CHAPTER V

DISCUSSION

Shikimate and dehydroshikimate have important roles in industries, therefore the development of their production is necessary. An effective method for the production of shikimate and dehydroshikimate has been studied and developed. Molecular cloning is one of effective methods that has been used to produce new clones of bacteria with shikimate and dehydroshikimate-producing properties. This work aims at cloning of *dqd* and *skdh* genes from *G. oxydans* 621H, homologusly and heterologously overexpressing these genes by using suitable expression vectors and optimizing the expression conditions.

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

PCR method is used for *in vitro* synthesis the interesting gene or gene fragment. This method requires a pair of primers which is specific to the target gene. In this experiment, a pair of primer was designed by using nucleotide sequence from *G. oxydans* 621H genome database. Because the genomic DNA is large, three genes were cloned into pGEM-T Easy vector to prevent non-specific product from 5'-restriction site of primer binding. The PCR result (Figure 4.1) shows that non-specific band was rarely produced, suggesting that primers were specific to *dqd*, *skdh* (GOX0859) and *skdh* (GOX1959) genes from *G. oxydans* 621H. From sequencing result in Figure 4.2-4.4, nucleotide sequences of PCR product (620 bp for *dqd*, 971 bp for *skdh* (GOX0859) and 863 bp for *skdh* (GOX1959)) were amplified with no mutation from the genomic DNA of *G. oxydans* 621H.

5.2 Subcloning of *dqd* and two of *skdh* genes into pET-21a expression vector

Expression systems were designed to produce many copies of a desired protein within a host cell. In order to accomplish this, the expression vector containing all of the genetic coding necessary to produce the protein, including an appropriate promoter to the host cell, a sequence which terminates transcription, and a sequence which codes for ribosome binding was inserted into a host cell. The pET expression system was developed in 1986 (Studier and Moffatt, 1986). It is widely used because of its ability to mass-produce proteins, the specificity involved in the T7 promoter which only binds T7 RNA polymerase, and also the design of the system which allows for the easy manipulation of how much of the desired protein is expressed and when that expression occurs. Moreover, 6xHis-tag is facilitates purification of the target protein. Therefore, pET-21a vector (Appendix E) was selected to express *dqd* and two of *skdh* genes. *E. coli* BL21 (DE3) was used for host expression because this strain has gene encoding bacteriophage T7 RNA polymerase, which is integrated into the chromosome of BL21. To insert *dqd* and two of *skdh* gene fragments into pET-21a at the right position, the gene fragment was amplified with the 5' end primer containing *Nde*I restriction site and 3' end primer that containing *Xho*I restriction site.

Nucleotide and deduced amino acid sequence of PCR products were compared with nucleotide and deduced amino acid sequence of two of *skdh* genes from *G. oxydans* 621H genome database to check any error, which may occur from DNA polymerase. From sequencing result in Figure 4.6-4.8, nucleotide and deduced amino acid sequence of *dqd* and two of *skdh* gene fragments PCR product of 489, 855 and 846 bps, respectively were similar to that of the database. The difference between

gene fragment in pGEM-T Easy (section 5.1) and the subcloned fragment in pET-21a was the smaller gene fragment containing only open reading frame was cloned into pET-21a and the stop codon of three genes was removed to express His-tag on C-terminal.

5.3 Gene expression in *E. coli* BL21 (DE3)

Since the gene fragments did not have their own promoter, they were expressed under T7*lac* promoter on the pET-21a vector. In the pET system, T7 RNA polymerase was under the control of *lac*UV5 promoter, and the plasmid vector equipped with a bacteriophage T7*lac* promoter upstream of the gene. Both promoters contain the *lac* operator (*lac*O) in such position that binding of a *lac* repressor to the operator site blocks transcription. IPTG can bind to the repressor which results in the transcription of T7 RNA polymerase. Therefore, the target gene in the vector was transcribed by adding IPTG. A final concentration of 1 mM IPTG is recommended for full induction with vector having T7*lac* promoter (Novagen, 2003).

5.3.1 Expression of *dqd* gene in *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. From Table 4.1, the DQD activity of the transformant was 2.61 μmole/min.mg protein. This activity is much higher (~37 fold) than DQD activity (0.07 μmole/min.mg protein) previously reported in a recombinant *E. coli.* AB2848/pUC18-*dqd* derived from *Helicobactor pyroli* (Bottomley *et al.*, 1996). However, the expressed DQD in our case at 37°C resulted in inclusion body according to the SDS-PAGE (Figure 4.9). From previous report, increased growth temperature is one parameter that promotes inclusion body formation caused by

hydrophobic interaction between protein chains. A higher temperature increases hydrophobic interaction and might also expose hydrophobic stretchs of amino acid that are normally exposed (Strandberg and Enfors, 1991). The expression at low temperature (30°C) was carried out in order to decrease the inclusion body. The DQD activity of the transformant when expressed at 30°C was as high as 10.80 U/mg (Table 4.2) and more soluble. From SDS-PAGE result, the inclusion body formation was decreased (Figure 4.10).

5.3.2 Expression of *skdh* genes in *E. coli* BL21 (DE3)

In 2005, *G. oxydans* 621H genome sequence was done (Prust *et al.*, 2005). Two genes, *skdh* (GOX0859) and *skdh* (GOX1959), were annotated as shikimate dehydrogenase (SKDH). The *skdh* (GOX0859) gene consisting of 849 bp (282 amino acid) and the *skdh* (GOX1959) gene consisting of 840 bp (279 amino acid). Amino acid sequence identity of *skdh* (GOX0859) and *skdh* (GOX1959) is 27%. In *E. coli*, it has two types of SKDH, AroE and YdiB. It may be possible that the *G. oxydans* has two types of SKDH. Therefore, *skdh* (GOX0859) and *skdh* (GOX1959) genes were cloned and overexpressed to prove this assumption.

5.3.2.1 Expression of *skdh* (GOX0859) gene in *E. coli* BL21 (DE3) and purification of SKDH (GOX0859) using Ni-NTA agarose column chromatography

The *E. coli* BL21 (DE3)/pET-GOX0859 was cultured on a shaking incubator at 37°C until OD_{600} reach 0.2 and, then induced by 1mM IPTG for 5 hours. From activity result, the SKDH activity of the transformant (0.047 μmole/min.mg protein) and of *E. coli* BL21 (DE3) (0.043 μmole/min.mg protein) were not different. The molecular weight of SKDH (GOX0859) from the deduced amino acid including

6xHis-tag (30.7 kDa) was different from SDS-PAGE analysis (36.3 kDa). Molecular weight determinations based solely on SDS-PAGE may be misleading, since some proteins migrate anomalously (Bollag *et al.*, 1996). Nonetheless, from SDS-PAGE result, most of the expressed protein was inclusion body. The inclusion body formation is might be caused by hydrophobic interaction between protein chain (Strandberg and Enfors, 1991) as described in section 5.3.1. Moreover, decreasing the expression level by lowering the concentration of inducer may enchanced the soluble yield of some target proteins (Novagen, 2003). Therefore, the transformant was cultured at lower temperature, 30°C. IPTG concentration was decreased to 0.5 mM. From the activity result, the SKDH activity of the transformant when cultured at 30°C was not different from that of the *E. coli* BL21 (DE3). From SDS-PAGE result, most of the expressed SKDH (GOX0859) was still formed as inclusion body. Therefore, the transformant was culture at 20°C to decrease inclusion body formation. From the activity result, the SKDH activity of the transformant when culture at 20°C was still similar to that of the *E. coli* BL21 (DE3). The SDS-PAGE analysis showed that expression level at 20°C was lower than that of 30°C. As a possible reason for these results is a slowed transcription and translation machinery of *E. coli* at low temperatures (Pflug *et al.*, 2007). Therefore, an efficient protein expression vector based on the low-temperature expression promoter gene of *E. coli*, pCold I vector (Takara), was used.

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

In pCold I system, gene was expressed under the control of *csp*A promoter, which derived from cold-shock gene promoter. At the downstream of the *csp*A promoter, *lac* operator is inserted so that the expression can induce by adding 0.1 mM IPTG. Moreover, it has 6xHis-tag on N-terminal. After SKDH activity assay, SKDH activity of the transformant (0.063 U/mg protein) and the *E. coli* BL21 (DE3) (0.054 U/mg protein) was not much different. From SDS-PAGE result in Figure 4.13, almost all of the expressed SKDH (GOX0859) was still formed as inclusion body. From previous report, *E. coli* OrigamiB/pCold III-*Cal*B, harboring lipaseB gene from *Candida antarctica* (CalB), has a lipase activity 5-fold (11μg/mg) higher than that of *E. coli* Origami2 (DE3)/pET32-*Cal*B (Liu *et al.*, 2006). The pCold III vector is only different from pCold I vetor in lacking 6xHis-tag. The cause of inclusion body formation in our case may be the internal microenvironment of *E. coli* cells which may differ from the original source of the gene. Mechanisms for folding a protein may also be absent, and hydrophobic residues that normally would remain buried may be exposed and available for interaction with similar exposed sites on other proteins. It has been reported that chaperone co-expression may help protein to fold correctly (Schrodel *et al.*, 2005).

Co-expression of skdh (GOX0859) with chaperone vector, pG-KJE8

The *E. coli* BL21 (DE3)/pCold I-GOX0859 was co-expressed with pG-KJE8 chaperone vector. The pG-KJE8 vector is express DnaK, DnaJ, GrpE, GroES and GroEL chaperone protein. In *E. coli*, it has two molecular chaperone machines, DnaK-DnaJ-GrpE and GroEL-GroES. DnaK and its co-factors, DnaJ and GrpE, have

been proposed to interact with nascent polypeptides and to either directly facilitate the proper folding of the newly synthesized proteins or to mediate their transfer to the GroEL-GroES chaperonins system (Thomas and Baneyx, 1996). From the SKDH activity result in Table 4.6, the *E. coli* BL21 (DE3)/pCold I-GOX0859/pG-KJE8 showed very low SKDH activity which was fairly similar to that of the *E. coli* BL21 (DE3). It is indicated that the *skdh* (GOX0859) gene may not be the gene encoding for shikimate dehydrogease. From the SDS-PAGE result, it showed that the expressed protein was more soluble (Figure 4.14). From previous report, *E. coli* OrigamiB/pCold III-*Cal*B, harboring lipaseB gene from *Candida antarctica* (CalB) was co-expressed with pG-KJE8 chaperone vector. A lipase activity was 1.6-fold (18 μg/mg) higher than that of *E. coli* OrigamiB/pCold III-*Cal*B (Liu *et al.*, 2006).

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

Immobilized-metal affinity chromatography was used to purify proteins using the chelating ligand nitrilotriacetic acid (NTA). NTA occupies four of the six ligand binding sites in the coordination sphere of the Ni^{2+} ion, leaving two sites free to interact with the 6xHis-tagged recombinant protein. The SKDH (GOX0859) was eluted from column by 50 mM sodium phosphate buffer pH 8.0 with 250 mM imidazole buffer containing 0.5 M NaCl. Each fraction was measured absorbance at 280 nm and determined SKDH activity. From the result, SKDH activity was not observed in any fraction. Therefore, protein peak in fraction number 3 and 4 were pooled and analyzed by SDS-PAGE. From SDS-PAGE result, the expressed SKDH (GOX0859) could be detected, although there were unwanted proteins remained. According to *G. oxydans* 621H genome database, SKDH (GOX0859)

consisting of 282 amino acid has a molecular weight at 29.6 kDa which similar to AroE (29.4 kDa). However, in this study the expected molecular weight of SKDH (GOX0859) from *E. coli* BL21 (DE3)/pCold I-GOX0859 was 33.1 kDa. Since *skdh* (GOX0859) was inserted 48 bp downstream of the start codon of pCold I vector and the stop codon of this gene was removed, protein translation was initiated at the start codon of the vector and terminated by using stop codon of the vector (48 bp and 45 bp downstream of the gene). Therefore, the SKDH expressed from *E. coli* BL21 (DE3)/ pCold I-GOX0859 was larger than that of *E. coli* BL21 (DE3)/ pET-GOX0859. The molecular weight of SKDH (GOX0859) from the molecular weight standard curve was larger than expected molecular weight. According to Bollag *et al.* (1996), the molecular weight determination using SDS-PAGE colud be misleading due to anomalous protein migration.

5.3.2.2 Expression of *skdh* (GOX1959) gene in *E. coli* BL21 (DE3) and purification of SKDH (GOX1959) by Ni-NTA agarose column chromatography

The *E. coli* BL21 (DE3)/pET-GOX1959 was cultured on a shaking incubator at 37°C until OD_{600} reached 1.0. Without induction by IPTG, the expression of *skdh* (GOX1959) was occurred because there was some expression of T7 RNA polymerase from the *lacUV5* promoter in the λDE3 lysogen from *E. coli* genome (Novagen, 2003). The transformant showed an activity with shikimic acid and $NADP^+$ at 92.49 μmole/min.mg protein. This SKDH expression is comparatively higher than that previously reported in *Mycobacterium tuberculosis* H37Rv using pET-23a vector (0.46 μmole/min.mg protein) (Fonseca *et al.*, 2006). The condition for *E. coli* BL21 (DE3)/pET23-*skdh* expression was grown for 24 hour at 37°C without IPTG induction. However, from SDS-PAGE analysis, some of SKDH (GOX1959) was partly formed inclusion body.

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

The SKDH (GOX1959) was purified by Ni-NTA agarose column chromatography. Purification fold and percent yield of SKDH from pET-GOX1959 were 7.33 fold and 37.59% yield. In 2006, *M. tuberculosis* H37Rv SKDH from *E. coli* BL21 (DE3)/pET23a-*skdh* was purified for several steps, having the purification fold and percent yield of 8.5 fold and 8.9% yield. Purified SKDH (GOX1959) was determined molecular weight by SDS-PAGE. The molecular weight of SKDH (GOX1959) including 6xHis-tag from the calibration curve (30.9 kDa) was nearly with the approximate molocular weight from deduced amino acid (30.2 kDa) while that of *E. coli* BL21 (DE3)/pET23a-*skdh* was 27 kDa. The molecular weight of SKDH (GOX1959) from deduced amino acid without 6xHis-tag was 29.1 kDa. In *E. coli*, two types of SKDH, AroE and YdiB, molecular weight were 29.4 kDa and 31.2 kDa, respectively.

Determination of kinetic parameters of SKDH (GOX1959)

SKDH activity was employed to determine the kinetic parameters. Shikimic acid and $NADP^+$ were used as the substrates as described in section 3.14. The initial reaction rate of the purified SKDH (GOX1959) was measured at various concentrations of each substrate and the results were analyzed by using Lineweaver-Burk plot. Table 5.1 summarizes kinetic parameters of purified SKDH (GOX1959) from *E. coli* BL21 (DE3)/pET-GOX1959 obtained in this study and compared with AroE (EC 1.1.1.25), YdiB (EC 1.1.1.282) from *E. coli*, SKDH-L from *Heamophilus influenzae* and SKDH from *M. tuberculosis* H37Rv (Michel *et al.*, 2003, Singh *et al.*, 2005, Fonseca *et al.*, 2006). The SKDH (GOX1959) had high affinity with $NADP^+$.

This result was similar to AroE from *E. coli*, SKDH-L from *H. influenzae* and SKDH from *M. tuberculosis* H37Rv. From k_{cat} value, the SKDH (GOX1959) had the highest k_{cat} value for $NADP^+$ and shikimic acid. This high k_{cat} value can be attributed to substrate and cofactor specificity, indicating that shikimate and $NADP^+$ may be the biological substrate and cofactor for SKDH (GOX1959). The deduced amino acid of SKDH (GOX1959), AroE, YdiB, SKDH-L and SKDH from M. *tuberculosis* H37Rv were aligned and percent similarity was shown in Table 5.2. From the kinetic parameter and percent similarity result, the SKDH (GOX1959) had the highest identity with AroE (35%).

Table 5.1 Kinetic parameters of shikimate dehydrogenases from different microorganisms

Substrate	**This study**		**(Michel *et al.*, 2003)**		**(Michel *et al.*, 2003)**		**(Singh *et al.*, 2005)**		**(Fonseca *et al.*, 2006)**	
	SKDH (GOX1959)		**AroE**		**YdiB**		**SKDH-L**		**SKDH from *M. tuberculosis* H37Rv**	
	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
$NADP^+$ at shikimate saturation	52.6	1.16×10^5	56	1.42×10^4	100	7	37	0.2	22	5.9
NAD^+ at shikimate saturation	-	-	-	-	87	3	-	-	-	-
$NADP^+$ at quinate saturation	-	-	-	-	500	3	-	-	-	-
NAD^+ at quinate saturation	-	-	-	-	116	3	-	-	-	-
Shikimate at $NADP^+$ saturation	170	8.47×10^5	-	1.42×10^4	120	7	234	0.2	50.18	8.2
Quinate at $NADP^+$ saturation	-	-	-	-	555	3	-	-	-	-
Shikimate at NAD^+ saturation	-	-	-	-	20	3	-	-	-	-
Quinate at NAD^+ saturation	-	-	-	-	41	3	-	-	-	-

Table 5.2 %Identity of shikimate dehydrogenases from different microorganisms

SKDH type	**%Identity**			
	AroE	**YdiB**	**SKDH-L**	**SKDH from *M. tuberculosis* H37Rv**
SKDH (GOX0859)	24	28	21	24
SKDH (GOX1959)	35	31	29	31
AroE	-	27	22	27
YdiB	24	-	27	28
SKDH-L	22	27	-	23
SKDH from *M. tuberculosis* H37Rv	27	28	23	-

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

To inserted *gdh* gene into pET-GOX1959, the gene fragment was amplified with the 5' end primer and 3' end primer containing containing *Xho*I restriction site. The gdh PCR pruducts were ligated into pET-GOX1959. After ligation, the recombinant plasmid was sequenced to check error which may occur from DNA polymerase and *gdh* direction. Nucleotide and deduced amino acid sequence of PCR products were compared with nucleotide and deduced amino acid sequence of *gdh* gene from *G. oxydans* 621H genome database. From sequencing result in Figure 4.25, nucleotide and deduced amino acid sequence of 875-bp *gdh* gene fragments PCR product (29 bp upstream region, 801 bp *gdh* gene, 45 bp downstream) were as same as that in the database.

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

From SKDH reaction : shikimate + $NADP^+ \rightarrow$ 3-dehydroshikimate +NADPH + H^+, shikimate and $NADP^+$ molar ratio is 1:1. k_{cat} value (turn over number) is equivalent to the number of substrate molecules converted to product in a given number of time (Lehninger *et al.*, 2000). From Table 4.10, k_{cat} values for shikimate and $NADP^+$ were 8.74 x 10^5 and 1.16 x 10^5 min^{-1}, respectively. The activity result showed that the GDH activity (0.55 µmole/min.mg protein) was comparatively low when compared with SKDH activity (109.04 µmole/min.mg protein). Therefore, $NADP^+$ regeneration may not be sufficient. In bacteria, the controls for translational efficiency are exerted through processes such as initiation elongation and termination. The initiation of translation is one of the major rate-limiting steps in protein synthesis under normal situations (Thanaraj and Pandit, 1989). For bacterial mRNA, ribosome binding site and start codon play important roles for translation initiation. Base pairing interactions between an mRNA's Shine-Dalgano sequence (ribosome binding site) and the 16S rRNA apparently permit the ribosome to select the proper initiation codon (Voet and Voet, 1995). Because the *gdh* gene was cloned from 23 base pairs upstream of start codon, the *gdh* gene fragment consists of the ribosome binding site of *G. oxydans* 621H. In transcription process, *skdh* (GOX1959) and *gdh* genes were co-transcribed in the same mRNA strand. However, these two genes were translated to different protein strands. The *skdh* gene was translated by using pET-21a vector ribosome binding site while the *gdh* gene was translated by using *G. oxydans* 621H ribosome binding site. This may affected to base pairing interactions between an mRNA's ribosome binding site and the 16S rRNA resulting the decrease of GDH translation. Therefore, the GDH activity was low. From previous report, *E. coli* BL21

(DE3)/pET28-P450 BM3-*gdh* harboring P450 monooxygenase and *gdh* gene from *Bacillus megaterium*, respectively were co-expressed. Under the optimized conditions, when grown in a 25-ml LB medium at 30°C and induced with 0.5 mM IPTG at the mid stage of the log phase, the maximum P450 BM3 and GDH activities amounted to 8173.13 pmole/min.mg protein and 0.045 μmole/ min.mg protein, respectively. The highest yield (2.9 mM) of indigo was obtained after 8-hour incubation when the substrate (indole) concentration was 5 mM while that of *E. coli* BL21(DE3)/pET28-P450 BM3 was only 0.001 mM when using 0.5 mM indole (Lu and Mei, 2007). From another report, plasmid pQE30-gdh223 harboring *gdh* gene from *B. megaterium* AS1.223 and pQE30-ALR harboring NADPH-dependent aldehyde reductase gene were co-expressed in *E. coli* M15. The highest GDH activity (58.7 μmole/min.mg protein) was obtained when culture in a rich medium (MMBL) at 37°C and induced with 0.3 mM IPTG at late log phase. The yield of R-CHBE catalyzing by ALR reach 100% when *E. coli* M15/ pQE30-gdh223/ pQE30-ALR was used (Xu *et al.*, 2007).

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

5.6.1 Effect of medium volume

The medium volume is an important parameter reflecting the dissolved oxygen level in flask, and can influence the metabolic process of the cell and hence influence the target proteins expression (Lu and Mei, 2007). To study the effect of medium volume on the enzymes expression, cultivation was carried out in a 250-ml flasks with various LB medium volume (25, 50, 75, 100 and 125 ml). From the activity result in Table 4.12, the highest SKDH and GDH activity were obtained when the medium volume was 100 ml. This result indicated that the optimal culture volume was 100 ml in which dissolved oxygen was sufficient for both cell growth and expression of

fusion protein. It was also seen that the 25 ml medium volume could not improve but even reduced the expression level. One possible reason was that the fermentation broth would become very thick due to water evaporation when very little media were in the flask, which might bring about some inhibition effects on cell growth and protein expressions (Zhong *et al.*, 2006). On the other hand, *E. coli* BL21 (DE3)/pET28-P450 BM3-*gdh* has highest activities when cultured in 25-ml LB medium (Lu and Mei, 2007). It is indicated that gene expression in this system required high amount of dissolved oxygen.

5.6.2 Effect of IPTG concentration

For the pET system, the proteins expression was induced by adding IPTG to the culture. Final IPTG concentration should be optimized because of its great contribution to recombinant protein expression and potential harm to cell growth (Lu and Mei, 2007). In this study, IPTG was added to final concentration of 0.2, 0.5, 0.8 and 1.0 mM, respectively. From the activity result in Table 4.13, the highest SKDH and GDH activity were obtained when induced with 0.5 and 0.2 mM, respectively. However, the GDH activity was not significantly when induced with different IPTG concentrations. At high IPTG concentration SKDH and GDH activity were decreased because high IPTG concentration may be harm to cell growth resulting in gene expression inhibition (Zhong *et al.*, 2006). In *E. coli* BL21 (DE3)/pET28-P450 BM3-*gdh* expression, the highest specific activities were obtained when induced with 0.5 mM IPTG (Lu and Mei, 2007).

5.6.3 Effect of induction temperature

Temperature is an important factor in recombinant protein expression. The transformant was cultured in a 100-ml LB medium at 28, 30, 34, 37 and 40°C,

respectively. From activity result in Table 4.14, the GDH activity was not significantly different when cells were grown at temperature ranging from 28°C to 37°C while the highest SKDH activity was obtained at 30°C. Nonetheless, the SKDH and GDH activity were decreased significantly at 40°C since The *E. coli* cells can not grew well at 40°C (Wen *et al.*, 2005). Then, protein expression was decreased significantly. On the other hand, the favorable for the cells growth (37°C for *E. coli*) resulted in the production of large amounts of nascent protein. The formation of large amounts of expressed protein in a short period of time may effect to protein folding and formed aggregates of an inactive form of enzyme (Wen *et al.*, 2005). From the *E. coli* BL21 (DE3)/pET28-P450 BM3-*gdh* expression(Lu and Mei, 2007), the specific activity of P450 BM3 was highest and the specific activity of GDH was lower than that at 25°C very little. However, at a higher temperature, both the specific activities of P450 BM3 and GDH decreased. Therefore, the optimal temperature was 30°C.

5.6.4 Effect of cell-growth stage

In the process of recombinant protein production in *E. coli*, IPTG induction is the turning point between cell growth and recombinant protein synthesis. The addition of IPTG means the beginning of the transcription of the foreign gene on the plasmid and can bring great changes to the metabolism of host cell by initiating the translation of heterologous protein (Lu and Mei, 2007). From the activity result in Table 4.15, the highest SKDH and GDH activities were obtained when induced at OD_{600} = 0.5400 (mid log phase). The SKDH and GDH activities were decreased when induced at OD_{600} = 1.1270 (stationary phase). It may be from the increase of cell density and the viability of induced *E. coli* cells was not balanced (Zhong *et al.*, 2006). The *E. coli* BL21(DE3)/pET28-P450 BM3-*gdh* expression in previous report showed that highest

expression of P450 BM3 and GDH in *E. coli* BL21 was obtained when the induction timing was set at the mid stage of the log phase (Lu and Mei, 2007).

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

The pET-GOX1959 was co-expressed with compatible plasmid pACGD harboring *gdh* gene from *Bacillus megaterium* in *E. coli* BL21 (DE3). From the activity result, only the SKDH activity (65.79 U/mg) was observed. After SDS-PAGE analysis, most of GDH was in precipitates. From previous report, pACGD vector could be co-expressed with pKAR harboring aldehyde reductase (AR) in *E. coli* JM109. The GDH activity was 16 µmole/mg while the AR activity was 1.12 µmole/mg (Kataoka *et al.*, 1999). It is indicated that the expression system in *E. coli* BL 21(DE3) may not suitable for pACGD expression.

Expression of pACGD in E. coli JM109

The transformant was cultured in LB medium at 37°C. When the OD_{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. After GDH activity assay,it was showed that the GDH activity was low (0.14 U/mg). From SDS-PAGE result, most of GDH was formed as inclusion body. This may be from the effect of cell-growth stage. Since, IPTG is the turning point between cell growth and recombinant protein synthesis. The addition of IPTG means the beginning of the transcription of the foreign gene on the plasmid and can bring great changes to the metabolism of host cell by initiating the translation of heterologous protein (Lu and Mei, 2007). From previous report, 0.1 mM IPTG was added when OD_{600} reach 0.6 for E. coli JM109/pKAR/pACGD expression

(Kataoka *et al.*, 1999). Therefore, adding IPTG at OD_{600}=0.2 may be not suitable for GDH expression.

5.8 Expression of *skdh* (GOX1959) gene in *G. oxydans* IFO3244 using pSG8 vector

A *Gluconobacter-E. coli* shuttle vector, pSG8 vector, was constructed by ligating pAG5 plasmid with an *E. coli* vector pUC18 at the *Hind*III site (Tonouchi *et al.*, 2003). The foreign gene is expressed under *lac* promoter. To insert *skdh* (GOX1959) gene fragments into pSG8 at the right position. The gene fragment was amplified with the 5' end primer containing *Sac*I restriction site and 3' end primer that containing *Xba*I restriction site. Nucleotide and deduced amino acid sequence of PCR products were compared with nucleotide and deduced amino acid sequence of *skdh* (GOX1959) gene from *G. oxydans* 621H genome database to check any error of which may occur from DNA polymerase. From sequencing result in Figure 4.40, nucleotide and deduced amino acid sequence of *skdh* (GOX1959) gene fragment PCR product was as same as in the database.

From previous report, *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. From the activity result in Table 4.19, the SKDH activity of the transformant was 10-fold (0.54 U/mg) higher than that of wild type. However, the protein band between the transformant and the wild type from SDS-PAGE result was not different. Because the sensitivity of Coomassie Blue staing is 0.1-1 μg/band, the

SKDH (GOX1959) may be lower than 0.1 μg. Therefore, SDS-PAGE could not discriminate between the protein bands.

Attempt to optimize this expression was carried out by increasing IPTG concentration. From the activity result in Table 4.20, the activity of the transformant when induced with 0.2 mM IPTG was not different from the transformant with no IPTG induction. *Gluconobacter* system does not have lac*I* gene. Therefore, IPTG does not have an effect to gene expression in *Gluconobacter*.