CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Autoclave: MLS-3020, Sanyo Electric, Japan

Autopipette: Biopipette^{TM}, U.S.A.

DNA electrophoresis: Gelmate 2000, Toyobo, Japan

Electrophoresis unit: Model Mini-protein II Cell, Bio-Rad, USA.

Fraction collector: Redi Frac, Pharmacia LKB, Sweden

French press cell disrupter: Thermo Electron Corporation, USA.

Gene Pulser^{R}: Bio-Rad, U.S.A.

Gene Amp PCR system: Model 2400, Perkin Elmer, USA.

Incubator shaker: Innova 4000, New Brunswick Scientific, USA.

Refrigerated centrifuge: 5804R, Eppendorf, USA.

Spectrophotometer DU 800: Beckman Coulter, USA.

3.2 Chemicals

Acrylamide: Merck, Germany.

Agar: Scharlau Microbiology, Spain.

Agarose: SeaKem, USA.

Ammonium persulphate: Merck, Germany.

Bovine serum alubumin (BSA): Sigma, USA.

Bromophenol blue: BDH, England.

Calcium carbonate: Fluka, Switzerland.

Calcium chloride: BDH, England.

Chloroform: Sigma, USA.

Coomassie brilliant blue R-250: Sigma, USA.

DNA marker: 1Kb Ladder (#SM 0311 lot.4801), Fermentas, USA.

Ethidium bromide: Sigma, USA.

Ethylenediamine tetraacetic acid (EDTA): Fluka, Switzerland.

FastPlasmid Mini Kit: Eppendorf, USA.

Folin-Ciocalteu's reagent: Carlo Erba Reagenti, France

Glacial acetic acid: BDH, England.

Glucose: Fluka, Switzerland.

Glycerol: Univar, Australia.

Hydrochloric acid: Merck, Germany.

6xLoading dye: Toyobo, Japan.

Magnesium chloride: Merck, Germany.

β-Nicotinamide adenine dinucleotide (β-NAD): Fluka, Switzerland.

β-Nicotinamide adenine dinucleotide phosphate (β-NADP): Fluka, Switzerland.

N, N ,N',N'-Tetramethylene ethylene diamine (TEMED): BDH, England.

Phenol: BDH, England.

Protein molecular weight marker (#SM 0431 lot.1811): Fermentas, USA.

Quinic acid: Merck, Germany.

QIAprep® Spin Miniprep Kit (Cat. No. 27104): QIAGEN, U.S.A.

QIAquick® Gel Extraction Kit (Cat. No. 28704): QIAGEN, U.S.A.

Shikimic acid: Sigma, U.S.A.

Sodium chloride: BDH, England.

Sodium hydroxide: Merck, Germany.

Tris (hydroxymethyl) aminomethane: USB, U.S.A.

Tryptone: Merck, Germany.

Yeast extract: Scharlau Microbiology, Spain.

The other common chemicals were reagent grade from BDH, Carlo Erba, Fluka, Merck and Sigma.

3.3 Bacteria strains and plasmids

Gluconobacter oxydans 621H and *Gluconobacter oxydans* IFO3244 were a gift from Professor Hirohide Toyama (Yamaguchi University, Japan).

Escherichia coli DH5α, genotype: ∅80d*lacZ*ΔM15Δ (*lacZY* A-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^- m_k^+) *deo*R *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1, was used as a host for pGEM®-T Easy vector.

Escherichia coli BL21(DE3), genotype: *omp*T gal dcm lon *hsd*S_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]).

Escherichia coli JM109, genotype: *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(rK^- mk^+), $e14^-$(*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F'[*traD*36, *proAB*$^+$, *lac* I^q, *lacZ*ΔM15]

Escherichia coli XL1-blue, genotype: *rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 (r_k^- m_k^+) *sup*E44 *rel*A1 λ^-*lac*$^-$ [F' *pro*AB *lac*I^qZΔM15 Tn10(tet)]

pACGD vector (Kataoka *et al.*, 1999) was used as an expression vector.

pBluescript II SK (-) vector was used as an expression vector in *E. coli* JM109.

pCold I vector from Takara was used as an expression vector in *E. coli* BL21 (DE3) and *E. coli* BL21.

pG-KJE8 vector fron Takara was used as a chaperone vector in *E. coli* BL21.

pGEM®-T Easy vector from Promega was used as a cloning vector.

pET-21a vector was used as an expression vector in *E. coli* BL21(DE3).

pSG8 vector was used as an expression vector in *G. oxydans* IFO3244.

3.4 Media preparation

3.4.1 Luria-Bertani broth (LB medium)

LB medium consisting of 1% (w/v) Tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl.

3.4.2 LB agar

LB agar consisting of 1% (w/v) Tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl and 1.5% Agar.

3.4.3 LB-Amp-IPTG-X-gal Agar

LB-IPTG-X-gal Agar consisting of1% (w/v) Tryptone, 0.5% (w/v) yeast extract, 0.5% w/v) NaCl and 1.5% (w/v) Agar supplemented with 100 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-gal.

3.4.4 Potato medium

Potato medium was consisted of 0.5% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) polypeptone, 2% (w/v) glycerol and potato extract 10 ml. For solid medium, 2% (w/v) agar and 0.5% (w/v) $CaCO_3$ were added.

3.4.5 Basal medium

Basal medium was consisted of 0.1% (w/v) glycerol, 0.3% (w/v) yeast extract and 0.1% (w/v) polypeptone. For dehydroquinate production, 0.2% (w/v) quinic acid was added and the medium was adjusted to pH 4.0.

3.4.6 Glycerol medium

Glycerol medium was consisted of 1% (w/v) glecerol, 0.3% (w/v) polypeptone and 0.3% (w/v) yeast extract.

3.5 General techniques in genetic engineering

3.5.1 Genomic DNA extraction

G. oxydans 621H was cultured in 100-ml potato medium pH 7.0 at 30°C, 250 rpm, for 16 hours. Cells were collected by centrifugation at 9,000 rpm, 4°C, for 10 minutes. Cells were then washed with sterile distilled water and centrifuged at 9,000 rpm for 10 minute at 4°C. The pellet was resuspend in 5 ml of TNE solution (0.1M Tris base, 10 mM EDTA and 2 M NaCl, pH 7.4). Thirty microlitres of RNase A (10 mg/ml) and 20 mg lysozyme were added and incubated for 1 hour at 37°C. The DNA solution was incubated at -20°C for 1 hour. After incubation, 1/10 volume of 10% SDS and 10 mg proteinase K were added and incubated at 60°C until solution became clear. Then 1/10 volume of 10% CTAB in 0.7 M NaCl was added. The DNA solution was incubated for 65°C for 10 minutes and then cooled down. The DNA was extracted with an addition of an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 V/V), mixed gently and centrifuged at 12,000 rpm for 20 minutes. A viscous fluid formed at the upper layer was carefully transferred to a new tube and 1/10 volume of 3 M Sodium acetate pH 5.2 was added. The DNA was extracted with an addition of an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 V/V) to ensure the complete extraction of DNA prior to centrifugation at 12,000 rpm for 20 minutes. The upper layer was transferred to a new tube. DNA was precipitated by the addition of 2.5 volume of cold 100% ethanol, gently mixed by inversion and

centrifuge at 12,000 rpm for 20 minutes. The DNA pellet was washed with cold 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer.

3.5.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate, identify and purify fragments of DNA using 0.8% or 1% agarose depending on DNA fragment size in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) (Ausubel *et al.*, 2002). DNA samples in 1X loading buffer were loaded into the wells. The gel was run at 100 volts until bromophenol blue reached the bottom of the gel. After electrophoresis, the gel was stained by immersion in H_2O containing ethidium bromide (0.5 μg/ml) for 15-30 minutes at room temperature. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was estimated from the intensity and relative mobility of the standard DNA marker.

3.5.3 Preparation of *E. coli* competent cells for electroporation method

A single colony of *E. coli* cells was inoculated into 5 ml LB medium and grown at 37°C, shaking at 250 rpm overnight. 1 ml of cell inoculum was transferred to 100 ml LB medium and grown at 37°C, 250 rpm until OD_{600} reach 0.6. The cells were collected by centrifugation at 5,000 rpm 4°C for 10 minutes. A supernatant was discarded. The pellet was resuspended with equal volume of 10% (w/v) glycerol and centrifuge at 5,000 rpm 4°C for 10 minutes. This step was repeated twice. The pellet was resuspend with 0.4 ml of 10% glycerol and aliquot 40 μl/0.6 ml microcentrifuge tube on ice prior to immediate drop into liquid N_2. The competent cells were stored at -80°C until use.

3.5.4 Preparation of *E. coli* competent cells for heat shock method

A single colony of *E. coli* cells was inoculated into 5 ml LB medium and grown at 37°C 250 rpm overnight. 1 ml of inoculum was transferred to 100 ml LB medium and grown at 37°C, 250 rpm until OD_{600} reach 0.3-0.4. The inoculum was incubated on ice for 30 minutes. The cells were collected by centrifugation at 4,000 rpm 4°C for 10 minutes. The pellet was resuspended in 40 ml 0.1M $MgCl_2$ and centrifuge at 5,000 rpm 4°C for 10 minutes. The pellet was resuspended in 10 ml cold 0.1 M $CaCl_2$ and incubated on ice 30 minutes. After incubation, the cell suspension was centrifuged at 5,000 rpm 4°C for 10 minutes. The pellet was resuspended in 1.8 ml 0.1 M $CaCl_2$ and 0.4 ml sterile glycerol. The cell suspeision was aliquot 100 µl/0.6 ml microcentrifuge tube on ice prior to immediate drop into liquid N_2. The competent cells were stored at -80°C until use.

3.5.5 Preparation of *Gluconobacter oxydans* competent cells

A single colony of *G. oxydans* was inoculated into 5 ml potato medium and grown at 30°C overnight. 1 ml of inoculum was transferred to 100 ml sorbitol medium and grown at 30°C 250 rpm until OD_{600} reach 0.3-0.4. The inoculum was incubated on ice for 30 minutes. The cells were collected by centrifugation at 4,000 rpm 4°C for 10 minutes. The pellet was resuspend with equal volume of 10% glycerol and centrifuge at 4,000 rpm 4°C for 10 minutes. This step was repeated twice. The pellet was resuspend with 0.4 ml of 10% glycerol and aliquot 40 µl/0.6 ml microcentrifuge tube on ice prior to immediate drop into liquid N_2. The competent cells were stored at -80°C until use.

3.5.6 Transformation into host cell *E. coli* by electroporation

The recombinant plasmids were transformed into competent cells of *E. coli* DH5α or *E. coli* BL21 (DE3). The DNA was mixed with cold cell suspension, transferred to a chilled cuvette and place on ice for 1 minute. The mixture was electroporated in a cold 0.2-cm electrode gap cuvette with apparatus as follows; 25 μF, 200 Ω of the pulse controller unit and 250 kV time constant 4.8-4.6 msec. After electroporation, the cell suspension was transferred into a new microcentrifuge tube containing 1 ml of LB broth and incubated at 37°C for 1 hour. The cell suspension was spreaded onto the LB agar plate containing 100 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-gal. The plate was incubated at 37°C overnight.

3.5.7 Transformation into host cell *E. coli* by heat shock

The DNA was mixed with cold cell suspension in microcentrifuge tube and place on ice for 1 hour. The mixture was incubated at 42°C for 90 seconds and placed on ice for 5 minutes. The cell suspension was transferred into a new microcentrifuge tube containing 1 ml of LB broth and incubated at 37°C for 1 hour. The cell suspension was spreaded onto the LB agar plate containing 100 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-gal. The plate was incubated at 37°C overnight.

3.5.8 Plasmid preparation

Plasmid-harboring cells were cultured in 5-ml LB broth at 37°C 250 rpm overnight and harvested by centrifugation at 12,000 rpm at room temperature for 1 minute. The cell pellet was used to extract plasmid by Fast plasmid mini Kit from Eppendorf (USA) or QIAprep® Spin Miniprep Kit from Qiagen (USA).

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

3.6.1 Preparation of the *dqd* gene using the PCR technique

A pair of primer was designed by using the nucleotide sequence from *dqd* (GOX0437) gene of *G. oxydans* 621H. The sequence of forward primer (dqd_F) was (location 462168) 5'-GATCGCACCAGATAGCACAA-3' and the sequence of reverse primer (dqd_R) was (location 461549) 5'-CCGTCAGAATATCGGCCAAT-3' (Appendix A).

Twenty five microliters reaction mixture contained 2 U of *Taq* DNA polymerase, 0.2 mM dNTPS, 1X Reaction buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% v/v Tween20), 1.5 mM $MgCl_2$, 50 ng DNA template and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as followed.

The first round : Predenaturation at 94°C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 54°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle;
before maintaining at 4°C

The PCR products were electrophoresed through agarose gel. Finally, the *dqd* (GOX0437) gene fragment was harvest from agarose gel by QIAquick® Gel Extraction Kit.

3.6. 2 Preparation of the *skdh* (GOX0859) gene using the PCR technique

A pair of primer was designed by using the nucleotide sequence from *skdh* (GOX0859) gene of *G. oxydans* 621H. The sequence of forward primer (GOX0859_F) was (location 929339) 5'-ACAGGCACAGATCCGAGGAG-3' and the sequence of reverse primer (GOX0859_R) was (location 928369) 5'-AGCGGC TTCTTCGCCAAGGT-3' (Appendix B).

Twenty five microliters reaction mixture contained 2 U of *Taq* DNA polymerase, 0.2 mM dNTPS, 1X Reaction buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% v/v Tween20), 1.5 mM $MgCl_2$, 50 ng DNA template and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as followed.

The first round : Predenaturation at 94°C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 53°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle;
before maintaining at 4°C

The PCR products were electrophoresed through agarose gel. Finally, the *skdh* (GOX0859) gene fragment was harvest from agarose gel by QIAquick® Gel Extraction Kit.

3.6.3 Preparation of the *skdh* (GOX1959) gene using the PCR technique

A pair of primer was designed by using the nucleotide sequence from GOX0859 gene of *G. oxydans* 621H. The sequence of forward primer (GOX1959_F)

was 5'-GCGCATGATTGACGGTCACA-3' (location 2147827) and the sequence of reverse primer (GOX1959_R) was 5'-CCGGTCAGACCAATGATCTT-3' (location 2148689) (Appendix C).

Twenty five microliters reaction mixture contained 2 U of *Taq* DNA polymerase, 0.2 mM dNTPS, 1X Reaction buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% v/v Tween20), 1.5 mM $MgCl_2$, 50 ng DNA template and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as follow.

The first round : Predenaturation at 94°C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 53°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle;
before maintaining at 4°C

The PCR products were electrophoresed through agarose gel. Finally, the *skdh* (GOX1959) gene fragment was harvest from agarose gel by QIAquick® Gel Extraction Kit.

3.6.4 Ligation of the PCR product with the plasmid and transformation

PCR product was ligated with pGEM®-T easy vector (Appendix D). The ligation mixture of 10 μl containing 3 μl PCR products, 50 ng of pGEM®-T easy vector, 1x ligation buffer and 3 units of T4 DNA ligase, was incubated overnight at 16°C and then transformed into *E. coli* DH5α by electroporation. After incubation on LB-Amp-IPTG-X-gal agar plate for 16 hours, the white colonies were selected. The present of insert in the recombinant plasmids were confirmed by equencing.

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

3.7.1 PCR amplification of *dqd* gene

A pair of primer was designed by using the nucleotide sequence from *dqd* (GOX0437) gene of *G. oxydans* 621H. The sequence of forward primer (dqdpET_F) was 5'-**CA/TATG**ACGGCTCCGAAAGTGCT-3' with *NdeI* site (underlined). The sequence of reverse primer (dqdpET_R) was 5'-**C/TCGAG**TCCTTCGTCTTCG ATCAT-3' with *XhoI* site (Appendix A).

The PCR was done by using PuRe Taq Ready-To-Go PCR beads (Amersham Biosciences). Twenty five microliters reaction mixture contained, 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, stabilizers, BSA, 50 ng pGEM-T Easy vector harboring *dqd* gene and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as followed.

The first round	: Predenaturation at 94°C for 3 minutes; 1 cycle.
The second round	: Denaturation at 94°C for 1 minute;
	Annealing at 51°C for 1 minute;
	Extension at 72°C for 1 minute;
	30 cycles
The third round	: Final extension 72°C for 10 minutes; 1 cycle;
	before maintaining at 4°C.

The PCR products were run on 0.8% agarose gel. Finally, the *dqd* (GOX0437) gene fragment was harvested from agarose gel by QIAquick® Gel Extraction Kit.

3.7.2 PCR amplification of *skdh* (GOX0859) gene

A pair of primer was designed by using the nucleotide sequence from *dqd* gene of *G. oxydans* 621H. The sequence of forward primer (GOX0859pET_F) was 5'-**CA/TATG**AAGCCAGCAGAATTTCCGC-3' with *NdeI* site (underlined). The sequence of reverse primer (GOX0859pET_R) was 5'-**C/TCGAG**TGTCAGGCC GCGCCGAAAATA-3' with *XhoI* site (Appendix B).

The PCR was done by using PuRe Taq Ready-To-Go PCR beads (Amersham Biosciences). Twenty five microliters reaction mixture contained, 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, stabilizers, BSA, 50 ng pGEM-T Easy vector harboring GOX0859 gene and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as followed.

The first round : Predenaturation at 94°C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 55°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle;
before maintaining at 4°C

The PCR products were run on 0.8% agarose gel. Finally, the *skdh* (GOX0859) gene fragment was harvested from agarose gel by QIAquick® Gel Extraction Kit.

3.7.3 PCR amplification of *skdh* (GOX1959) gene

A pair of primer was designed by using the nucleotide sequence from *dqd* gene of *G. oxydans* 621H. The sequence of forward primer (GOX1959pET_F) was 5'-**CA/TATG**ATTGACGGTCACACGA-3' with *NdeI* site (underlined) and the sequence of reverse primer (GOX1959pET_R) was 5'-**C/TCGAG**CGCGTCAGT GCGCAGGCT-3' with *XhoI* site (underlined) (Appendix C).

The PCR was done by using PuRe Taq Ready-To-Go PCR beads (Amersham Biosciences). Twenty five microliters reaction mixture contained, 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, stabilizers, BSA,, 50 ng pGEM-T Easy vector harboring GOX1959 gene and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as follow.

The first round : Predenaturation at 94°C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 52°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle;
before maintaining at 4°C.

The PCR products were run on 0.8% agarose gel. Finally, the *skdh* GOX1959) gene fragment was harvested from agarose gel by QIAquick® Gel Extraction Kit.

3.7.4 Ligation of the *dqd* and two of *skdh* genes into pET-21a vector

The PCR products obtained from section 2.8.1, 2.8.2 and 2.8.3 were ligated into pGEM®-T Easy vector by the method form 2.7.4. The pET-21a vector (Appendix

E) and pGEM®-T Easy vector harboring insert were linearized with *Nde*I and *Xho*I. The reaction mixture containing of 1 µg pET-21a vector or pGEM®-T Easy vector harboring insert, 1X R buffer, 3 U of *Nde*I and 3 U of *Xho*I in total volume of 30 µl was incubated at 37°C overnight. The restriction enzyme was inactivated at 65°C for 15 minutes. Digested gene fragment was ligated to the digested pET-21a vector at molar ratio vector : insert at 1 : 3. The ligation mixture of 10 µl contained 50 ng of vector DNA, 150 ng of gene fragment and equal volume of ligation high from Toyobo. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) using electroporation. The transformed cells were grown in LB agar containing 50 µg/ml ampicillin at 37°C overnight.

3.7.5 Selection of positive recombinant

After incubation at 37°C overnight, the white colony was selected. The recombinant plasmids were confirmed to contain inserts by sequencing.

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

3.8.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 µg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.8.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 0.2, IPTG was added to final concentration of 1mM to induce *dqd* gene expression and cultivation was continued at 37°C for 5 hours. After cultivation, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C.

3.8.3 Preparation of intracellular enzyme

Bacterial cell mass from section 3.8.2 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was kept for DQD activity determination.

3.8.4 Dehydroquinate production

The *G. oxydans* IFO3244 colony was transferred into Potato medium and cultured on a shaking incubator at 30°C overnight. Starter inoculums (0.2%) was transferred into basal medium containing 0.2% quinic acid and cultured on a shaking incubator at 30°C for 20 hours. Bacterial cell mass was separated by centrifugation at at 9,000 rpm for 10 minutes. The supernatant was analyzed by paper chromatography (Appendix F) and kept for DQD activity assay.

3.8.5 Detection of dehydroquinate by paper chromatography

The supernatant from section 3.8.4 was spotted on chromatography paper and developed in the solvent system benzylalcohol: 2-butanol: 2-propanol: water = 3:1:1:1 (w/v) cotaining 2% (w/v) formic acid. The dried chromatogram was sprayed with freshly prepared solution of sodium metaperiodate (160 mg) in a mixture of each 12.5 ml of 1 M acetic acid and 1 M Na-acetate. About 20 minutes later, 3% (v/v) aniline in ethanol was sprayed over the paper (Adachi *et al.*, 2003a).

3.8.6 DQD activity assay

DQD activity was determined by monitoring the formation of dehydroshikimate at 234 nm (ε = 1.2 x 10^4 M^{-1} cm^{-1}) at 25°C. The standard assay mixture (1 ml) contained Tris-HCl buffer pH 9.0, dehydroquinate and intracellular crude enzyme.

One unit of enzyme was defined as the amount of enzyme that produces 1μmole of dehydroshikimate under the described condition.

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

3.9.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.9.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 0.2, IPTG was added to final concentration of 1mM to induce *skdh* gene expression and the cultivation was continued at 37°C for 5 hours. After cultivation, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C.

3.9.3 Preparation of intracellular enzyme

Bacterial cell mass from section 3.9.2 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was retained for determination of SKDH activity.

3.9.4 SKDH activity assay

One unit of enzyme is defined as the amount of enzyme catalyzing the formation of 1 μmole of NADPH or NADH per minute under indicated conditions. SKDH activity was determined by monitoring the formation of NADPH or NADH at 340 nm (ε for NADPH and NADH = 6.22×10^3 M^{-1} cm^{-1}) at 25°C. the SKDH assay

mixture (1 ml) consisted of 0.2 mM $NADP^+$ or NAD^+, 2mM shikimic acid or 2 mM quinic acid in 100 mM Tris-HCl pH 9.0.

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

3.10.1 Ligation of *skdh* (GOX0859) gene into pCold I expression vector

The 855 kb PCR products obtained from method 3.7.2 were ligated into pGEM®-T Easy vector by the method form 3.6.4. The pCold I expression vector (Appendix G) or pGEM®-T Easy vector harboring the insert were linearized with *Nde*I and *Xho*I. The reaction mixture containing of 1 µg pCold I vector, 1X R buffer, 3 U of *Nde*I and 3 U of *Xho*I in total volume of 30 µl was incubated at 37°C overnight. The restriction enzyme was inactivated at 65°C for 15 minutes. Digested gene fragment was ligated to the digested pET-21a vector at molar ratio vector : insert at 1 : 3. The ligation mixture of 10 µl containing 50 ng of vector DNA, 150 ng of gene fragment and equal volume of Ligation High from Toyobo. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) using electroporation. The transformed cells were grown in LB agar containing 50 µg/ml ampicillin at 37°C overnight.

3.10.2 Selection of positive recombinant

After incubation at 37°C overnight, the white colony was selected. The recombinant plasmids were cut check with *Nco*I and *EcoR*V. The presence of insert in the recombinant plasmid was confirmed by sequencing.

3.10.3 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 µg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.10.4 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 0.4, the inoculums was refrigerated at 15°C and leaved to stand for 30 minutes. IPTG was added to final concentration of 0.1mM to induce *skdh* (GOX0859) gene expression and cultivation was continued at 15°C for 24 hours. After cultivation, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C.

3.10.5 Preparation of intracellular enzyme

Bacterial cell mass from section 3.10.4 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was kept for SKDH activity determination by the method from section 3.9.4.

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

3.11.1 Transformation of pCold I/GOX0859 into *E. coli* BL21

The recombinant plasmids from section 3.10.1 were transformed into *E. coli* BL21 harboring a chaperone vector, pG-KJE8 (Appendix H) using electroporation. The transformed cells were grown in LB agar containing 100 μg/ml ampicillin and 20 μg/ml chloramphenicol at 37°C overnight. After incubation, the white colony was selected.

3.11.2 Starter inoculum

The *E. coli* BL21 transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol. The inoculum was cultured on a shaking incubator at 37°C overnight.

3.11.3 Enzyme production

Starter inoculums (1%) was transferred into LB medium containing 50 μg/ml ampicillin and 20 μg / ml chloramphenicol in Erlenmeyer flask and cultured on a shaking incubator at 37°C. For chaperone protein expression, 0.5 mg/ml L-arabinose and 1 ng/ml tetracycline was added. When the turbidity of the culture at 600 nm reached OD 0.4, the inoculum was refrigerated at 15°C and leaved to stand for 30 minutes. IPTG was added to final concentration of 0.1mM to induce *skdh* (GOX0859) gene expression and cultivation was continued at 15°C for 24 hours. After cultivation, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C.

3.11.4 Preparation of intracellular enzyme

Bacterial cell mass from section 3.11.3 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was kept for SKDH activity determination by the method from section 3.9.4.

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

3.12.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.12.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600

nm reached 1.2, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C.

3.12.3 Preparation of intracellular enzyme

Bacterial cell mass from section 3.12.2 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was kept for SKDH activity determination by the method from section 3.9.4.

3.13 Protein purification by Ni-NTA agarose column chromatography

Five milliliter of Ni-NTA agarose was resuspended with distilled water followed by native binding buffer twice. Intracellular enzyme from section 3.11.4 and 3.12.3 were applied to Ni-NTA agarose column chromatography. The sample was bound to the Ni-NTA agarose for 30-60 minutes using gentle agitation to keep the resin suspended in the supernatant. The resin was settled by gravity and washed with 10-ml native wash buffer three times to removed unbound protein. After washing, the resin was packed into the column. Bound protein was eluted by 75-ml native elution buffer (50 mM sodium phosphate buffer pH 8.0 with 250 mM imidazole buffer containing 0.5 M NaCl). Fractions of 1.0 ml were continuously collected. The protein and activity profile of the eluted fractions were monitored by measuring the absorbance at 280 nm and SKDH activity was assayed (section 3.9.4). Fractions with enzyme activity were pooled and dialyzed in 20 mM Tris-HCl buffer pH 8.0 for further determination.

3.14 Determination of kinetic parameter of SKDH

The purified protein from section 3.13 was used to determine the enzyme kinetic parameters (K_m and V_{max}) using shikimic acid and $NADP^+$ as the substrates. The SKDH activity was determined as describe in section 3.9.4 with varying concentration of shikimic acid and $NADP^+$: 10, 20, 30, 50, 100, 200 and 300 μM at saturating concentration of co-substrate 50 mM. Kinetic parameters, K_m and V_{max}, were obtained from the Lineweaver-Burk plot.

3.15 Cloning of NAD(P) - dependent *Glucose dehydrogenase* gene from *G. oxydans* 621H

3.15.1 Preparation of *gdh* gene by using PCR technique

A pair of primer was designed by using the nucleotide sequence from *gdh* (GOX2015) gene of *G. oxydans* 621H. The sequence of forward primer (GOX2015_F) was (location 2211301) 5'-T**C/TCGAG**TAACAAGGAGAGAGGT GAGGCCAGATG-3'and the sequence of reverse primer (GOX2015_R) was (location 2210448) 5'-T**C/TCGAG**TCGATCA GCAGAACGGTAAG-3' with *Xho*I site (Appendix I).

The PCR was done by using PuRe Taq Ready-To-Go PCR beads (Amersham Biosciences). Twenty five microliters reaction mixture contained, 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, stabilizers, BSA, 50 ng DNA template and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as followed.

The first round : Predenaturation at 94 °C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 56°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle.

The PCR products were run on 0.8% agarose gel. Finally, the *gdh* (GOX2015) gene fragment was harvest from agarose gel by QIAquick® Gel Extraction Kit and ligated into pGEM®-T easy vector by the method from section 3.6.4.

3.15.2 Ligation of the *gdh* gene into expression vector

The PCR products obtained from section 3.14.1 was ligated into pGEM®-T Easy vector by the method form 3.6.4. The pET-21a/GOX1959 and pGEM®-T Easy vector harboring *gdh* gene were linearized with *Xho*I. The reaction mixture containing of 1 μg pET-21a/GOX1959 or pGEM®-T Easy/*gdh*, 1X R buffer and 3 U of *Xho*I in total volume of 30 μl was incubated at 37°C overnight. The restriction enzyme was inactivated at 65°C for 15 minutes. Digested gene fragment was ligated to the digested pET-21a/GOX1959 at molar ratio vector : insert at 1 : 3. The ligation mixture of 10 μl contained 50 ng of vector DNA, 150 ng of gene fragment and equal volume of Ligation High from Toyobo. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) using electroporation. The transformed cells were grown in LB agar containing 100 μg/ml ampicillin at 37°C overnight.

3.15.3 Selection of positive recombinant

After incubation at 37°C overnight, the white colony was selected. The recombinant plasmids were cut check with *Nco*I. The presence of insert in the recombinant plasmid was confirmed by sequencing.

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

3.16.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.16.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 1.2, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C.

3.16.3 Preparation of intracellular enzyme

Bacterial cell mass from section 3.12.2 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was kept for SKDH activity determination by the method from section 3.9.4.

3.16.4 GDH activity assay

One unit of enzyme is defined as the amount of enzyme catalyzing the formation of 1 μmole of NADPH per minute under indicated conditions. Enzyme activity was determined by monitoring the formation of NADPH at 340 nm (ε for NADPH = 6.22×10^3 M^{-1} cm^{-1}) at 25°C. The GDH assay mixture (1 ml) consisted of 0.2 mM $NADP^+$, 2mM glucose in 100 mM Tris-HCl pH 9.0.

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

3.17.1 Effect of medium volume

3.17.1.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.17.1.2 Enzyme production

Starter inoculum (1%) was transferred into LB medium in 250-ml Erlenmeyer flask with the medium volume of 25, 50, 75, 100 and 125 ml. The inoculums were cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached 1.2, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C. Intracellular crude enzyme was prepared by the method from section 3.12.3. The activity of SKDH and GDH was determined by the method from section 3.9.4 and 3.12.4, respectively.

3.17.2 Effect of IPTG concentration

3.17.2.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.17.2.2 Enzyme production

Starter inoculums (1%) was transferred into 100 ml LB medium in 250-ml Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 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. Bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C. Intracellular crude enzyme was prepared by the method from section 3.12.3. The activity of SKDH and GDH was determined by the method from section 3.9.4 and 3.12.4, respectively.

3.17.3 Effect of induction temperature

3.17.3.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.17.3.2 Enzyme production

Starter inoculums (1%) was transferred into 100 ml LB medium in 250-ml Erlenmeyer flask and cultured on a shaking incubator at 28, 30, 34, 37 and 40°C, respectively. When the turbidity of the culture at 600 nm reached OD 0.2, IPTG was added to final concentration of 0.2 mM, respectively and cultivation was continued at each temperature for 5 hours. Bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C. Intracellular crude enzyme was prepared by the method from section 3.12.3. The activity of SKDH and GDH was determined by the method from section 3.9.4 and 3.12.4, respectively.

3.17.4 Effect of induction timing

3.17.4.1 Starter inoculum

The *E. coli* BL21 (DE3) transformant colony was transferred into LB medium containing 50 μg/ml ampicillin and cultured on a shaking incubator at 37°C overnight.

3.17.4.2 Enzyme production

Starter inoculums (1%) was transferred into 100 ml LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 0.2, 0.5, 1.0 and 1.1, IPTG was added to final concentration of 0.2 mM and cultivation was continued at 37°C for 5 hours to the final OD_{600} of 1.2. Bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C. Intracellular crude enzyme was prepared by the method from section 3.12.3. The activity of SKDH and GDH was determined by the method from section 3.9.4 and 3.12.4, respectively.

3.18 Transformation of pACGD vector into *E. coli* BL21 (DE3)

pACGD vector (Appendix J) harboring *gdh* gene from *Bacillus megaterium* (Kataoka *et al.*, 1999) was transformed into *E. coli* BL21 (DE3) by electroporation. The transformed cells were grown in LB agar containing 50 µg/ml kanamycin at 37°C overnight. After positive colony selection, transformant was used to prepare competent cell by the method from section 3.5.3.

3.19 Transformation of pET-21a/GOX1959 into *E. coli* BL21 (DE3)/pACGD

pET-21a/GOX1959 was transformed to *E. coli* BL21 (DE3)/pACGD from section 3.21 by electroporation. The transformed cells were grown in LB agar containing 50 µg/ml ampicillin and 50 µg/ml kanamycin at 37°C overnight. The positive colony was selected.

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

3.20.1 Starter inoculum

The transformant colony was transferred into LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37°C overnight.

3.20.2 Enzyme production

Starter inoculums (1%) was transferred into LB in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 0.2, IPTG was added to final concentration of 0.1 mM and cultivation was continued at 30°C for 6 hours. After cultivation, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C. Intracellular crude enzyme was prepared and assayed enzyme activity by the method from section 3.18.3.

3.21 Transformation of pACGD vector into *E. coli* JM109

pACGD vector was transformed into *E. coli* JM109 by electroporation. The transformed cells were grown in LB agar containing 50 μg/ml kanamycin at 37°C overnight. The positive colony was selected.

3.22 Expression of pACGD in *E. coli* JM109

3.22.1 Starter inoculum

The transformant colony was transferred into LB medium containing 50 μg/ml kanamycin and cultured on a shaking incubator at 37°C overnight.

3.22.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured on a shaking incubator at 37°C. When the turbidity of the culture at 600 nm reached OD 0.2, IPTG was added to final concentration of 0.1 mM and cultivation

was continued at 37°C for 6 hours. After cultivation, bacterial cell mass was collected by centrifugation at 9,000 rpm for 10 minutes at 4°C. Intracellular crude enzyme was prepared and assayed enzyme activity by the method from section 3.18.3.

3.23 Subcloning of *skdh* (GOX1959) into pSG8 vector

3.23.1 PCR amplification of *skdh* (GOX1959) gene

A pair of primer was designed by using the nucleotide sequence from *skdh* (GOX1959) gene of *G. oxydans* 621H. The sequence of forward primer (GOX1959pSG8_F) was 5'-TTC**GAGCT/C**GATGATTGACGGTCACACGA-3' with *Sac*I site (underlined) and the sequence of reverse primer (GOX1959pSG8_R) was 5'-GAC**T/CTAGA**TCACGCGTCAGTGCGCAG-3' with *Xba*I site (underlined) (Appendix A).

The PCR was done by using PuRe Taq Ready-To-Go PCR beads (Amersham Biosciences). Twenty five microliters reaction mixture contained, 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, stabilizers, BSA, 50 ng pGEM-T Easy vector harboring GOX1959 gene and 1 μM of each primer. Three rounds of temperature cycling for PCR were performed as follow.

The first round : Predenaturation at 94 °C for 3 minutes; 1 cycle.

The second round : Denaturation at 94°C for 1 minute;
Annealing at 57°C for 1 minute;
Extension at 72°C for 1 minute;
30 cycles

The third round : Final extension 72°C for 10 minutes; 1 cycle.

The PCR products were run on 0.8% agarose gel. Finally, the *skdh* (GOX1959) gene fragment was harvest from agarose gel by QIAquick® Gel Extraction Kit.

3.23.2 Ligation of *skdh* (GOX1959) genes into pSG8 vector

The PCR products obtained from method 3.7.2 were ligated into pGEM®-T Easy vector by the method form 3.6.4. The pSG8 vector or pGEM®-T Easy vector harboring insert were linearized with *Sac*I and *Xba*I. The reaction mixture containing of 1 μg pET-21a vector or pGEM®-T Easy vector harboring the insert, 1X TA buffer, 3 U of *Sac*I and 3 U of *Xba*I in total volume of 30 μl was incubated at 37°C overnight. The restriction enzyme was inactivated at 65°C for 15 minutes. Digested gene fragment was ligated to the digested pSG8 vector (Appendix K) at molar ratio vector : insert at 1 : 3. The ligation mixture of 10 μl contained 50 ng of vector DNA, 150 ng of gene fragment and equal volume of ligation high from Toyobo. The recombinant plasmids were transformed into *G. oxydans* IFO3244 using electroporation. The transformed cells were grown in LB agar containing 100 μg/ml ampicillin at 37°C overnight.

3.23.3 Selection of positive recombinant

After incubation at 37°C overnight, the white colony was selected.

3.24 Expression of pSG8-GOX1959 in *G. oxydans* IFO3244

3.24.1 Starter inoculum

The *G. oxydans* IFO3244 transformant colony was transferred into potato medium containing 100 μg/ml ampicillin and cultured on a shaking incubator at 30°C overnight.

3.24.2 Enzyme production

Starter inoculums (1%) was transferred into glycerol medium in Erlenmeyer flask and cultured on a shaking incubator at 30°C for 26 hours.

3.24.3 Preparation of intracellular enzyme

Bacterial cell mass from section 2.25.2 was suspended in 20 mM Tris-HCl pH 8.0 and disrupted by French pressure cell press at 14,000 lb/in^2. The cell debris was separated by centrifugation at 9,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was retained for determination of SKDH activity by the method from section 3.9.4.

3.25 Effect of IPTG to pSG8-GOX1959 expression

3.25.1 Starter inoculum

The *G. oxydans* IFO3244 transformant colony was transferred into potato medium containing 100 μg/ml ampicillin and cultured on a shaking incubator at 30°C overnight.

3.25.2 Enzyme production

Starter inoculums (1%) was transferred into glycerol medium in Erlenmeyer flask and cultured on a shaking incubator at 30°C. When the turbidity of the culture at 600 nm reached OD 0.3, IPTG was added to final concentration of 0.2 mM and cultivation was continued at 30°C for 26 hours. Intracellular crude enzyme was prepared and the SKDH activity was determined by the method from section 3.9.4.

3.26 Protein determination

Protein concentration was determined by a modified Lowry's method (Lowry *et al.*, 1951) with bovine serum albumin as the standard protein. The protein standard curve was show in Appendix L.

Reagents

Solution A: 2% sodium carbonate in 0.1M sodium hydroxide containing 0.5% SDS

Solution B: 0.5% copper sulphate in 1% potassium sodium tartrate

Solution C: Phenol reagent (Folin-Ciocalteu's reagent)

After preparation of sample (0.2 ml), 2 ml of mixed solution A and B (A:B, 50:1) were added and rapidly mixed. The mixture was incubated at 30°C for 10 minutes. Subsequently, 0.2 ml of solution C was added, rapidly mixed and incubated at 30°C for 30 minutes. Finally, the absorbance of clear blue-color solution was measured at 750 nm at room temperature. The reagent preparation was presented in Appendix M.

3.27 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing gel was carried out with 0.1% (w/v) SDS in 12.5% (w/v) and 5.0% (w/v) stacking gels with Tris-glycine buffer pH 8.0 containing 0.1% SDS as electrode buffer (see Appendix N). Samples to be analyzed were treated with sample buffer and boiled for 5 minutes prior to the application to the gel. The electrophoresis was performed at constant current of 10 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

3.28 Detection of proteins on SDS-PAGE by Coomassie blue staining

Gels were stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for at least 30 minutes. The slab gels were destained with a destaining solution (10% methanol and 10% acetic acid) for 1-2 hours several times until the gel background was clear.

Table 3.1 The summary of primers used in this study

Primer name	Primer sequence
dqd_F	5'-GATCGCACCAGATAGCACAA-3'
dqd_R	5'-CCGTCAGAATATCGGCCAAT-3'
dqdpET_F	5'-**CATATG**ACGGCTCCGAAAGTGCT-3'
dqdpET_R	5'-**CTCGAG**TCCTTCGTCTTCGATCAT-3'
GOX0859_F	5'-ACAGGCACAGATCCGAGGAG-3'
GOX0859_R	5'-AGCGGCTTCTTCGCCAAGGT-3'
GOX0859pET_F	5'-**CATATG**AAGCCAGCAGAATTTCCGC-3'
GOX0859pET_R	5'-**CTCGAG**TGTCAGGCCGCGCCGAAAATA-3'
GOX1959_F	5'-GCGCATGATTGACGGTCACA-3'
GOX1959_R	5'-CCGGTCAGACCAATGATCTT-3'
GOX1959pET_F	5'-**CATATG**ATTGACGGTCACACGA-3'
GOX1959pET_R	5'-**CTCGAG**CGCGTCAGTGCGCAGGCT-3'
GOX1959pSG8_F	5'-TTC**GAGCTC**GATGATTGACGGTCACACGA-3'
GOX1959pSG8_R	5'-GAC**TCTAGA**TCACGCGTCAGTGCGCAG-3'
GOX2015_F	5'-T**CTCGAG**TAACAAGGAGAGAGGTGAGGCCAGATG-3'
GOX2015_R	5'-T**CTCGAG**TCGATCA GCAGAACGGTAAG-3'