CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Shikimic acid

Shikimic acid, more commonly known as, shikimate its anionic form, is an important biochemical intermediate in plants and microorganisms. Its name comes from the Japanese flower *shikimi* (シキミ, *Illicium anisatum*), from which it was first isolated. The chemical structure of shikimate is showed in figure 2.1.

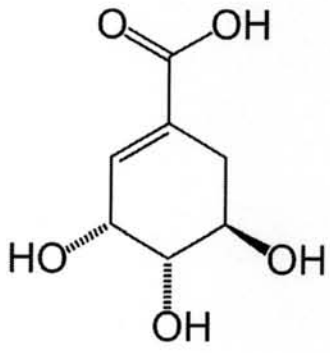

Figure 2.1 : The chemical structure of shikimate

(http://en.wikipedia.org/wiki/Image:Shikimic-acid-skeletal.png)

Shikimic acid can be extracted from the Chinese star anise with the yield ranging from 3-7% by weight, whereas it can be extracted from the seeds of the sweetgum fruit, abundant fruit in North America, with the yield of about 1.5% by weight. Shikimic acid has been widely used in several industries. In the pharmaceutical industry, shikimic acid from the Chinese star anise is used as a base material for production of Tamiflu (oseltamivir), the most promising drug to treat influenza A and influenza B including bird flu (H5N1). Oseltamivir is neuraminidase

inhibitor. It acts as a transition-state analogue inhibitor of influenza neuraminidase, preventing new viruses from emerging from infected cells. In agricultural industry, shikimic acid is used in herbicide production as glyphosate. Glyphosate is one of the most widely used herbicides. It is effective in killing a wide variety of plants, including grasses, broadleaf, and woody plants.

Because of shikimate has an important role in several industries, shikimate production has been developed for a long time. Recently, metabolic engineering of biosynthetic pathways in *E. coli* have been enhanced to allow the organism to accumulate enough material to be used commercially.

2.2 3-Dehydroshikimate (DHS)

3 – Dehydroshikimate (synonymous to 3 - dehydroshikimic acid, 5 - dehydroshikimate and 5-dehydroshikimic acid) is an intermediate in the common pathway of aromatic amino acid biosynthesis as well as an intermediate in the catabolism of shikimic acid and quinic acid by way of the β-ketoadipate pathway. Most of DHS was produced by microorganisms. The chemical structure of dehydroshikimate ($C_7H_8O_5$) was shown in Figure 2.2.

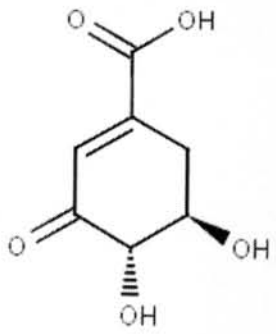

Figure 2.2 : The chemical structure of 3-dehydroshikimate

(www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:30918)

DHS has been used in several industries. In food industry, DHS is used as an antioxidant to extend food product shelf life and to improve food safety and quality. It is inhibits a development of undesirable flavor, aroma, and color compounds triggered by peroxidation of lipids. Moreover, there is an evidence to suggest that many naturally derived antioxidants provides additional health benefits as antimutagenic, anticarcinogenic, or antiinflammatory agents (Chang *et al.*, 2003).

Beside of food industry, DHS is a catalyst for syntheses of adipic acid, catechol, and vanillin (Li and Frost, 1999). The main use of adipic acid is as monomer for the production of nylon by a polycondensation reaction with hexamethylene diamine forming 6,6-nylon, the most common form of nylon. Other uses are including a monomer for production of Polyurethane, reactant to form plasticizers and lubricant components and food Ingredient as a flavorant and gelling aid. Cathechol (pyrocatechol) has been used as a film developing chemical. Vanillin is most prominent as the principal flavor and aroma compound in vanilla. At smaller concentrations, vanillin contributes to the flavor and aroma profiles of foodstuffs as diverse as olive oil, butter, and raspberry and lychee fruits. In other foods, heat treatment evolves vanillin from other chemicals. In this way, vanillin contributes to the flavor and aroma of coffee, maple syrup, and whole grain products including corn tortillas and oatmeal.

2.3 Shikimate pathway

The shikimate pathway was discovered as the biosynthetic route to the aromatic amino acids (phenylalanine, tyrosine, and tryptophan). This pathway has been found only in microorganisms and plants. Phenylalanine and tryptophan are essential components of animal diets, while animals can synthesize tyrosine using a single step from phenylalanine.

Bacteria generally spend more than 90% of their total metabolic energy on protein biosynthesis. Consequently, the bacterial shikimate pathway serves almost exclusively to synthesize the aromatic amino acids. The shikimate pathway enzymes in bacteria are seven individual polypeptides, each possessing a single enzyme activity, which are encoded by separate genes.

In contrast, higher plants use these amino acids not only as protein building blocks but also as precursors for a large number of secondary metabolites, among them plant pigments, compounds to defend against insects and other herbivores, UV light protectants, and lignin. Under normal growth condition, 20% of the carbon fixed by plants flows through the shikimate pathway. The biosynthesis and regulation of shikimate pathway-derived secondary metabolites is very extensive among plant species (Herrmann, 1995). Plants have a molecular organization of the shikimate pathway enzymes similar to bacteria, i.e. separate enzymes encoded by separate genes, with the exception of dehydroquinate dehydratase and shikimate dehydrogenase which have been shown to be present as separate domains on a bifunctional polypeptide. Plant enzymes, although nuclear encoded, are largely active in the chloroplast and accordingly possess an N-terminal transit sequence.

Shikimate pathway enzymes of fungi are differrent from bacteria and plants. All fungi examined to date have monofunctional 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthases and chorismate synthases and a pentafunctional polypeptide termed AROM. The AROM polypeptide has domains analogous to the bacterial enzymes: dehydroquinate synthase, 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, shikimate kinase, DHQase and shikimate dehydrogenase (Campbell *et al.*, 2004).

Figure 2.3 outlines the seven steps of the shikimate pathway. In the first step, phosphoenol pyruvate (the glycolytic intermediate) and erythrose-4-phosphate (the pentose phosphate pathway intermediate) are condensed to a seven-carbon six-membered heterocyclic compound, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). In the second step, the ring oxygen is exchanged for the exocyclic C7 of DAHP to form a highly substituted cyclohexane derivative, 3-dehydroquinate. The remaining five steps serve to introduce a side chain and two of the three double bonds that convert this cyclohexane into the benzene ring, chorismate, the building block of aromatic compounds.

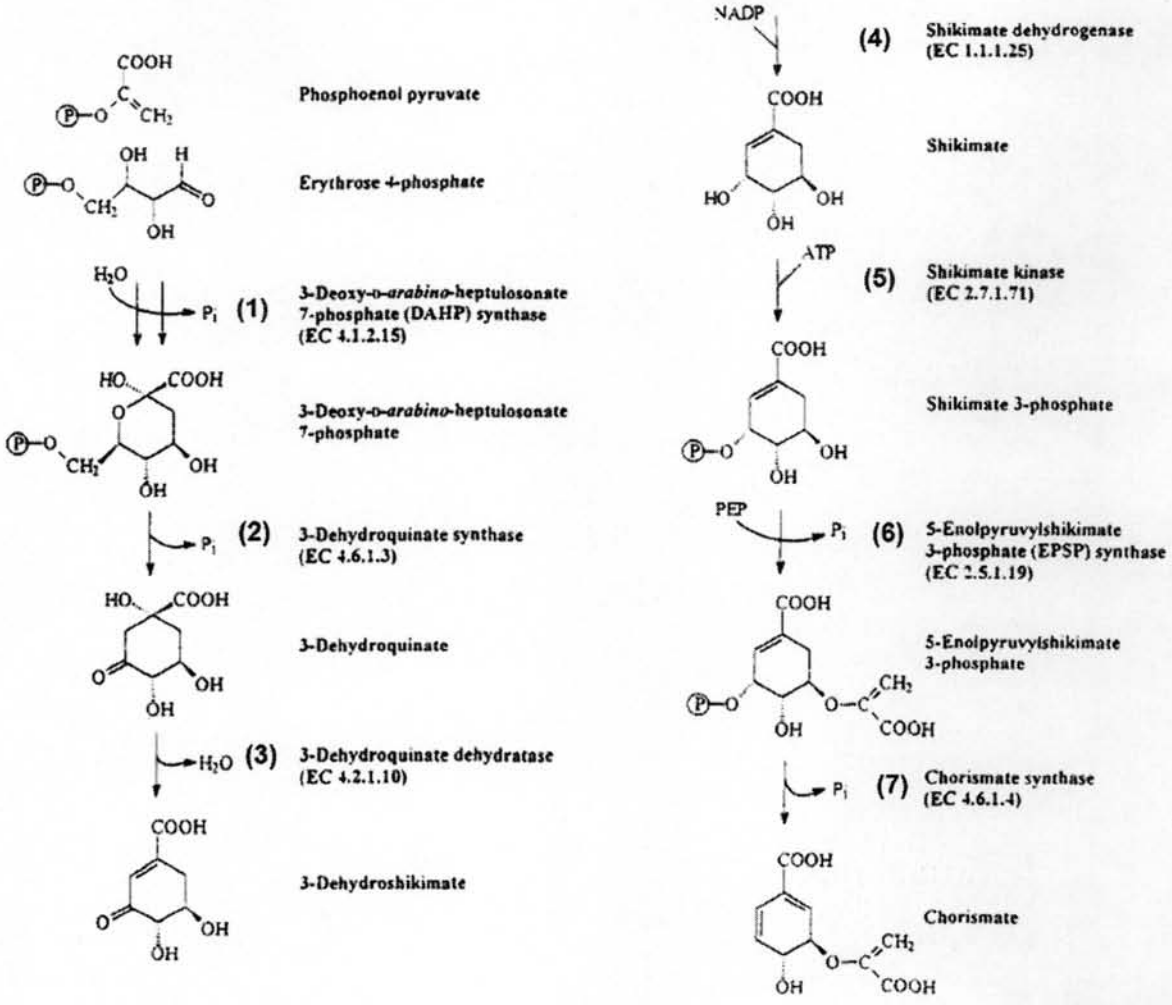

Figure 2.3 : The shikimate pathway in plant (Herrmann, 1995)

2.4 Metabolic engineering of shikimate pathway for the production of shikimate pathway intermediates in *E. coli*

Shikimate production from the Chinese star anise is difficult and costly. An alternative method in shikimate production was thus developed. Shikimate production process from glucose presents an excellent alternative to meet the current market volume at a competitive price level. However, shikimate is further converted to aromatic amino acid resulting in difficulty for large-scale production of shikimic acid. In order to improve shikimic acid's availability, metabolic engineering of shikimate pathway was done in *E. coli*.

The shikimate pathway in *E. coli* starts with the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) by DAHP synthase. In *E. coli*, three different DAHP synthase isoenzymes encoded by the *aro*F, *aro*G and *aro*H genes (step 1 in Figure 2.4). Dehydroquinate synthase, encoded by *aro*B (step 2 in Figure 2.4), converts DAHP into 3-dehydroquinate (DHQ). DHQ dehydratase, encoded by *aro*D (step 3 in Figure 2.4), catalyzes the elimination of H_2O to obtain 3-dehydroshikimate (DHS). Subsequently, shikimate dehydrogenase, encoded by *aro*E (step 4 in Figure 2.4), reduces DHS to shikimic acid whereby NADPH is consumed. The shikimate pathway proceeds via formation of shikimate-3-phosphate (S3P), which is catalyzed by two shikimate kinase isoenzymes encoded by *aro*K and *aro*L (step 5 in Figure 2.4), and 5-enolpyruvylshikimate-3-phosphate (EPSP), which is catalyzed by EPSP synthase encoded by *aro*A (step 6 in Figure 2.4). Finally, EPSP is catalzyzed to pathway branch point compound chorismic acid by chorismate synthase encoded by *aro*C (step 7 in Figure 2.4).

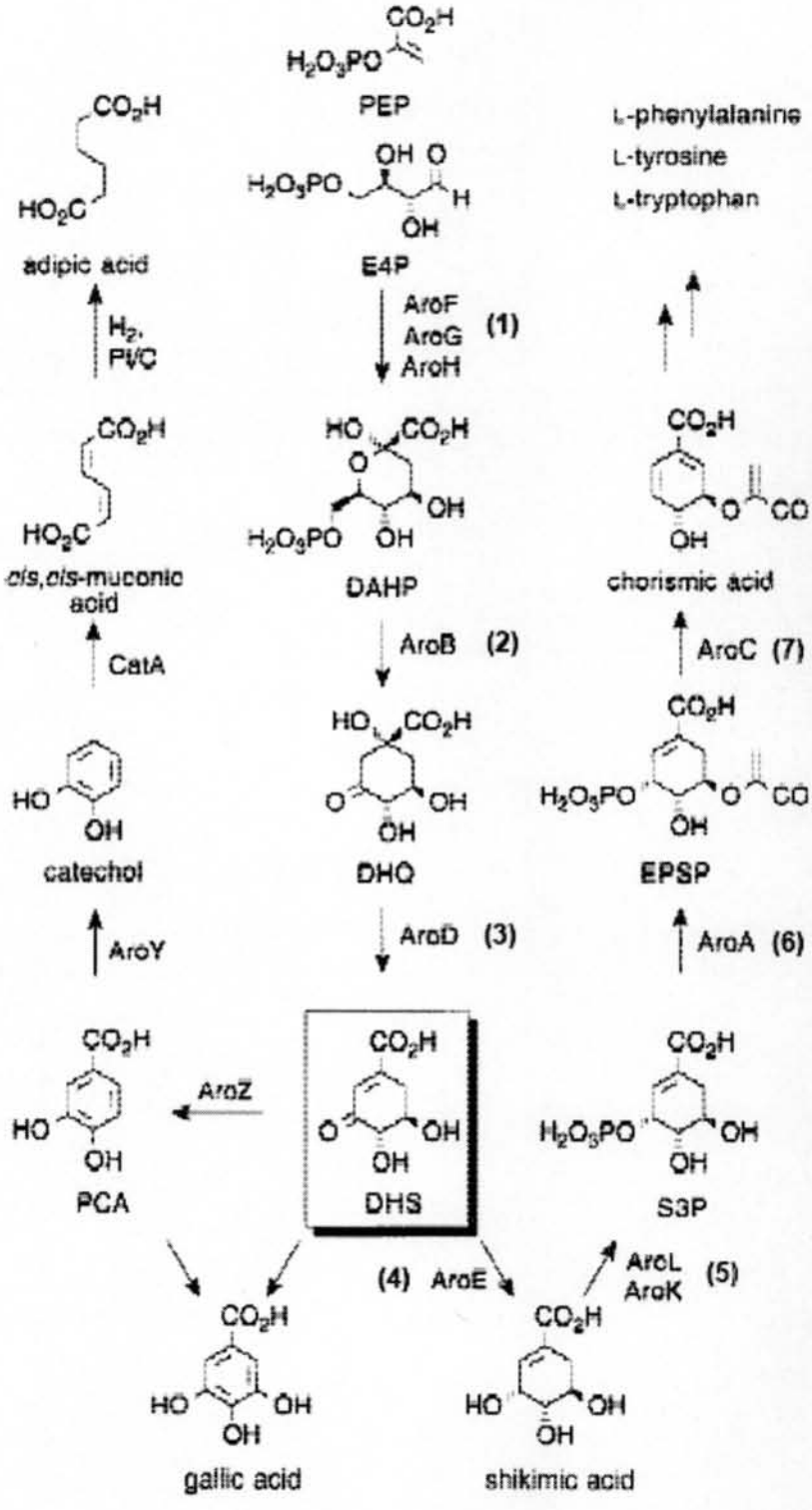

Figure 2.4 : Schematic representation of the aromatic amino acid biosynthesis pathway in *E. coli* (Li *et al.*, 1999).

2.4.1 3-Dehydroshikimate production

It has been repoted that DAHP syntase (step 1 Figure 2.4) activity dictates the amount of cellular carbon directed into DHS synthesis. Transcriptional repression and feedback inhibition of DAHP synthase (step 1 Figure 2.4) by aromatic amino acids have been viewed as the regulatory mechanisms that control the catalytic activity of this enzyme. Therefore, a mutant isozyme of DAHP synthase which is insensitive to feedback inhibition (*aroF*^{fbr}) by aromatic amino acids was constructed. The amount of DAHP synthesized in *E.* coli could reflect the rate at which E4P is produced. Since transketolase catalyzes the interconversion of D-fructose 6-phosphate and E4P, DAHP synthesis may be influenced by increased transketolase activity resulting from expression of extrachromosomal transketolase-encoding gene (*tktA*). Recombinant plasmid pKL4.130B harboring *tktA* and *aroF*^{fbr} genes was transformed into shikimate dehydrogenase (*aroE*) deficient *E. coli* mutant, *E. coli* KL3. DHS titers of 69 g/L were synthesized in 30% yield (mol/mol) Significant concentrations of 3-dehydroquinic acid (6.8 g/L) and gallic acid (6.6 g/L) were synthesized in addition to DHS (Li *et al.*, 1999).

The mechanism employed to transport carbohydrate starting material from the culture medium into the cytoplasm is an important determinant of the yield of a microbially synthesized product. In a wide variety of microbes that employ the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), one molecule of phosphoenolpyruvate is converted into pyruvate for each molecule of glucose transported from the culture medium into the cytoplasm and phosphorylated to form glucose 6-phosphate. Subsequent conversion of pyruvate to CO_2 results in a maximum theoretical yield of 43% (mol/mol), for the conversion of glucose into

DHS. The impact of recycling pyruvate generated by the PTS back to phosphoenolpyruvate on the yield of 3-dehydroshikimic acid synthesized by *E. coli* from glucose was determined. Recombinant plasmid harboring PEP synthase (*ppsA*), *tktA* and $aroF^{fbr}$ genes was transformed into *E. coli* KL3. Shikimate pathway byproducts 3-deoxy-D-*arabino*-heptulosonic acid, 3-dehydroquinic acid, and gallic acid were also generated. the total yield of 3-dehydroshikimic acid and shikimate pathway byproducts synthesized from glucose was 51% (mol/mol) (Yi *et al.*, 2002).

2.4.2 Shikimate production

Microbial production of shikimate was improved by metabolic engineered *E. coli* strains, which carried disrupted *aroL* and *aroK* genes encoding shikimate kinase (step 5 Figure 2.4). In order to increase the carbon flux from the central metabolism into the aromatic amino acid pathway the feedback resistant DAHP synthase gene $aroF^{fbr}$ (step 1 figure 2.4) was introduced. This gene was combined with the *aroB* gene encoding DHQ synthase (step 2 Figure 2.4) in order to circumvent polar effects caused by *aroK* disruption and to overcome the rate-limiting DHQ synthase step. Furthermore, *aroE* gene coding for shikimate dehydrogenase (step 5 Figure 2.4 was introduced.The fermentative production of shikimate was associated with the formation of quinic acid as a side product (Draths *et al.*, 1999).

Approaches to further improve shikimic acid production by *E. coli,* by increasing the availability of PEP and E4P, which condensate at the beginning of the shikimate pathway have also been made. Glucose uptake in *E. coli* is maintained by the PEP consuming phosphoenolpyruvate-phosphotransferase system (PTS). In order to increase the availability of PEP, the PTS was inactivated and replaced by a PEP independent, but ATP dependent uptake and phosphorylation system consisting of the

glucose facilitator (*glf*) and the glucokinase (*glk*) from *Zymomonas mobilis*. In combination with an overexpressed *tktA* gene this resulted in a high shikimic acid titer (71 g/L) and yield (0.27 mol/mol based on glucose) (Chandran *et al.*, 2003).

Although various metabolic engineering approaches increase the production yield of shikimate, many of these, in turn, slow down the growth in *E. coli*. Therefore, the transfer of promising results from lab-scale experiments to industrial processes is still difficult. To solve these problems, many sciencetists attempt to develop a new approach for dehydroshikimate and shikimate production.

Recently, a new approach for dehydroshikimate and shikimate production using quinate has been developed.

2.5 Shikimate production through quinate pathway

Quinate can be utilized by some bacteria such as *Pseudomonas* and other aerobic bacteria. It is used as a growth substrate by fungi as well as by bacteria (Adachi *et al.*, 2003b). Quinate pathway is shown in Figure 2.5. Quinate is oxidized to 3-dehydroquinate, 3-dehydroshikimate and subsequently, to the production of shikimate.

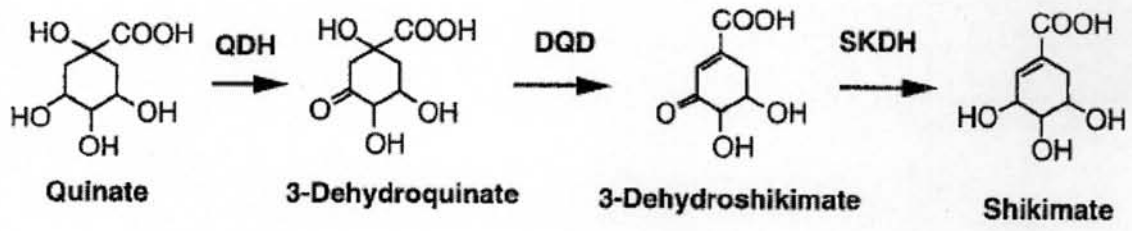

Figure 2.5 : Metabolic map of quinate pathway (Adachi *et al.*, 2003a)

In the first step quinate is oxidized to 3-dehydroquinate by quinate 5-dehydrogenase (synonymous to NAD(P)-dependent quinate dehydrogenase, EC 1.1.1.24) (**QDH**). Then, 3-dehydroquinate is oxidized to 3-dehydroshikimate by dehydroquinate dehydratase (synonymous to 5-dehydroquinase, EC 4.2.1.10) (**DQD**). Finally, 3-dehydroshikimate is oxidized to shikimate by shikimate 5-dehydrogenase (synonymous to 5-dehydroshikimate reductase, EC 1.1.1.25) (**SKDH**).

2.6 Dehydroshikimate and shikimate production in *Gluconobacter* strains

Aerobic microorganisms usually oxidize their carbon sources completely to carbon dioxide and water. During this degradative process, energy and intermediary metabolites required for biosynthesis are generated. Only under special circumstances e.g., excess of carbon substrates, abnormal physiological conditions, or the presence of inhibitory compounds, the oxidation of the substrate sometimes is incomplete. In contrast, some microorganisms oxidize their substrates incompletely even under normal growth conditions. The genus *Gluconobacter*, a member of acetic acid bacteria is known for their rapid and incomplete oxidation of a wide range of sugars and alcohols. The corresponding products are excreted almost completely into the medium. These organisms are able to grow in highly concentrated sugar solutions and at low pH-values. High oxidation rate correlating with low biomass production makes *Gluconobacter* strains interesting organisms for industrial applications.

2.6.1 Dehydroshikimate production in *Gluconobacter*

DHS production from quinate by oxidative fermentation with Gluconobacter strains of acetic acid bacteria was analyzed in 2003 (Adachi *et al.*, 2003a). Dried cells of *Gluconobacter* IFO3244 were found to be useful catalysts for DHS production with 11% of the final yield. The dried cells (1mg/ml) were put into 1% dehydroquinate solution (3 ml) in a test tube, shaken at 30°C (200rpm) for 24 hours. However, shikimate and protochatechuate were always detected.

A method for enzymatic preparation of DHS was established according to quinate pathway (figure 2.5) by controlling the enzyme activity of 3-dehydroquinate dehydratase. For DHS preparation, dried membrane of *G. oxydans* IFO3244 (5 g) was incubated with 1g of quinate in 100 ml McIlvaline buffer pH 8.0 at 30°C with shaking. The final conversion rate from quinate to DHS was more than 90% (Adachi *et al.*, 2006a).

2.6.2 Shikimate production in *Gluconobacter*

Shikimate production by single cell system has been studied (Figure 2.6). Dried cells (3mg) were poured into a 3 ml reaction mixture containing 30 mg dehydroquinate in a test tube, shaken at 30°C (200 rpm) for 24 hours. Shikimate production was proportional to dehydroquinate concentration. Any enzyme yielding NADPH can be coupled as NADPH generating enzyme. Therefore, NADP-dependent glucose dehydrogenase and excess glucose was added to regenerate NADP (Adachi *et al.*, 2003a).

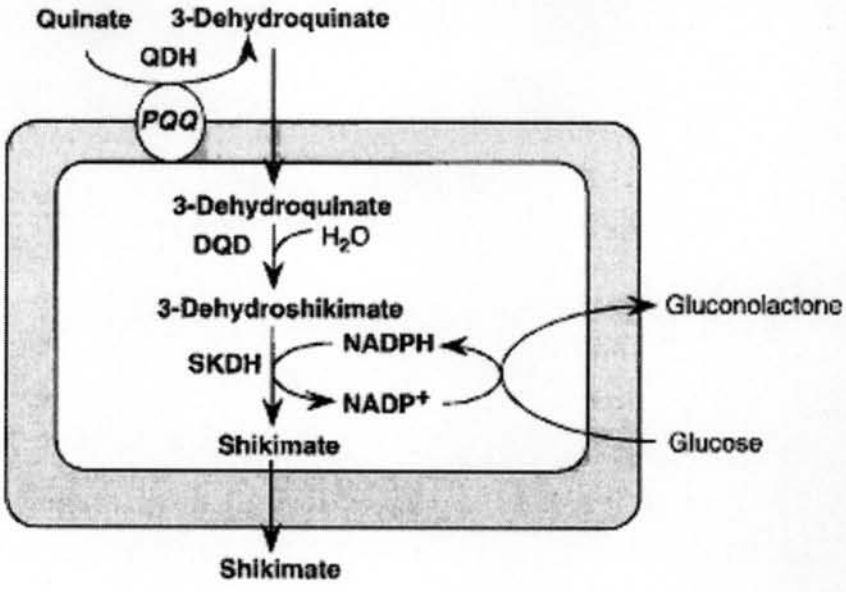

Figure 2.6 : Schematic diagram of shikimate production from quinate by single-cellular system (Adachi *et al.*, 2003a).

In 2006, shikimate was produced by a coupling system using two enzymes, SKDH and NADP-dependent glucose dehydrogenase (GDH), with DHS as a starting material. An excess D-glucose was also added to the reaction mixture as a substrate for GDH for NADP regeneration. The reduction of DHS to shikimate in this reaction at 25°C was completed in 75 minutes yielding approximately 100% shikimate (Adachi *et al.*, 2006b).

2.7 Enzymes in quinate pathway

2.7.1 Quinate dehydrogenase (QDH)

The QDH (NAD(P)-dependent quinate dehydrogenase, EC 1.1.1.24) is an enzyme that catalysts the bioconversion of quinate to 3-dehydroquinate.

The first report of a quinate-oxidizing enzyme in acetic acid bacteria was done by Whiting and Coggins (1967). They indicated that quinate is oxidized to

dehydroquinate by NAD(P)-independent quinate dehydrogenase (QDH) (EC 1.1.99.25). van Kleef and Duine (1988) suggested that QDH is a quinoprotein in which pyrroloquinoline quinine (PQQ) is involved.

The higher QDH activity was detected in a membrane fraction of *Gluconobacter* strains while relatively lower activities were obtained in *Acinetobacter* strains (Vangnai *et al.*, 2004).

2.7.2 Dehydroquinate dehydratase (DQD)

The DQD (EC 4.2.1.10) is an enzyme that catalyzes the dehydration of 3-dehydroquinate to 3-dehydroshikimate (step 3 in Figure 2.4). This reaction is common to two metabolic pathways: the shikimate pathway for the synthesis of aromatic compounds such as aromatic amino acids and ubiquinone and the quinate pathway of fungi, which allows the organism to utilize quinate as a carbon source via the β-oxoadipate pathway.

Two distinct types of enzyme have been described with DQD activity. Type I enzymes, which involve a *syn* elimination reaction, usually catalyze the biosynthetic reaction. In contrast type II enzyme, which can catalyze either the biosynthetic or catabolic reactions, involves an *anti* elimination reaction. The two types of DQD are quite distinct from one another in terms of both amino acid sequences and three-dimensional structures. Type I enzyme is dimers of 27 kDa subunits which contain an $(\alpha/\beta)_8$ fold, whereas type II enzyme is dodecameric with the 16 kDa subunits arranged as tetramers of trimers. Each type II DQD subunit adopts a flavodoxin-type fold, consisting of a five-stranded parallel β-sheet core flanked by four α-helices. In terms of catalytic mechanism, type I enzymes are known to proceed *via* an *imine* intermediate whereas type II enzymes are believed to involve an *enol* intermediate (Evans *et al.*, 2002).

2.7.3 Shikimate dehydrogenase (SKDH)

SKDH (EC 1.1.1.25) catalyzes the fourth reaction in the shikimate pathway (figure 2.4), the NADP-dependent reduction of DHS to shikimate. Whereas dehydrogenases usually form oligomers, SKDH is present as a monomer in most bacteria. In higher organisms this activity is part of a multifunctional enzyme. In plants shikimate dehydrogenase is associated with type I dehydroquinase to form a bifunctional enzyme, whereas in fungi, such as *Neurospora crassa*, this enzyme forms the fifth domain of the pentafunctional AROM polypeptide, which catalyzes five of seven steps of the shikimate pathway.

E. coli has two types of SKDH, AroE and YdiB (Michel *et al.*, 2003). Both enzymes display a similar architecture with two α/β domains separated by a wide cleft. The AroE oxidized shikimic acid using $NADP^+$ as cofactor. The kinetic parameters are very similar for both the cofactor and the substrate (Table 2.1).

In contrast, YdiB is able to oxidize shikimic acid by using either $NADP^+$ or NAD^+ as cofactor. At saturation of shikimate, YdiB displays similar kinetic parameters for both cofactors (Table 2.1). The K_m values significantly differ for the shikimic acid, according to the type of cofactor used at saturation (Table 2.1). Contrary to AroE, YdiB also displays a clear activity on quinic acid, with either $NADP^+$ or NAD^+ as a cofactor. At saturation of quinate, YdiB displays a five times lower K_m for NAD^+ than for $NADP^+$ (Table 2.1). This phenomenon is accentuated for the K_m of quinic acid, which is 10 times lower at saturation of NAD^+ than at saturation of $NADP^+$ (Table 2.1) (Michel *et al.*, 2003).

Recently, a novel class of shikimate dehydrogenase term shikimate dehydrogenase-like (SKDH-L) from *Haemophilus influenzae* has been identified. Phylogenetic and kinetic analyses show that SDH-L is distinct from both AroE and YdiB, yet, all three classes have similar three-dimensional structures. This class of shikimate dehydrogenase was annotated by sequencing projects as shikimate dehydrogenase-like because of the lack of supporting biological and biochemical data. SKDH-L catalyzes the shikimate in the presence of $NADP^+$. The kinetic parameters are showed in Table 2.2. The catalytic rate for shikimate was 1000-fold lower than *E. coli* AroE , but it was comparable with the *E. coli* YdiB (Singh *et al.*, 2005)

Table 2.1 Kinetic parameters of AroE and YdiB

Substrate	AroE		YdiB							
			Quinate saturation		Shikimate saturation		NAD^+ saturation		$NADP^+$ saturation	
	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} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
quinate	-	-	-	-	-	-	41	3	555	3
shikimate	65	1.42x 10^5	-	-	-	-	20	3	120	7
NAD^+	-	-	116	3	87	3	-	-	-	-
$NADP^+$	56	1.42x 10^5	500	3	100	7	-	-	-	-

Table 2.2 Kinetic parameter of SKDH-L

Substrate	SKDH-L	
	K_m (μM)	k_{cat} (s^{-1})
shikimate	234±14	0.2
$NADP^+$	37±6	0.2

2.8 *Gluconobacter* strains

Members of the genera *Gluconobacter*, *Gluconacetobacter* and *Acetobacter* belonging to the family *Acetobacteriaceae* have been used since historic times for vinegar production. All of them are Gram-negative, aerobic, and rod-shaped acidophilic bacteria belonging to the α-subclass of the *Proteobacteria*. *Gluconobacter* strains are distinguished from the genus *Acetobacter* and most *Gluconacetobacter* species by their inability to oxidize acetate and lactate to carbon dioxide. While the production of acetic acid from ethanol is efficient with *Acetobacter* strains, the oxidative activity towards sugars is more pronounced in *Gluconobacter* strains. Another difference between these groups of organisms is that *Gluconobacter* possesses ubiquinone-10, whereas *Acetobacter* strains contain ubiquinone-9 (Deppenmeier *et al.*, 2002).

Gluconobacter strains are strict aerobes. They use a variety of sugars, alcohols and polyols as substrates that are oxidized incompletely. Usually, they grew on media containing high amounts of sugars or polyols supplemented with yeast extract or tryptone. The optimal growth temperature is 25–30°C and the optimal pH is 5.5–6 (Deppenmeier *et al.*, 2002).

2.8.1 *Gluconobacter oxydans*

G. oxydans is a member of the *Acetobacteraceae* family within the alpha proteobacteria and can be isolated from flowers, fruits, and fermented beverages. *G. oxydans* is an obligate aerobe, having a respiratory type of metabolism using oxygen as the terminal electron acceptor. This organism uses membrane-associated dehydrogenases to incompletely oxidize a wide variety of carbohydrates and alcohols. Oxidation occurs in the periplasm with the products being released into the medium via outer membrane porins and the electrons entering the electron transport chain. Since oxidation occurs in the periplasm, alcohols and carbohydrates do not need to be transported into the cytoplasm, therefore *G. oxydans* is able to oxidize large amounts of substrates, making it useful for industrial purposes. Among other applications, it has been used to produce 2-ketogluconic for iso-ascorbic acid production, 5-ketogluconic acid from glucose for tartaric acid production, and L-sorbose from sorbitol for vitamin C synthesis.

2.8.2 *Glucnobacter oxydans* 621H

The *G. oxydans* 621H (DSMZ 2343) genome was determined by a whole-genome shotgun approach using plasmid and cosmid libraries (Prust *et al.*, 2005). The chromosome consists of 2,702,173 base pairs and contains 2,432 open reading frames. In addition, five plasmids were identified that comprised 232 open reading frames. On the basis of the genomic sequence of *G. oxydans*, the process of incomplete oxidation and the physiology of organisms were described. The organism contains many membrane-bound dehydrogenases that are parts of their strategy to thrive and to survive in nutrient-rich environments. The oxidized compounds are taken up and reduced in the cytoplasm, the reactions being catalyzed by a soluble set of oxidoreductases. Therefore, this deposit and withdrawal system of sugars and sugar

alcohols is a clever strategy adopted by *G. oxydans* to survive in mixed microbial populations. Furthermore, incomplete oxidation of glucose and other aldoses, which are abundant in the natural habitats of *G. oxydans*, lead to the formation of sugar acids and to a decrease in the pH value, thereby preventing propagation of many other microorganisms.

The respiratory chain is designed to accelerate incomplete oxidation of glucose and other aldoses because proton-translocating abilities are limited. This prevents an increase in the electrochemical membrane potential that would otherwise lead to the inhibition of membrane-bound redox reactions. However, the low energy-transducing efficiency results in very low growth yields. The inability to degrade glucose and other sugars via the Embden-Meyerhof pathway and the incomplete citrate cycle contribute to the inadequate utilization of the substrates. In summary, *G. oxydans* reveals an extreme adaptation to its nutrient rich habitats by outcompeting other microorganisms. Furthermore, the unique metabolism makes it an ideal organism for microbial process development (Prust *et al.*, 2005).

2.8.3 *Gluconobacter oxdans* IFO3244

In 2003, *G. oxydans* IFO3244 has been studied. They found that QDH in *G. oxydans* IFO3244 was inducible by quinate and was not constitutively expressed in the absence of quinate. The purification of holo-form of QDH to nearly homogeneity was achieved. The purified QDH appears to have two subunits of approximately 65 and 21 kDa, which could be the result of proteolysis of single polypeptide. Kinetic analysis indicated that the purified enzyme is much more specific to quinate than QDH from *Acinetobacter calcoaceticus* (Vangnai *et al.*, 2004).

In 2006, DHS and shikimate production from *G. oxydans* IFO3244 has been studied as decribed above.

2.9 NADPH regeneration system

Many oxidoreductases utilise coenzymes (e.g. NAD^+, $NADP^+$, NADH, NADPH), which must be regenerated as each product molecule is formed. Although these represent many of the most useful biological catalysts, their application is presently severely limited by the high cost of the coenzymes and difficulties with their regeneration. These problems may be overcome if the coenzyme can regenerate *in situ*. A simple way of regenerating coenzymes would be to use whole-cell systems and these are, of course, in widespread use. Normally yeast cells are good catalysts for bioreduction since they are capable of high regeneration of NADPH or NADH. However, when they catalyze the chiral compound synthesis, mixtures of stereoisomeric alcohols are also produced in many cases. To be useful in regenerating coenzymes, enzymic processes must utilise cheap substrates and readily available enzymes and give non-interfering and easily separated products.

The new strategy to regenerate cofactor is subject to a lower effficiency of nicotinamide cofactor generation and regeneration in *E. coli*. Since these nicotinamide cofactors are too expensive to be directly added in large-scale bioprocesses, it is necessary to develop some processes to generate and regenerate $NADP^+$ cofactor effectively in *E. coli* system. Some previous work has been done to produce NADPH by using cytoplasmic enzymes, such as glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), malic enzyme (ME), and NADP-dependent isocitrate dehydrogenase (NADP-IDH), etc. (Barrosoa *et al.*, 1999). Among them, GDH is preferred for the construction of a low-cost NADPH-regenerating system because it catalyzes the oxidation of cheap D-glucose to D-glucono-δ-lactone with concomitant reduction of $NADP^+$ (Figure 2.7).

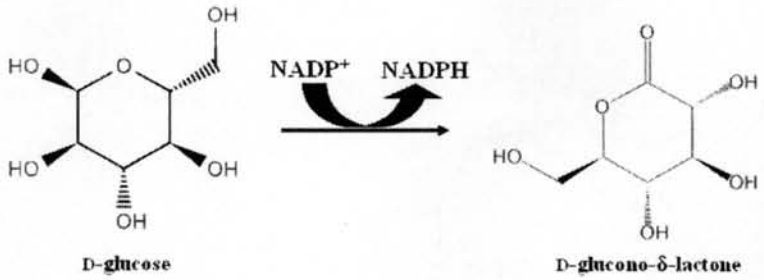

Figure 2.7 : NADP-dependent GDH reaction.

Since the GDH activity in these natural strains cannot meet the demand of its increasing applications, the molecular technique was used to improve GDH activity. In 1999, *E. coli* cells expressing both NADPH-dependent aldehyde reductase (AR) gene from *Sporobolomyces salmonicolor* and the *gdh* gene from *Bacillus megaterium* was studied (Kataoka *et al.*, 1999). The specific activity of AR (pKAR vector) and GDH (pACGD vector) were 1.12 and 16.0 U/mg, respectively. The *E. coli* JM109/pKAR/pACGD was employed in the asymmetric bioreduction of ethyl 4-chloro-3-oxobutanoate (COBE) to (*R*)-4-chloro-3-hydroxybutanoate ethyl ((*R*)-CHBE), which was catalyzed by AR. The CHBE production was occurred in a water-n-butyl acetate two-phase system reaction pH 6.0 with 1820 mM COBE, 0.127 mM $NADP^+$, 1110 mM glucose, and 2.0 g (wet weight) of washed cells of the tramsformant. The (R)-CHBE formed in the organic phase amounted to 1610 mM, with a molar yield of 94.1% and an optical purity of 91.7% enantiomeric excess. In 2007, two glucose dehydrogenase (E.C. 1.1.1.47) genes, *gdh*223 and *gdh*151, were cloned from *Bacillus megaterium* AS1.223 and AS1.151, and were inserted into pQE30 to construct the expression vectors, pQE30-*gdh*223 and pQE30-*gdh*151, respectively. The transformant *E. coli* M15 with pQE30-*gdh*223 gave a much higher GDH activity (2.08 U/mg) than that with the plasmid pQE30-*gdh*151 (0.21 U/mg). Whole cells of M15 (pQE30-*gdh*223) was employed in the bioreduction of COBE to (*R*)-CHBE resulting in the yield of (*R*)-CHBE reach 100% (Xu *et al.*, 2007).