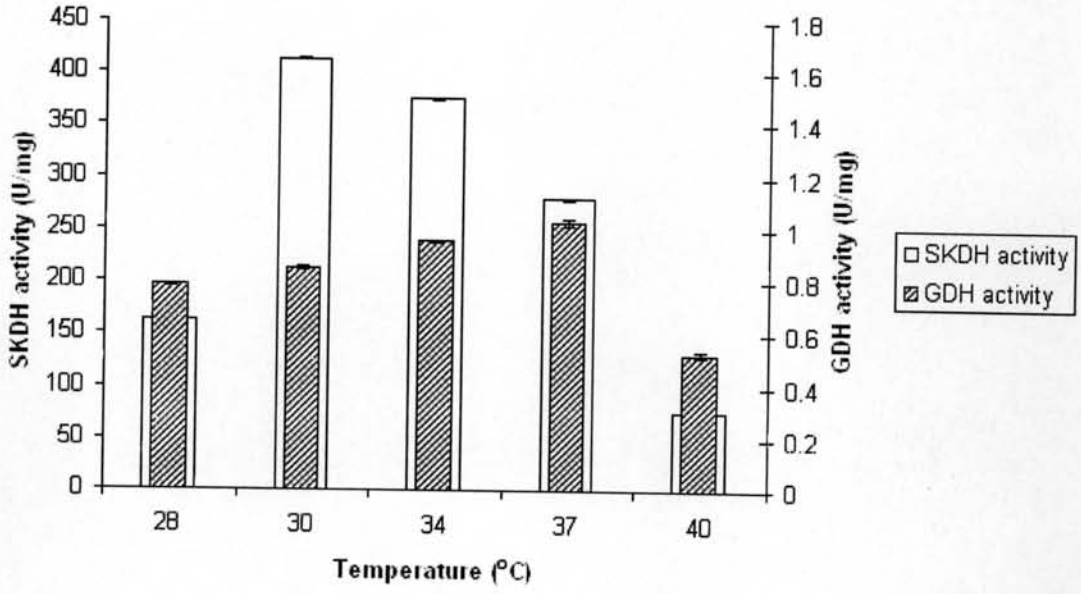


Figure 4.32 : SKDH and GDH activities of 100-ml cells induced with 0.2 mM IPTG and grown at different temperatures

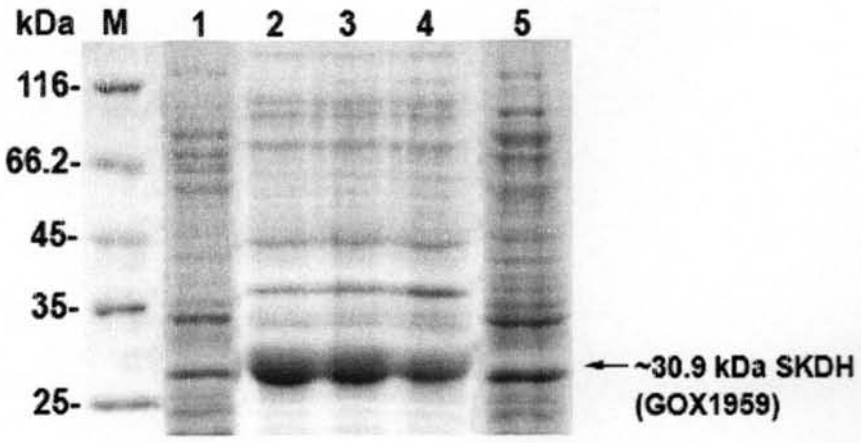


Figure 4.33 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 cell free extract when cultured at different temperatures

Lane M : Protein molecular weight marker

β-galactosidase	116 kDa
BSA	66.2 kDa
Ovalbumin	45 kDa
Lactate dehydrogenase	35 kDa
REase Bsp98I	25 kDa

Crude enzyme from a 100-ml cell culture induced with 0.2 mM IPTG when grown at different temperatures:

Lane 1 : 28°C (30 μg protein)

Lane 2 : 30°C (30 μg protein)

Lane 3 : 34°C (30 μg protein)

Lane 4 : 37°C (30 μg protein)

Lane 5 : 40°C (30 μg protein)

4.13.4 Effect of cell-growth stage on SKDH and GDH activities

In the cultivation process of recombinant E. coli, IPTG induction is the turning point of recombinant protein synthesis and can bring great change to cell growth and its metabolism(Zhong *et al.*, 2006). Then, the effect of cell-growth stage on SKDH and GDH activities was evaluated. The *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 was cultured in 100 ml LB medium at 37°C. When the turbidity of the culture at 600 nm reached 0.2 (early log phase), 0.5 (mid log phase), 1.0 (stationary phase) and 1.1 (stationary phase) (Appendix O), IPTG was added to final concentration of 0.2 mM and cultivation was continued at 37°C for 5 hours. Intracellular crude enzyme was prepared as described in section 3.12.3 and used to determine SKDH and GDH activities. Table 4.15 and Figure 4.34 show SKDH and GDH activity. The highest SKDH and GDH activity were obtained when cells at at OD_{600} =0.5400 were induced. Crude enzyme was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme was subjected to electrophoresis on 12.5% SDS-PAGE. From the result in Figure 4.35, the intensity of major protein band was corresponded to the level of enzyme activity.

Table 4.15 SKDH and GDH activities of cells at different stage of growth when induced with 0.2 mM IPTG and grown at 37°C in a 100-ml LB medium

OD_{600} and cell-growth stage	**Crude enzyme from *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015**	
	SKDH activity (U/mg)	**GDH activity (U/mg)**
OD_{600} =0.1894 (early log phase)	117.70±0.61	0.47±0.006
OD_{600} =0.5400 (mid log phase)	227.92±0.48	0.79±0.012
OD_{600} =1.007 (stationary phase)	137.09±0.73	0.60±0.006
OD_{600} =1.1270 (stationary phase)	90.34±0.97	0.49±0.010

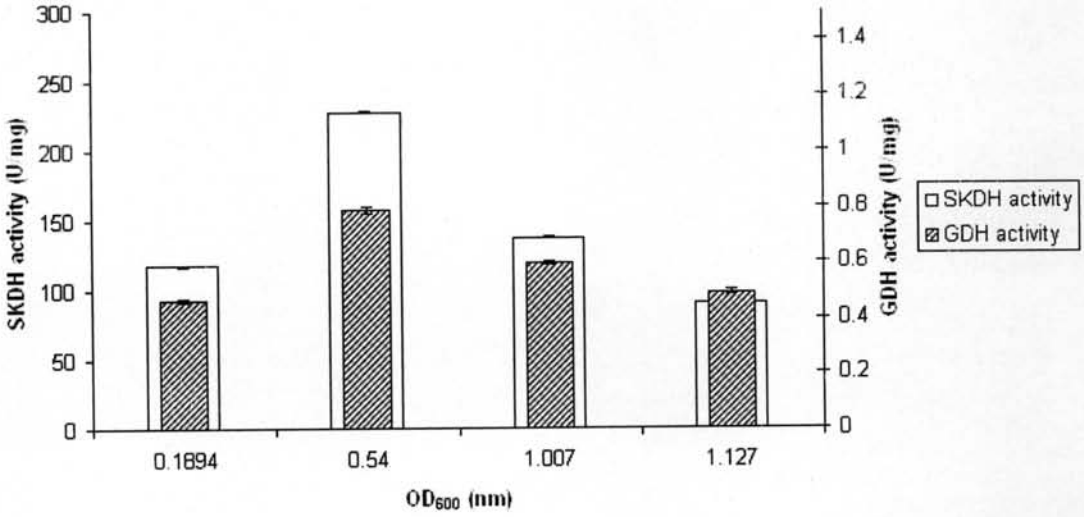


Figure 4.34 : SKDH and GDH activities of 100-ml cells at different stage of growth when induced with 0.2 mM IPTG and grown at 37°C

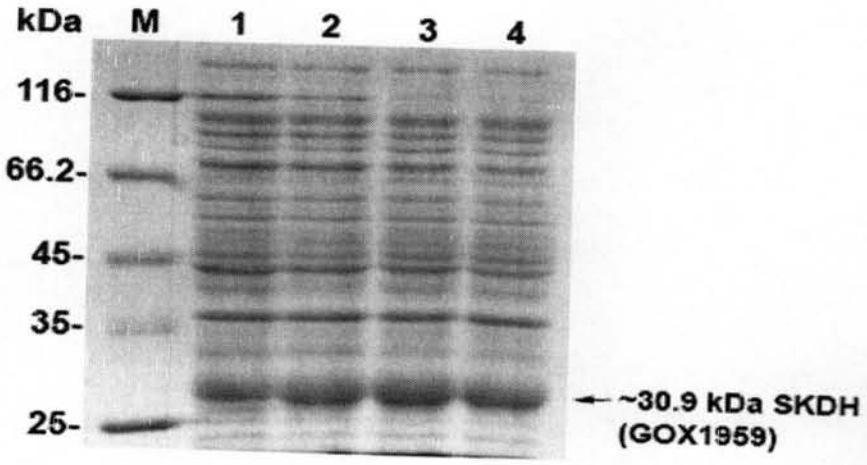


Figure 4.35 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX1959-GOX2015 cell free extract at different stage of growth

Lane M : Protein molecular weight marker

β-galactosidase	116 kDa
BSA	66.2 kDa
Ovalbumin	45 kDa
Lactate dehydrogenase	35 kDa
REase Bsp98I	25 kDa

Crude enzyme from cells at different stage of growth when induced with 0.2 mM IPTG and grown at 37°C in a 100-ml LB medium:

Lane 1 : OD_{600} = 0.1894 (30 μg protein)

Lane 2 : OD_{600} = 0.5400 (30 μg protein)

Lane 3 : OD_{600} = 1.007 (30 μg protein)

Lane 4 : OD_{600} = 1.1270 (30 μg protein)

4.14 Co-expression of pET-GOX1959 with pACGD vector in *E. coli* BL21 (DE3)

From previous report (Kataoka *et al.*, 1999), pACGD vector containing *gdh* gene (1-kb gene encoding) from *Bacillus megaterium* could be coexpressd with pKAR harboring aldehyde reductase in *E. coli* JM109. Therefore, pET-GOX1959 was co-expressed with pACGD in *E. coli* BL21 (DE3). Intracellular crude enzyme was prepared as described in section 3.18.3 and used to assay enzyme activity. From the result, SKDH activity was high but no GDH activity was observed (Table 4.16).

Intracellular crude enzyme and precipitate were analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme was subjected to electrophoresis on 12.5% SDS-PAGE. Since the expected size of GDH is approximately 30.4 kDa, the result in Figure 4.36 showed that most of the expressed GDH was included in inclusion body. It may be the expression system in *E. coli* BL21 (DE3) is not suitable for pACGD expression. Therefore, pACGD vector was expressed in *E. coli* JM109.

Table 4.16 SKDH and GDH activities of *E. coli* BL21 (DE3)/pET-GOX1959 co-expressed with pACGD

Samples	SKDH activity (U/mg)	GDH activity (U/mg)
E. coli BL21(DE3)	0.017±0.0006	Not detected
E. coli BL21(DE3)/pET-GOX1959/pACGD	65.79±0.18	Not detected

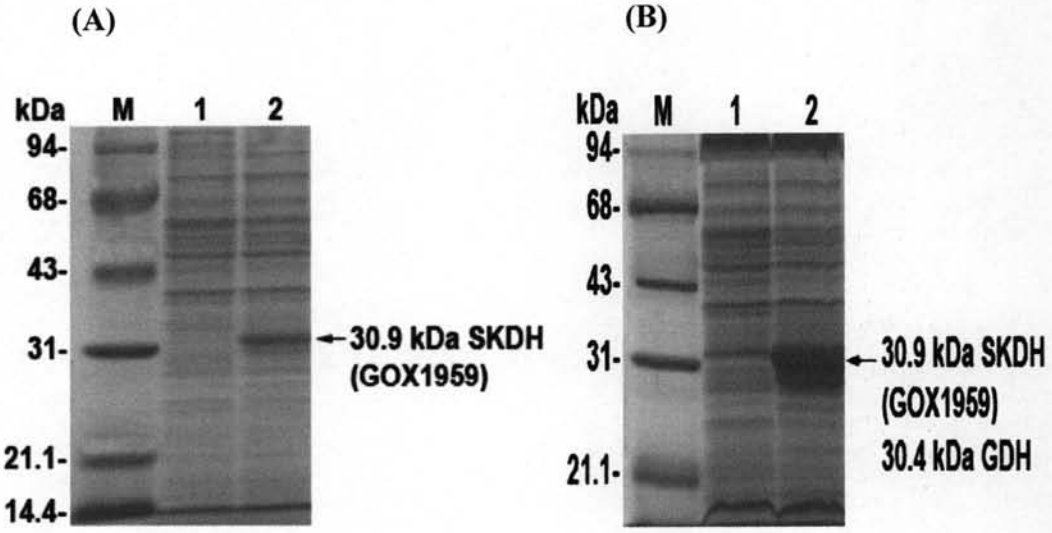


Figure 4.36 : SDS-PAGE analysis of *E. coli* BL21 (DE3)/pET-GOX1959/pACGD **(A)** cell free extract and **(B)** precipitate

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* BL21 (DE3) (30 µg protein)

Lane 2 : *E. coli* BL21 (DE3) /pET-GOX1959/pACGD (30 µg protein)

4.15 Expression of pACGD in *E. coli* JM109

pACGD vector was transformed into *E. coli* JM109 to dertermine GDH expression. The *E. coli* JM109/pACGD was cultured in 100 ml LB medium at 37°C. When the turbidity of the culture at 600 nm reached 0.2, IPTG was added to final concentration of 0.1 mM and cultivation was continued at 37°C for 6 hours. Intracellular crude enzyme was prepared as described in section 3.18.3 and used to determine GDH activity. From the result, the GDH activity was low as shown in Table 4.17. Intracellular crude enzyme and precipitate was analyzed by SDS-PAGE. Ten microliters of precipitates and 30 μg protein of crude enzyme were subjected to electrophoresis on 12.5% SDS-PAGE. From SDS-PAGE analysis result in Figure 4.37, it was found that most GDH were inclusion bodies.

Table 4.17 GDH expression of pACGD in *E. coli* JM109

Samples	GDH activity (U/mg)
E. coli JM109	Not detected
E. coli JM109/pACGD	0.14±0.006

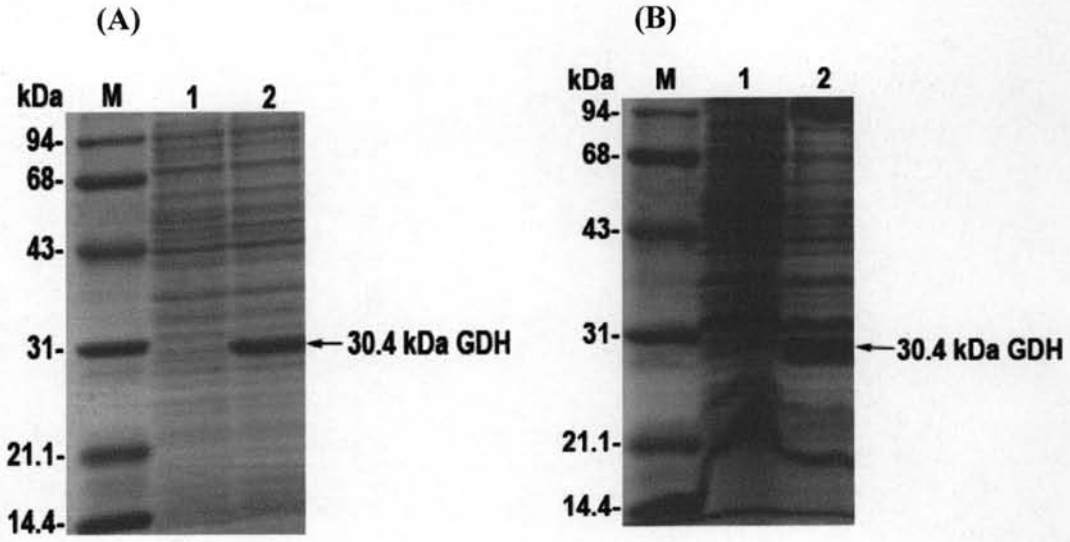


Figure 4.37 : SDS-PAGE analysis of *E. coli* JM109/pACGD **(A)** cell free extract **(B)** precipitates

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *E. coli* JM109 (30 µg protein)

Lane 2 : *E. coli* JM109/pACGD (30 µg protein)

4.16 Subcloning of *skdh* (GOX1959) gene into pSG8 vector

G. oxydans has an important role in several industries. Many compounds including shikimate are produced with *G. oxydans*. For efficient production, strain improvement by molecular technique has been done. In this experiment, pSG8 was chosen as an expression vector because it was constructed from E. coli vector pUC18 and *G. oxydans* vector pAG5. *skdh*' (GOX1959) gene including stop codon was amplified according to the method from section 3.23. To prepare the amplified gene product for insertion into pSG8, the 5'-end primer comprised of *Sac*I restriction site and 5' base of *skdh* (GOX1959) gene. The 3'-end primer comprised of *Xba*I restriction site and 3' base of *skdh* (GOX1959) gene were designed. The PCR products were analyzed on 0.8% agarose gel (Figure 4.38), the major band of *skdh* (GOX1959) PCR product was observed at 850 bps, respectively. After that the PCR products were ligated into pET-21a vector and the recombinant plasmids were sequenced. From sequencing result in Figure 4.39, the insert size *skdh* (GOX1959) gene was 859 bps (10 bp upstream region, 840 bp coding region, and 9 bp downstream region).

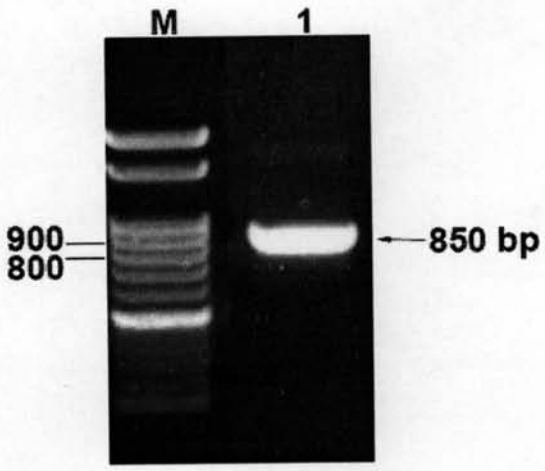


Figure 4.38 : *skdh* (GOX1959) PCR products for pSG8 on 0.8% agaraose gel

Lane M : 100 bps ladder

Lane 2 : *skdh* (GOX1959) PCR product

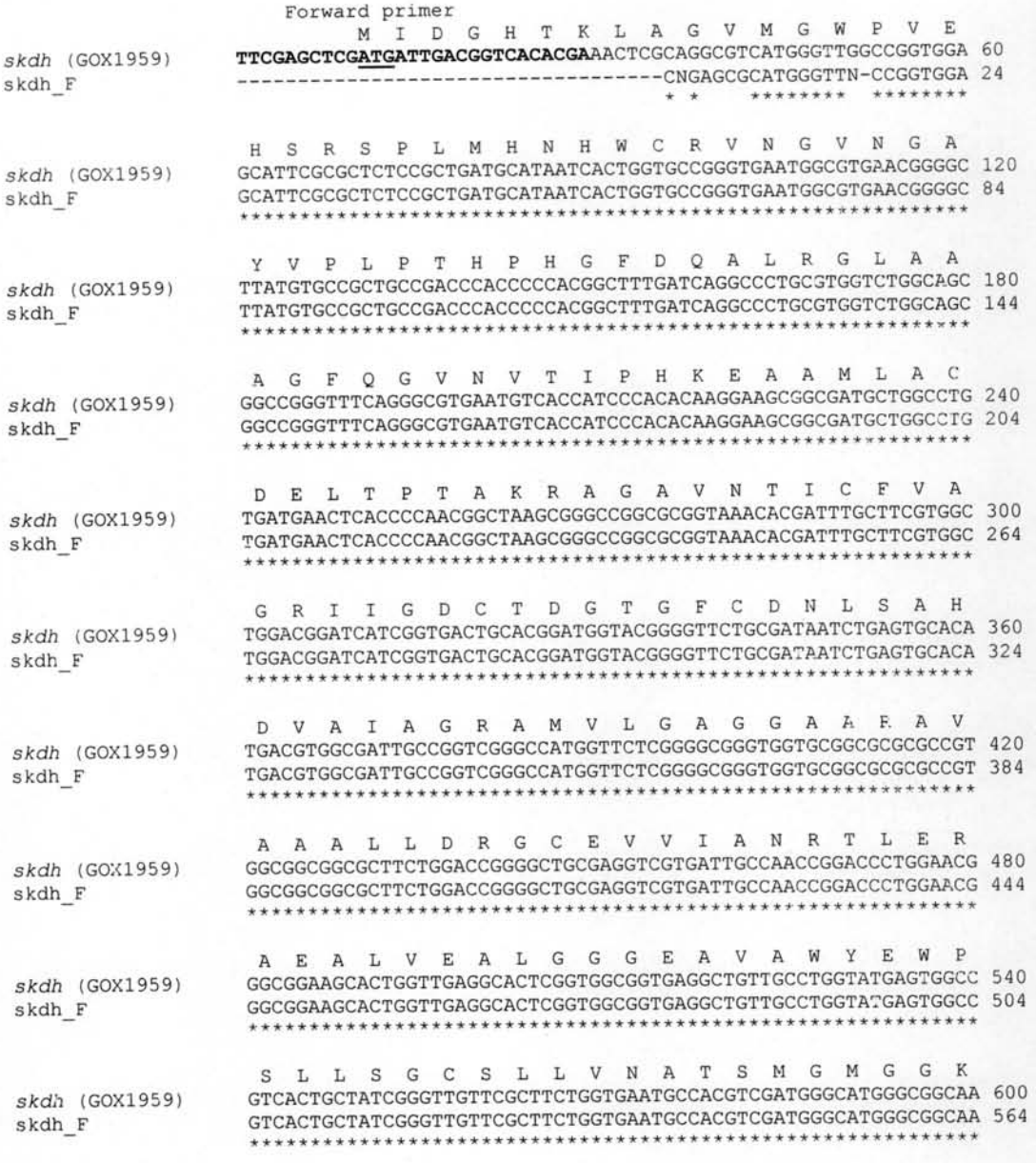


Figure 4.39 : Alignment of 859-bp *skdh* (GOX1959) PCR product for pSG8 sequencing result (skdh_F) with *skdh* (GOX1959) gene from *G. oxdans* 621H genome sequence. The deduced amino acid sequence is also shown.

```
                 A  G  L  D  W  D  A  A  L  R  E  A  A  P  G  L  C  V  T  D
skdh (GOX1959)   GGCTGGTCTGGACTGGGATGCGGCTCTTCGTGAGGCCGCGCCGGGCCTGTGCGTGACGGA 660
skdh_F           GGCTGGTCTGGACTGGGATGCGGCTCTTCGTGAGGCCGCGCCGGGCCTGTGCGTGACGGA 624
                 ************************************************************

                 I  V  Y  T  P  R  E  T  P  L  L  L  A  A  Q  A  R  G  L  R
skdh (GOX1959)   TATTGTCTACACGCCGCGCGAGACGCCGCTTCTGCTGGCCGCCCAGGCACGGGGACTGCG 720
skdh_F           TATTGTCTACACGCCGCGCGAGACGTCGCTTCTGCTGGCCGCCCAGGCACGGGGACTGCG 684
                 ************************* ********************************

                 T  V  D  G  L  G  M  L  V  H  Q  A  R  A  G  F  R  A  W  F
skdh (GOX1959)   GACCGTGGATGGTCTGGGGATGCTGGTTCATCAGGCGCGGGCCGGGTTTCGGGCATGGTT 780
skdh_F           GACCGTGGATGGTCTGGGGATGCTGGTTCATCAGGCGCGGGCCGGGTTTCGGGCATGGTT 744
                 ************************************************************

                 G  V  D  P  Q  A  D  R  T  T  F  D  L  L  A  A  S  L  R  T
skdh (GOX1959)   CGGCGTTGATCCGCAGGCCGACCGGACGACATTCGATCTGCTGGCGGCGAGCCTGCGCAC 840
skdh_F           CGGCGTTGATCCGCAGGCCGACCGGACGACATTCGATCTGCTGGCGGCGAGCCTGCGCAC 804
                 ************************************************************
                   Reverse primer
                  D  A
skdh (GOX1959)   TGACGCGTGATCTAGAGTC---------------------- 859
skdh_F           TGAN-CGTGATCTAAATCGACCTGCAGGCATGCAACTTTGAA 845
                 ***  ********* *
```

Figure 4.39 : (continue)

4.17 pSG8-GOX1959 expression in *G. oxydans* IFO3244

G. oxydans IFO3244 has a high quinate dehydrogenase (QDH) activity (Adachi *et al.*, 2003b). Moreover, the *G. oxydans* IFO3244 can produce dehydroshikimate in a higher yield when compare with another strains (Adachi *et al.*, 2003a). It may be possible to produce shikimate from quinate. Therefore, the *G. oxydans* IFO3244 was used as a host for pSG8-GOX1959 expression. The *G. oxydans* IFO3244/pSG8-GOX1959 was grown in glycerol medium with 100 μg/ml ampicillin for 26 hours. Intracellular crude enzyme was prepared as described in section 3.9.4 and used to assay SKDH activity. From the result in Table 4.18, SKDH activity in transformant was 10-fold higher than that of wild type.

Crude enzyme was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme was subjected to electrophoresis on 12.5% SDS-PAGE. From SDS-PAGE analysis result, although the SKDH activity in the transformant was significantly higher protein bands between the transformant and wild type was not different (Figure 4.40). Explanation was described in discussion (section 5.20).

Table 4.18 SKDH (GOX1959) activity expressed in *G. oxydans* IFO3244/pSG8-GOX1959

Samples	SKDH activity (U/mg)
G. oxydans IFO3244	0.052±0.001
G. oxydans IFO3244/pSG8-GOX1959	0.54±0.006

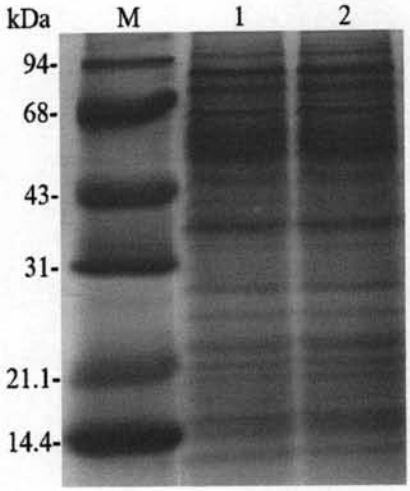


Figure 4.40 : SDS-PAGE analysis of *G. oxydans* IFO3244/pSG8-GOX1959 cell free extract

Lane M : Protein molecular weight marker

Phosphorylase	94	kDa
BSA	68	kDa
Ovalbumin	43	kDa
Carbonic anhydrase	31	kDa
Trypsin inhibitor	21.1	kDa
Lysozyme	14.4	kDa

Lane 1 : *G. oxydans* IFO3244 (30µg protein)

Lane 2 : *G. oxydans* IFO3244/pSG8-GOX1959 (30µg protein)

4.18 Effect of IPTG to pSG8-GOX1959 expression in *G. oxydans* IFO3244

G. oxydans IFO3244 harboring pSG88-GOX1959 was grown in glycerol medium at 30°C. When the turbidity of the culture at 600 nm reached 0.3, IPTG was added to final concentration of 0.2 mM and cultivation was continued at 30°C for 26 hours (to the stationary phase). Intracellular crude enzyme was prepared as described in section 3.9.4 and used to determined SKDH activity. The SKDH activity is shown in Table 4.19, IPTG induction did not affect to protein expression in *G. oxydans* IFO3244. Crude enzyme was analyzed by SDS-PAGE. Thirty micrograms protein of crude enzyme was subjected to electrophoresis on 12.5% SDS-PAGE. From the result in Figure 4.46, the protein bands between wild type and transformant were not different.

Table 4.19 : SKDH (GOX1959) activity expressed in *G. oxydans* IFO3244/pSG8-GOX1959 when induced with 0.2 mM IPTG

Samples	SKDH activity (U/mg)
No IPTG induction	0.79±0
0.2 mM IPTG induction	0.79±0

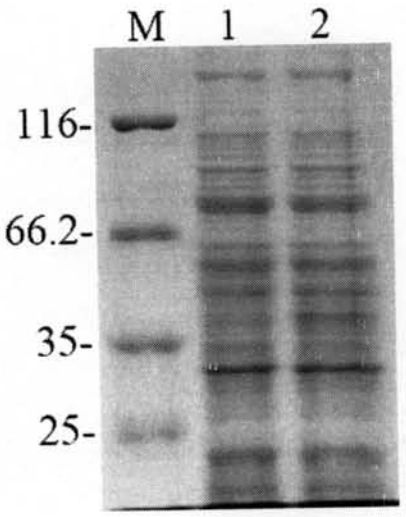


Figure 4.41 : SDS-PAGE analysis of cell-free extract of *G. oxydans* IFO3244/pSG8-GOX1959 with and without IPTG induction

Lane M : Protein molecular weight marker

β-galactosidase	116 kDa
BSA	66.2 kDa
Ovalbumin	45 kDa
Lactate dehydrogenase	35 kDa
REase Bsp98I	25 kDa

Lane 1 : no IPTG induction (30 μg protein)

Lane 2 : 0.2 mM IPTG (30 μg protein)