CHAPTER I
INTRODCUTION

1.1 Amino acids

Amino acids are simple organic compounds containing at least one amino group (NH_2) and one carboxylic acid group (COOH). They have served an important role as the building blocks of life since they constitute the biopolymer proteins in all organisms (Wendisch, 2007). Amino acids are compounds of considerable industrial importance, which used as feed and food additives, taste and aroma enhancers, pharmaceuticals or building blocks for drugs, dietary supplements, nutraceuticals and ingredients in cosmetics (Bongaerts et al., 2001).

There are 22 proteinogenic L-amino acids that are genetically encoded for cellular protein synthesis. The essential L-amino acids: L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan and L-valine are not synthesized by humans and animals, but have to be ingested with food or feed. In addition, infants required more amino acids: L-arginine and L-histidine (Wendisch, 2007). Owing to its specific characteristics of L-amino acid especially, chirality, it is greatly important and of interest for chemical, pharmaceutical, cosmetic and agricultural industries. In global market for fermentation products, the largest generating product is ethanol followed by antibiotics. The amino acids are the third most important product (Leuchtenberger, Huthmacher, and Drauz, 2005).

The global market volume of amino acids is increasingly progressive with annual growth rates of 5-7%. The largest segment of the total amino acid in worldwide market is the animal feed amino acids (L-lysine, DL-methionine, L-threonine, and L-tryptophan). The flavoring segment or food segment holds a smaller share. The food sector consists of overall three amino acids L-glutamic acid, L-aspartic acid and L-phenylalanine. L-glutamic acid is particularly used in form of a flavor enhancer monosodium glutamate (MSG). The last two amino acids (L-aspartic acid and L-phenylalanine) are starting materials for the peptide artificial sweetener called as Aspartame (Leuchtenberger et al., 2005).

The remaining proteinogenic amino acids can be synthesized by itself organism with interconversion from each uptaken amino acid or from other metabolic intermediates. These amino acids are required in the pharmaceutical and cosmetics industries and are also ideal raw materials for synthesis of chiral active ingredients. In recent years, the productions of proteinogenic amino acids are widely established by the fermentation processes using high performance strains of *Corynebacterium glutamicum* and *Escherichia coli* from sugar sources such as molasses, sucrose, or glucose while the use of enzyme and whole cell biocatalysts are still remained the valuable preferred production method for nonproteinogenic amino acids and amino acid derivatives (Leuchtenberger et al., 2005).

1.2 Aromatic amino acids

The three proteinogenic aromatic amino acids are L-tryptophan (L-Trp), L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr). They commonly have an aromatic ring structure as a core side group. These aromatic amino acids specially absorb UV light with maximum absorption around 260 nm for L-Phe or 280 nm for L-Tyr and L-Trp. This is the specific basis of an easy spectrophotometric measurement of protein concentrations at wavelength 280 nm (A_{280}) (Layne, 1957). The three aromatic amino acid together averagely constitute less than 10% of proteins. L-Trp is the largest and the rarest of the 20 different proteinogenic amino acids (Sprenger, 2007a).

1.2.1 L-Tryptophan

L-Trp is produced at a multiple thousand-ton scale and predominantly used as animal feed and pharmaceutical purposes as described in Table 1.1. On account of its low abundance in protein compositions and its lability during acidic hydrolytic processes, L-Trp cannot be provided from acidic protein hydrolysates. It can be prepared through biotransformation with *E. coli* cells by catalytic reaction of tryptophanase (Kawasaki, Yokota, and Tomita, 1995) and tryptophan synthase (Bang et al., 1983). However, the majority of L-Trp production is generated by microbial fermentations mainly with *Corynebacterium* strain and *E. coli* (Bongaerts et al., 2001).

Table 1.1 Market volumes of three aromatic amino acids

	Use	Methods of production	Main producers	Market size[#]
L-Tryptophan	Feed additive	Microbial fermentations Biotransformation	Ajinomoto (55% feed-market share)	Feed-use: 6,000 tons per year (tpa)
	Sleep aid (precursor of 5-hydroxytryptophan, serotonin), nutritional therapy, intravenous solutions, dietetic foods, antidepressant	Enzymatic methods Protein hydrolysates	Mitsui Chem. Tanabe Seiyaku Kyowa Hakko Kogyo Archer Daniels Midland Amino Rexim / Degussa	
L-Phenylalanine	Aspartame precursor	Microbial fermentations	Nutrasweet Kelco	Aspartame: 24,000-25,000 tpa (Aspartame 35-40% share)
	Flavour enhancer, infusion fluids	Chemical synthesis	Ajinomoto	
	Dietetical and nutraceutical building block for pharma (rennin inhibitors, HIV protease inhibitor, anti-inflammatory drugs)	Enzymatic syntheses	Miwon HSC (aspartame process) Kyowa Hakko Several Chinese companies	
L-Tyrosine	Raw material for L-DOPA production, treatment of Basedow's disease, dietary supplement	Microbial fermentations Protein hydrolysates	Ajinomoto Kyowa Hakko Daebong LS Tanabe Seiyaku	100 ~ 200 tpa

[#]Estimations for 2011. Source: modified from Sprenger, 2007a; Ajinomoto, 2011: online.

1.2.2 L-Tyrosine

L-Tyr is not an essential amino acid because it can be synthesized in mammals from essential amino acid L-Phe. In the presence of L-Phe source, the conversion of L-Phe to L-Tyr by phenylalanine hydroxylase is taken place. The production scale of this amino acid is rather low (< 200 tons per year, Table 1.1). L-Tyr can be formed via biotransformation by activity of tyrosine phenol-lyase in *Erwinia herbicola* (Lloyd-George and Chang, 1995). Moreover, L-Tyr fermentation has been established by microbial production strain, *Corynebacterium* strain (Ikeda, 2006). L-tyr is potential use for the production of the anti-Parkinson's drug L-3,4-dihydroxyphenylalanine (L-DOPA), for the treatment of Basedow's disease and as a dietary additive.

1.2.3 L-Phenylalanine

L-Phe can be obtained by chemical synthesis from benzaldehyde, glycine and acetic acid anhydride, through extraction from protein hydrolysates, or via enzymatic or microbial processes. L-Phe synthesis by enzyme processes either with whole cells or purified enzymes have been approached by amination of *trans*-cinnamic acid catalyzed by phenylalanine ammonia-lyase (PAL) or transamination of phenylpyruvate catalyzed by aromatic amino acid aminotransferase (AAT) or reductive amination of phenylpyruvate catalyzed by phenylalanine dehydrogenase (PheDH) (de Boer and Dijkhuizen, 1990).

Microbial approach for L-Phe production involves either biotransformations of phenylpyruvate and aspartate by recombinant *E. coli* cells with elevated levels of aminotransferases and phospho*enol*pyruvate (PEP) carboxykinase or fermentations from glucose, sucrose, or molasses (Bongaerts et al., 2001; Ikeda, 2006; Sprenger, 2007a).

Most amino acids are produced by fermentation. This process ensures that only the biologically active L-forms are synthesized (Burkovski and Krämer, 2002). Industrial use of microbial production strains for L-Phe fermentation has been focused especially recombinant strains of *E. coli* and *C. glutamicum* (Bongaerts et al., 2001). L-Phe is an important building block in the pharmaceutical and food additive industries (Bongaerts et al., 2001; Sprenger, 2007a; Wang et al., 2011) as shown in

Table 1.1. Aspartame (α-L-aspartyl-L-phenylalanine methyl ester) is synthesized by condensation biotransformation reaction of two starting amino acids, L-aspartate and L-phenylalanine methylester, catalyzed by thermolysin as shown in Figure 1.1. This enzyme has been isolated from *Bacillus stearothermophilus* and *Thermoactinomycetes thalpophilus* (Majumder and Kanekar, 2012). Aspartame is a low-caloric sweetener which is approximately 200 times sweeter than sucrose and is widely used in foods and beverages in more than 130 countries and regions. (Leuchtenberger et al, 2005; Raval, Vaswani, and Majumder, 2012) with an estimated total market volume of 23,000-24,000 tons in 2011 (Table 1.1). The demand for free L-Phe is steadily growing in recent years to satisfy the needs for its main use in synthesis of aspartame. The beneficial use of L-Phe has resulted in an increased demand for L-Phe and has prompted research to improve its production mainly in *E. coli* (Takors et al., 2001; Liu et al., 2004; Wang et al., 2011).

1.3 Phenylalanine dehydrogenase

Phenylalanine dehydrogenase (PheDH, EC 1.4.1.20) is one of the most interesting amino acid dehydrogenases, which catalyzes the reversible pyridine nucleotide-dependent reductive amination of phenylpyruvate to form L-Phe as shown in Figure 1.2 (Brunhuber and Blanchard, 1994). Much attention has been paid to this enzyme because it is useful as an industrial catalyst in the asymmetric synthesis of L-Phe and related L-amino acids from their keto analogs, and as a clinical reagent for the selective determination of L-Phe and phenylpyruvate (Asano, Nakazawa, and Endo, 1987a).

The PheDH activity was first discovered in *Brevibacterium* species in 1984 (Hummel, Weiss, and Kula, 1984). Consequently, it was found that the enzyme activity was very narrowly distributed in aerobic spore-forming, gram-positive bacteria, *Rhodococcus* sp. M4 (Hummel et al., 1987), *Sporosarcina ureae* (Asano et al., 1987a), *Bacillus sphaericus* (Asano et al., 1987a), *Bacillus badius* (Asano et al., 1987b), *Rhodococcus maris* (Misono et al., 1989) and *Nocardia* sp. 239 (de Boer et al., 1989). The main disadvantage of PheDH activity from mesophilic microorganisms is that its stability is not enough for industrial and clinical application. Therefore, thermophilic microorganisms predominantly conferred

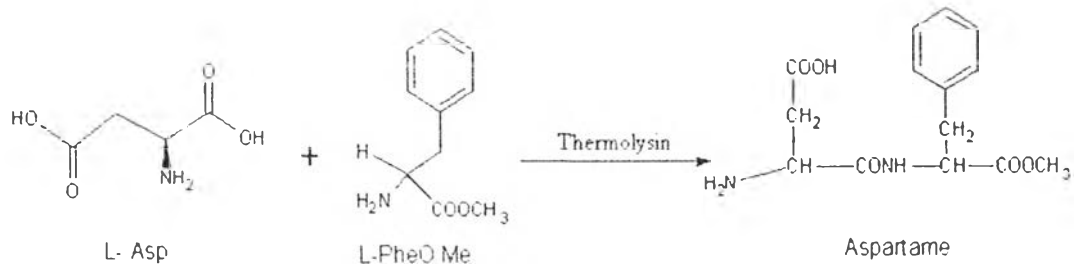

Figure 1.1 Synthesis of Aspartame by thermolysin

Source: Raval et al., 2012

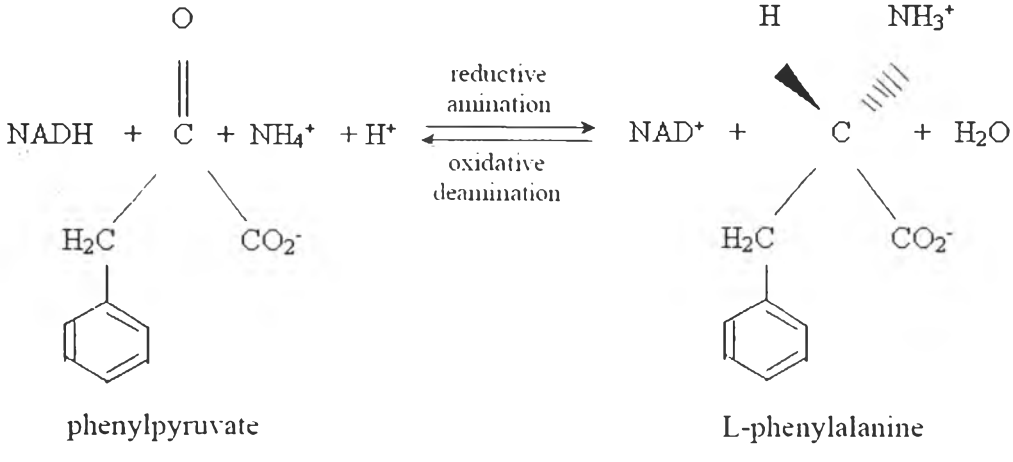

Figure 1.2 The reaction of phenylalanine dehydrogenase

a promising source for biocatalysts with higher stability. The appearance of thermostable PheDH has been reported in *Thermoactinomyces intermedius* (Ohshima et al., 1991).

In 2005, our research group screened for thermotolerant PheDH-producing bacteria from soil samples. Among them, an isolate showing a high activity of PheDH was selected and further identified as *Bacillus lentus*. Later on, the enzyme was purified and characterized for its properties and kinetic mechanism. The enzyme had a molecular mass of about 340,000 Da and consisted of 8 identical subunits. The enzyme showed high substrate specificity in the oxidative deamination on L-Phe and the reductive amination on phenylpyruvate. The optimum pHs for the oxidative deamination and the reductive amination were 10.4 and 8.5 and optimum temperatures were 50 °C and 55 °C, respectively. The enzyme was stable over a broad pH range of 6.0 and 12.0. Furthermore, no loss of the enzyme activity was observed upon incubation at 50 °C, pH 7.4 for 4 h. The enzyme retained 50% of the activity after incubation at the same temperature for 3 days (Inkure, 2005). Once the nucleotide sequence of *B. lentus phedh* was obtained, the gene was cloned into pET-17b resulting in pBLpheDH (Figure 1.3) and transformed into *E. coli* BL21(DE3). The optimum condition for *phedh* gene expression was induction with 0.2 mM IPTG for 8 h. The specific activity of crude recombinant enzyme was 77.9 fold higher than that of the enzyme from *B. lentus* (Thongchuang, 2006).

From the properties of *B. lentus* PheDH, the enzyme is of interest to be used in the industrial synthesis of various amino acids due to its high substrate specificity. However, the use of isolated enzyme increases process cost, especially substrate cost. To overcome this problem, a complete fermentative route using whole cells with renewable and sustainable resources is of commercial interest. In general, the use of renewable waste substrates is an environmental friendly choice that contributes to the reduction of waste treatment costs and increases the economic value of by-products (da Silva, Mack, and Contiero, 2009).

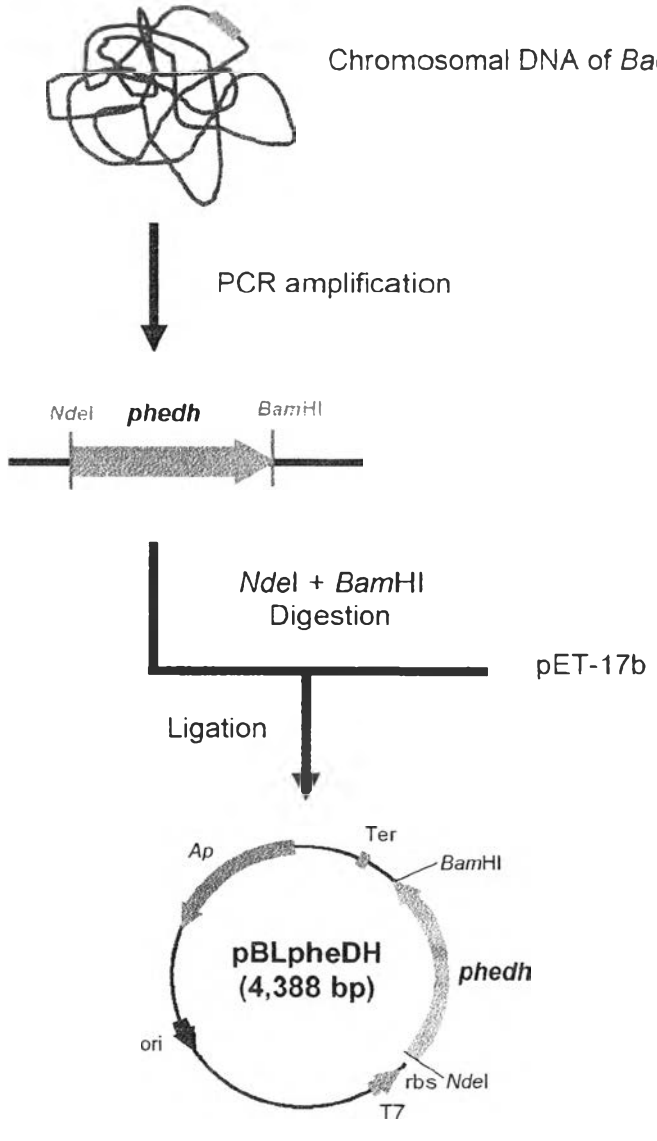

Figure 1.3 The construction of recombinant plasmid pBLpheDH

Source: Thongchuang, 2006

1.4 Use of glycerol as carbon source

Since petroleum oil (or fossil fuel) availability is limited, searching for the new renewable bioenergy source is of major interest. Biodiesel, the most promising source for the substitution of fossil fuels, is produced from vegetable oils such as from rape seeds, soybeans, sunflower seeds or animal fats through transesterification with alcoholysis (methanol or ethanol), generally catalyzed by NaOH or KOH. Inevitably, this process generates an abundant by-product glycerol with a ratio of 10% (w/w) relating to biodiesel produced. The formation of surplus glycerol leads to an environmental problem since it cannot be disposed (da Silva et al., 2009). The conversion of cheaper resources such as glycerol to value-added products can economically support viability of biodiesel industry. Glycerol is used as an ingredient for several productions including cream, food, feed, paper, cosmetic, paint pharmaceutic, textile and leather (Wang et al., 2001).

Nowadays, several microbial fermentations have been attractively adjusted to use glycerol as carbon and energy source based on whether the desired product has predominantly been carried out from glycerol as substrate. Besides, glycerol is higher degree of reduction per carbon than sugars allowing the opportunity to produce reduced chemicals at higher yields than those attained by sugars (Dharmadi, Murarka, and Gonzalez, 2006). Microbial biotechnological production from glycerol have been developed for the production of dihydroxyacetone, 1,2-propanediol, 1,3-propanediol, ethanol, succinic acid, citric acid, 2,3-butanediol, glyceric acid, propionic acid, polyhydroxyalcanoate, pigment, biosurfactant and amino acid.

The biosynthesis of L-glutamate, L-lysine, and L-Phe from glycerol has been reported in engineered *C. glutamicum* and *E. coli* producers. Presently, for *E. coli*, only L-Phe production from glycerol has been accomplished. This result may refer to capability of the other aromatic amino acid productions. The fermentation for L-Phe production was carried out from 1% glycerol using recombinant *E. coli* BL21(DE3) expressing PheDH from *Acinetobacter lwoffii*. A higher yield of L-Phe on glycerol (0.58 g/g) (Khamduang et al., 2009b) was obtained when compared to the use of glucose (0.25 g/g) (Ikeda, 2003). This supports that glycerol tends to be effective and alternative carbon source for microbial fermentation of amino acid biosynthesis. In principle, production of amino acids from glycerol should possibly approach

because relevant pathways for amino acid synthesis start from intermediates of glycolysis (L-valine from pyruvate; L-serine from 3-phosphoglycerate), intermediates of glycolysis and pentose phosphate pathway (aromatic amino acids L-Tyr L-Phe, and L-Trp from phospho*enol*pyruvate and erythrose 4-phosphate), and intermediates of tricarboxylic acid (TCA) cycle (L-glutamate, L-glutamine from 2-oxoglutarate; L-aspartate, L-lysine from oxaloacetate) (Sprenger, 2007a).

A number of microorganisms such as *Citrobacter freundii*, *Klebsiella pneumoniae*, *Clostridium pasteurianum*, *Clostridium butyricum*, *Enterobacter agglomerans*, *Enterobacter aerogenes*, *Lactobacillus reuteri*, *Saccharomyces cerevisia, Debaryomyces hansenii* and *Pichia sorbitophila* (da Silva et al., 2009; Wendisch, Linder, and Meiswinkel, 2011), can utilize glycerol as a sole carbon and energy source. The initial step of glycerol utilization (Figure 1.4) is the uptake of glycerol molecule into the cell. Although the small uncharged molecule can diffuse through membranes without a transport system, various microorganisms possess glycerol transporters. In *E. coli,* glycerol transport is mediated by the glycerol facilitator (GlpF) encoded by the *glpF* gene (Sweet et al., 1990; Voegle, Sweet, and Boos, 1993; Lu et al., 2003). Intracellular glycerol can enter to the central carbon metabolism in form of the glycolytic intermediate dihydroxyacetone-phosphate (DHAP). The routes for the formation of DHAP from glycerol have been divided into two pathways as the glycerol-3-phosphate (G3P) pathway and the dihydroxyacetone (DHA) pathway. In the former pathway, an imported glycerol is phosphorylated to G3P by ATP-dependent glycerol kinase encoded by *glpK* gene and G3P is subsequently oxidized to DHAP by G3P dehydrogenase. Glycerol kinase catalyzes the rate-limiting step in glycerol utilization (Pettigrew et al., 1996). The conversion of G3P to DHAP can be catalyzed by either quinone or FAD-dependent G3P dehydrogenase encoded by the *glpABC* operon in the absence of oxygen and presence of other electron acceptors such as fumarate or NAD-dependent G3P dehydrogenase encoded by *glpD* gene under aerobic condition. In the second pathway, glycerol is oxidized to DHA by glycerol dehydrogenase encoded by *gldA* gene before it is phosphorylated to DHAP by ATP- or phospho*enol*pyruvate (PEP)-dependent dihydroxyacetone kinase encoded by *dhaK* and *dhaKLM* genes, respectively (Gonzalez et al., 2008; Wendisch et al., 2011). In *E. coli,* the pathway via DHA

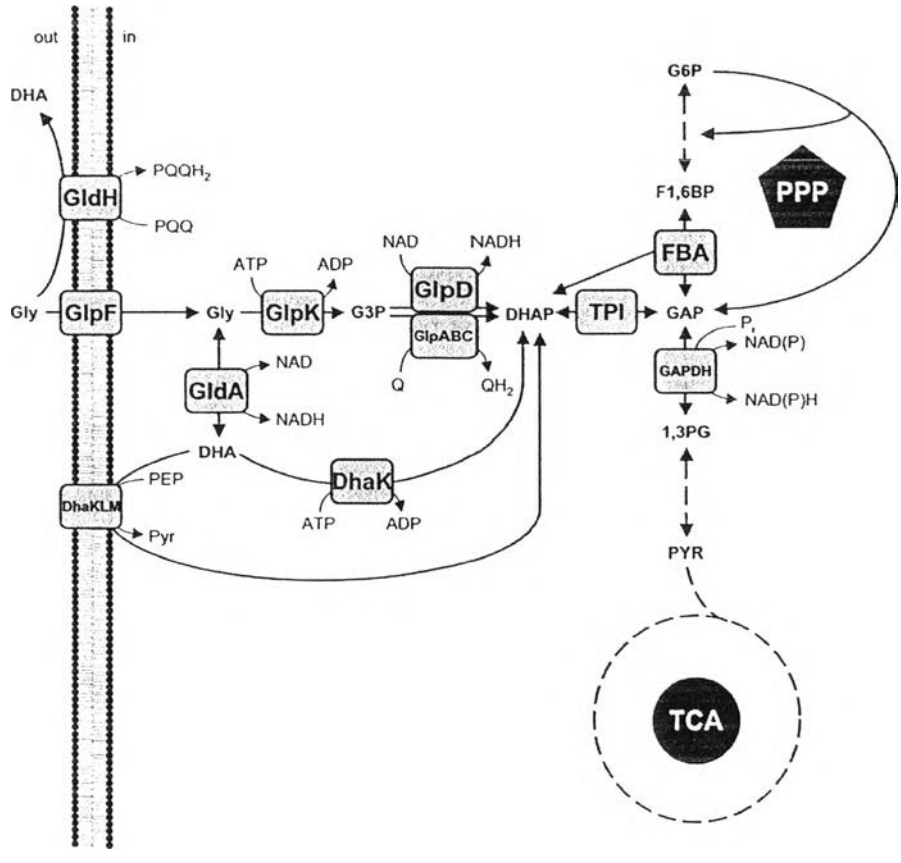

Figure 1.4 Pathways of glycerol utilization in *E. coli*

Abbreviations: 1,3PG, 1,3 phosphoglycerate; DHA, dihydroxyacetone; DhaK, ATP dependent dihydroxyacetone kinase; DhaKLM, PEP dependent dihydroxyacetone kinase; DHAP, dihydroxyacetone-phosphate; F1,6BP, fructose-1,6-bisphosphate; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; GAP; glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GldA, soluble glycerol dehydrogenase; GldH, membrane bound glycerol dehydrogenase; GlpABC, quinone dependent glycerol-3-phosphate dehydrogenase; GlpF, glycerol facilitator; GlpK, glycerol kinase; GlpD, glycerol-3-phosphate dehydrogenase; Gly, glycerol; PEP, phospho*enol*pyruvate; Pi, inorganic phosphate; PPP, pentose phosphate pathway; Pyr, pyruvate; TCA, tricarboxylic acid cycle; TPI, triosephosphate isomerase

Source: Wendisch et al., 2011

operates only under certain anaerobic conditions, while the glycerol-3-phosphate pathway is major under aerobic condition (Wendisch et al., 2011). Both DHAP and G3P intermediates are further metabolized in glycolysis to supply phospho*enol*pyruvate, an initial precursor, for the common aromatic amino acid biosynthetic pathway. Moreover, DHAP and G3P are metabolized via gluconeogenesis to form fructose-1,6-bisphosphate (F1,6BP) which is dephosphorylated by fructose-1,6-bisphosphatase to yield fructose 6-phosphate (F6P). F6P can be shunted from glycolysis to the non-oxidative branch of the pentose phosphate pathway to supply erythrose 4-phosphate, another initial precursor, of the common aromatic amino acid biosynthetic pathway.

1.5 An aromatic amino acid biosynthesis pathway

The biosynthesis of aromatic amino acids in most microorganisms, fungi, or plants is through the common aromatic (amino acid) biosynthetic pathway. This is generally called the shikimate pathway due to the formation of shikimate as an intermediate compound. However, the last common compound in this pathway is chorismate which is served as the precursor for all aromatic amino acids. The shikimate pathway does not appear in animals leading to the fact that L-Trp and L-Phe are essential amino acids for man and most livestock (Sprenger et al., 2007a).

The aromatic amino acid biosynthesis pathway in *E. coli* as depicted in Figure 1.5 is initiated by the condensation reaction between phospho*enol*pyruvate (PEP) from the glycolysis pathway and erythrose 4-phosphate (E4P) from the pentose phosphate pathway to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) catalyzed by three DAHP synthases. This reaction step is committed and most tightly regulated. These isoenzymes are encoded by the genes *aroF*, *aroG*, and *aroH*. The three enzymes (AroF, AroG and AroH) contribute to the overall activity of DAHP synthase. These are subjected to feedback inhibition by individual aromatic amino acids L-Tyr, L-Phe, and L-Trp, respectively. L-Phe-feedback inhibited DAHP synthase (AroG) usually accounts for about 80% of the total DAHP synthase activity and AroG demonstrates a significantly higher resistance to specific proteolysis than other DAHP synthases (Bongaerts et al., 2001). In the second step of the pathway, DAHP is

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converted to 3-dehydroquinate (DHQ) catalyzed by DHQ synthase (encoded by the *aroB* gene). Subsequently, DHQ dehydratase (encoded by *aroD* gene) catalyzes the water elimination from DHQ leading to 3-dehydroshikimate (DHS). DHS is reduced to shikimate (SHIK) by activity of shikimate dehydrogenase (encoded by *aroE* gene) in the following step. SHIK is phosphorylated to yield shikimate 3-phosphate (S3P) by shikimate kinase I and II. These two shikimate kinase isoenzymes are encoded by *aroK* and *aroL* genes, respectively. The *aroL* gene apparently encodes the predominant enzyme (isoenzyme II) with the highest affinity to its substrates. The apparent K_m of this isoenzyme for SHIK is approximately 100-fold lower than the K_m of shikimate kinase I (Defeyter and Pittard, 1986). A second PEP molecule enters the aromatic amino acid pathway in which PEP is condensed with S3P by 5-*enol*pyruvyl shikimate 3-phosphate (EPSP) synthase (encoded by *aroD*) to yield EPSP. The three-carbon fragment introduction of PEP is destined to create the side-chain of L-Phe and L-Tyr afterwards. The further step is catalyzed by chorismate synthase (encoded by *aroC* gene) with a *trans*-1,4 elimination of phosphate from EPSP to form chorismate (CHA). The rate-limiting enzymes for an aromatic amino acid pathway of *E. coli* were identified as DHQ synthase (encoded by *aroB*), shikimate kinase (encoded by *aroL* or *aroK*) by analysis of intermediate metabolite accumulation (Dell and Frost, 1993). The central pathway branches at CHA to permit the terminal pathways that are specific for relevant aromatic amino acid (L-Phe or L-Tyr or L-Trp).

1.5.1 L-Phenylalanine biosynthesis pathway

The pathways toward L-Phe or L-Tyr are diverged at prephenate (PPA) by the action of the bifunctional enzymes chorismate mutase/prephenate dehydratase for L-Phe (encoded by *pheA*) and chorismate mutase/prephenate dehydrogenase for L-Tyr (encoded by *tyrA*). In the terminal pathway of L-Phe biosynthesis, PheA catalyzes the conversion of chorismate to phenylpyruvate (PPY) via prephenate (PPA). It is the key determinant of L-Phe production (Hudson and Davidson, 1984). The PheA activity is feedback-inhibited by L-Phe. The activities of prephenate dehydratase and the chorismate mutase were inhibited by L-Phe with nearly 90% and 55%, respectively. Feedback-resistant mutant for AroG and PheA are well established

to be used in biotechnology for the L-Phe production. The last step of L-Phe biosynthesis is a transamination reaction onto the α-keto acids phenylpyruvate with amino donor glutamate to yield L-Phe, which is catalyzed by three aminotransferases encoded by *tyrB*, *aspC* and *ilvE* genes. Their catalytic reactions are different in detail. The aromatic aminotransferase encoded by *tyrB* is the main enzyme for L-Phe (and L-Tyr) biosynthesis from PPY (and 4-hydroxyphenylpyruvate (HPP)) under normal conditions. In cases of high phenylpyruvate and 4-hydroxyphenylpyruvate pools, the aspartate aminotransferase encoded by *aspC* contributes to the biosynthesis of these amino acids as well. The third aminotransferase, isoleucine aminotransferase encoded by *ilvE*, normally catalyzes the synthesis of L-isoleucine, L-valine, and L-leucine. In *aspC* and *tyrB* deletion mutants, isoleucine aminotransferase is able to perform the synthesis of L-Phe, but not L-Tyr (Bongaerts et al., 2001; Krämer et al., 2003; Müller et al., 2006; Yakandawala et al., 2008; Wendisch et al., 2011).

Regulation of the L-Phe pathway has been studied in detail, and three layers of regulation can be distinguished: feedback inhibition of pacemaker enzymes, repression at the transcriptional level and attenuation at the transcriptional/ translational interface. For L-Phe, the first committed step of aromatic biosynthesis (DAHP synthase) and the step of chorismate mutase/prephenate dehydratase (PheA) are controlled by feedback inhibition exerted by the final product, L-Phe. The intracellular level of the three DAHP synthases in *E. coli* is controlled by transcriptional repression through the repressors TyrR and TrpR, which bind the aromatic amino acids. Deletion of the *tyrR* and *trpR* genes alleviates the transcriptional control. Attenuation is a regulatory element in the biosynthesis of L-Phe where *pheL* gene encoding a leader region is found upstream of *pheA* (Sprenger, 2007b).

1.6 Metabolic engineering for aromatic amino acid production

Typically, the major steps of an attempt to develop a production strain consist of selection of a suitable host organism, deleting branched pathways leading to undesired by-products, deregulation of pathways at both the enzyme activity level and the transcriptional level, and overexpression of enzymes in the desired pathways.

These processes are extensively user-friendly in well-documented microorganisms such as *E. coli* (Patnaik and Liao, 1994). Mostly, the first target for engineering is the terminal pathway leading to the desired product. For example, competing pathway should be knocked out by gene deletions or disruptions resulting in an auxotrophic strain. L-Tyr auxotrophic strains usually have been used to obtain high L-Phe production and to exclude unwanted carbon flux into L-Tyr synthesis. Furthermore, genes encoding feedback-resistant key enzymes (i.e. $aroG^{fbr}$ and $pheA^{fbr}$ genes) have been constructed and overexpressed. Also, combinations of genes on expression plasmids that overcome limitations in pathways are included (Sprenger, 2007b). In addition, the manipulation of central metabolic pathways mediating more supply of necessary precursors and energy for biosyntheses can improve production rate and product yield leads to. As previously mentioned, the precursors of the common aromatic amino acid biosynthetic pathway, PEP and E4P, are derived from central metabolism (Figure 1.6). PEP is formed during glycolysis and the pentose phosphate pathway supplies E4P. The optimization of both the specific biosynthetic pathway and the carbon flux from central carbon metabolism has to be subjected to improve the production of aromatic compounds (Frost and Draths, 1995; Liao et al., 1994). Therefore, attempts to improve supply of either PEP or E4P or of both have been reported (Bongaerts et al., 2001; Ikeda, 2006; Sprenger, 2007b). In the nonoxidative pentose phosphate pathway, the key enzymes are transketolase (encoded by *tktA*) and transaldolase (encoded by *talB*). These enzymes catalyze the reactions mediating the interconversion of metabolic intermediates fructose 6-phosphate (F6P), glyceraldehyde 3-phosphate (GA3P) and E4P linking the glycolysis and the aromatic amino acid pathways. Overproduction of enzymes from the pentose phosphate pathway, such as transketolase or transaldolase, was found to improve E4P supply (Sprenger, 2007b). To improve E4P precursor supply, it was concluded that transketolase is more effective in directing the carbon flux to the aromatic pathway than transaldolase (Liao, Hou, and Chao, 1996). Another attempt to increase E4P availability is an inactivation of phosphoglucose isomerase (*pgi*) in order to block glycolysis, then carbon flux is diverted into the pentose phosphate pathway (Mascarenhas, Ashworth and Chen, 1991). An increasing the PEP supply was successfully approached by knocking out the genes of PEP consuming enzymes, PEP

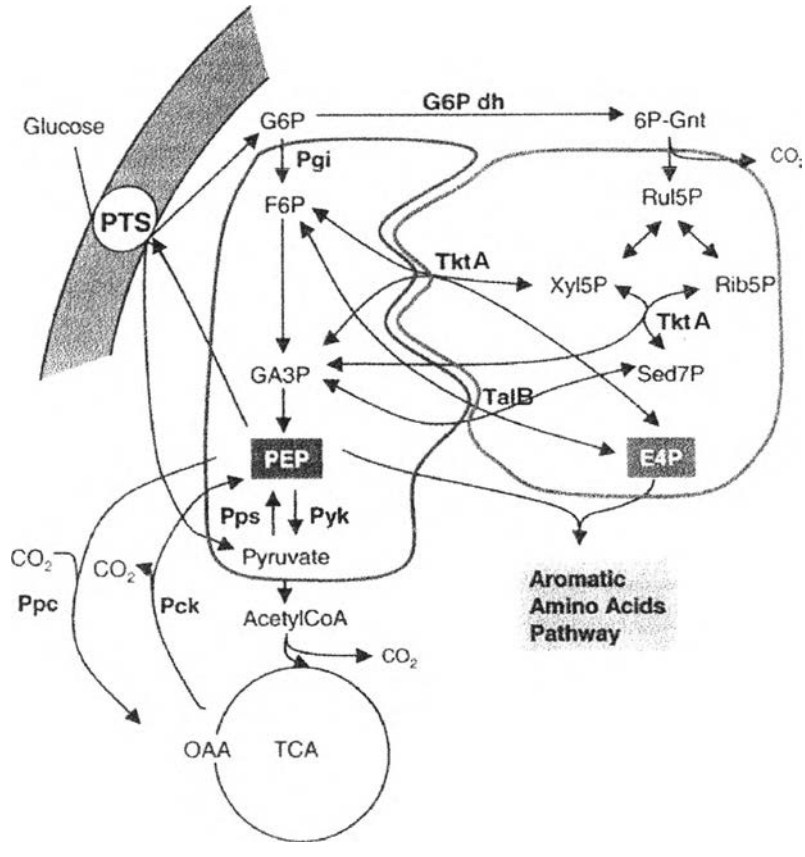

Figure 1.6 Schematic overview of reactions in the central carbon metabolism of *E. coli*. Abbreviations used: PTS, phospho*enol*pyruvate phosphotransferase system; G6P dh, glucose-6-phosphate dehydrogenase; TktA, transketolase; TalB, transaldolase; Pgi, phosphoglucose isomerase; Ppc, PEP-carboxylase; Pyk, pyruvate kinase; Pck, PEP carboxykinase; Pps, PEP synthetase; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; PEP, phospho*enol*pyruvate; OAA, oxaloactetate; 6P-Gnt, 6-phosphogluconate; Rul5P, ribulose 5-phosphate; Rib5P, ribose 5-phosphate; Xul5P, xylulose 5-phosphate; Sed7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate

Source: Bongaerts et al., 2001

carboxylase (*ppc*) or pyruvate kinase (*pyk*), in *E. coli* L-Phe producers (Patnaik and Liao, 1994) or combining PEP carboxylase gene (*ppc*) mutations with overproduction of the gluconeogenic PEP carboxykinase (*pck*) for improvement of carbon flux into the shikimate pathway (Sprenger, 2007b). However, the most limiting factor in PEP supply in *E. coli* is the PEP-dependent sugar phosphotransferase system (PTS), which is responsible for glucose uptake. Fifty percent of PEP generated in glycolysis during growth on glucose has been spent for uptake of glucose and is concomitantly converted into pyruvate. Thus, an exchange of the glucose-PTS for other transport systems, which do not depend on PEP, was studied in *E. coli* mutant strains deficient for the glucose-PTS (Chen et al., 1997). Saving PEP sugar uptake is possible either by activation of a galactose permease (*galP*), which transports glucose independently from PEP (Gosset et al., 1996), or by importing a glucose facilitator (*glf*) from *Zymomonas mobilis*, *E. coli* mutants with a PEP-independent glucose uptake have been reported (Weisser et al., 1995). Another approach to avoid PEP consumption during substrate uptake is to use a non-PTS carbon source, such as xylose, maltose and lactose (Bongaerts et al., 2001). Moreover, the synthesis of L-Phe requires an additional molecule of PEP. It was found that the concentration of E4P is the first limiting substrate for DAHP synthase, followed by PEP (Liao et al., 1996). Notably, the intense alterations of precursor supply could also bring about unwanted by-product formation (e.g. acetate, pyruvate) or growth impairment.

Apart from metabolic pathways and regulatory networks, transport systems are also pivotal for understanding amino acid metabolism and production in bacteria. Apart from substrate uptake, this transport reaction refers to product excretion as well as product re-uptake. These transport systems are relevant for understanding their significance and for providing a basis for rational metabolic design (Marin and Krämer, 2007).

Amino acid transport systems are ubiquitously found in eukaryotes and prokaryotes. The physiological advantage of the presence of amino acid uptake systems for bacterial cells is well studied. Externally available amino acids can be used directly for protein synthesis without spending energy for anabolism. Moreover, they can be served as carbon, energy and/or nitrogen source. This process might be a disadvantage to amino acid production because of re-uptake of the excreted product.

This may result in a waste of metabolic energy and a decreased production rate (Burkovski and Krämer, 2002).

Two of the enzymes, AroG and PheA, involved in the biosynthetic pathway of L-Phe are subject to feedback inhibition by L-Phe, the end product of the pathway. To overcome this, the genes encoding these enzymes have been successfully altered through mutagenesis to produce enzymes that are resistant to feedback inhibition (Nelms et al., 1992; Kikuchi, Tsujimoto, and Kurahashi, 1997; Ger et al., 1994). An alternative route to relieve the feedback inhibition is to reduce intracellular accumulation of L-Phe in *E. coli* by enhancing the cell's ability to export the aromatic amino acid. Among the ~4,500 open reading frames of the *E. coli* genome, approximately 900 (20%) are predicted to encode inner membrane proteins (Díaz-Mejía, Babu, and Emili, 2009). Beside passive diffusion, aromatic amino acids are actively transported by distinct carriers across the cytoplasmic membrane of *E. coli*. The *aroP* gene product transports L-Phe, L-Tyr, and L-Trp, while PheP is a high-affinity transporter specific for L-Phe (Burkovski and Krämer, 2002). Formerly, efflux of aromatic amino acid from producer cells is generally assumed to proceed via simple diffusion since all three compounds are hydrophobic enough to pass the membrane. In 2007, Doroshenko and coworkers reported that YddG protein from *E. coli* promoted export of aromatic amino acids. The *yddG* overexpression enhanced the production of L-Phe, L-Tyr or L-Trp by the respective amino acid-producing *E. coli* strains. On the other hand, the inactivation of *yddG* decreased the aromatic amino acid accumulation. The cells of the L-Phe-producing *E. coli* strain containing overexpressed *yddG* accumulated less L-Phe inside and exported the amino acid at a higher rate than the cells of the isogenic strain containing wild-type *yddG* (Doroshenko et al., 2007). More than 10 exporter proteins have been identified for various amino acids and their analogs such as exporter for L-cysteine, L-lysine, L-threonine, L-valine, L-arginine, aromatic amino acid (L-Tyr, L-Trp, L-Phe) and L-alanine. Many reports have linked overexpression of the genes encoding specific amino acid exporters with enhanced production of the relevant amino acid in *E. coli* (Kruse et al., 2002; Franke et al., 2003; Livshits et al., 2003; Kutukova et al., 2005; Yamada et al., 2006; Doroshenko et al., 2007; Park et al., 2007; Hori et al., 2011).

Up to now, metabolic engineering for the production of aromatic amino acids and derivative compounds has been extensively reported. Engineering of regulatory circuits, amplification and overexpression of genes of the common and terminal pathways, and improvements in the precursor supply have all been targeted (Bongaerts et al., 2001). Metabolic engineering of aromatic amino acid pathways requires first alleviation of all control levels (repression, attenuation and feedback inhibition), to identify and remove rate-limiting steps by the appropriate overproduction of enzymes of the common aromatic amino acid pathway, and then to reduce competing pathways and to improve and balance precursor supply both in the common pathway as well as in the specific branch (Sprenger, 2007b). If degrading enzymes exist (e.g. tryptophanase in *E. coli*), these have to be removed as well (Aiba Tsunekawa, and Imanaka, 1982). Transport and re-uptake of products can cause unwanted futile cycles. This trouble can be overcome by knockout of appropriate transporter genes as shown for L-Trp production in *C. glutamicum* (Ikeda and Katsumata, 1994). In L-Trp-producing *E. coli* strain, each of the three genes (*mtr*, *tnaB*, and *aroP*) of L-Trp uptake system was knocked out. The knockout mutants all showed lower L-Trp uptake activities and higher L-Trp production than their parent (Zhao et al., 2012).

1.7 Metabolic engineering for L-Phe production

Microbial production of L-Phe has been focused mainly on *E. coli*, *C. glutamicum* and *Brevibacterium* strains (de Boer and Dijkhuizen, 1990). Classic methods have been applied to screen for auxotrophs and mutants with feedback deregulated key enzymes. In many cases, the resistance relies on altered allosteric binding sites of the pacemaker enzymes or leads to deregulation of a pathway and product accumulation in the culture supernatant. For classical strain improvements by mutagenesis and selection, economically sufficient product titers for L-Phe had not been achieved for industrial productions (Ikeda, 2006).

L-Phe-producing strains of *E. coli* and *C. glutamicum* have also been constructed with the use of recombinant DNA technology. The strategies used for improved production include amplification of possible rate-limiting enzyme(s) and/or the first enzyme in the common pathway. The rational constructions of *E. coli* L-Phe

producers have been extensively reported via metabolic engineering. Sugimoto and coworkers used a temperature controllable expression vector to conditionally express the deregulated *aroF*^{fbr} and *pheA*^{fbr} genes in an L-Tyr-auxotrophic *E. coli* strain and obtained a maximal L-Phe titer of 16.8 g/L at the optimal temperature of 38.5 °C (Sugimoto et al., 1987). This process was further developed leading to titers of 46 g/L of L-Phe (Konstantinov et al., 1991). Backman and coworkers also engineered *E. coli* for L-Phe production with feedback-resistant PheA (*pheA*^{fbr}) and feedback-resistant AroF (*aroF*^{fbr}) genes, with which a titer of 50 g/L L-Phe with a yield of 0.25 (g L-Phe/g glucose) was obtained after 36 h (Backman et al., 1990). In addition to these strain constructions, central metabolism was also modified for improved L-Phe production. For example, inactivation of PEP carboxylase (*ppc*) has been shown to be effective for L-Phe production by *E. coli*, although such a modification was accompanied by unwanted by-products such as acetate and pyruvate (Miller et al., 1987). Tatarko and Romeo reported a unique approach to engineer a global regulatory network in central carbon metabolism for improved L-Phe production by *E. coli*. A global regulatory gene (*csrA*) was disrupted to cause both increasing flux of gluconeogenesis and decreasing flux of glycolysis resulting in an elevation of the intracellular PEP pool for L-Phe biosynthesis and a twofold increase of L-Phe was obtained (Tatarko and Romeo, 2001). Moreover, the construction of strains for L-Phe production can be approached through a precise chromosomal deletion. The gene cluster *pheA-aroF-tyrA* was removed together with the TyrR repressor site and *pheL* attenuator region to attain the basic double-auxotrophic (L-Phe and L-Tyr) strain F4, which served as host for various plasmid constructions. To enhance L-Phe production, two genes in altered forms (*aroF*^{fbr} and *pheA*^{fbr}) were introduced on a medium-copy-number vector under control of a *lacI*^{q}/Ptac control generating pJF119EH (Fürste et al., 1986). In shake-flask experiments, L-Phe formation was found. Consequently, trial fermentations in a 20-L fermenter (initial volume of 7.5 L; 37 °C) were performed (Gerigk et al., 2002). Two prominent by products of these fermentations were shikimic acid and 3-dehydroshikimic acid indicating that the flux through the aromatic pathway was impeded, most likely by insufficient activity of the shikimate kinases. Therefore, the *aroL* gene (encoding the major shikimate kinase) was cloned as third gene under the control of Ptac promoter to yield plasmid

pF46 (*aroF*^{fbr}-*pheA*^{fbr}-*aroL*). Indeed, the expression in strain F4 led to reduced production of shikimic acid and 3-dehydroshikimic acid. However, L-Phe yields were not improved, pointing to still other limitations in the pathway. Eventually, DAHP and its dephosphorylated derivative DAH were found in culture supernatants. This pointed to a suboptimal activity of the second step of aromatic biosynthesis catalyzed by dehydroquinate synthase (AroB). Therefore, *aroB* was added to the gene construct. Expression of the extra *aroB* gene abolished formation of DAHP and DAH (Rüffer et al., 2004). Although an addition of plasmid-borne extra copies of *aroL* and *aroB* significantly lowered formation of by-products, there was no concomitant increase in overall yields of L-Phe as could have been predicted (Sprenger, 2007b). Though the classical knowledge of strain improvement which states that the pacemaker enzymes should be feedback-resistant, the observations during long-term fermentations led to the idea that the feedback-resistant DAHP synthase should be exchanged with the wild-type AroF form. Expression plasmids that carried the wild-type *aroF* (*aroF*^{wt}) instead of that encoding the feedback-resistant enzyme (AroF^{fbr}) were constructed as plasmid pF69 (*aroF*^{wt}-*pheA*^{fbr}-*aroL*). When the tyrosine feedback-sensitive form was used, L-Tyr feeding had to be strictly limited in the production phase. In fed-batch fermentation with the aid of L-Tyr control, wild-type *aroF* (*aroF*^{wt}) could be used for L-Phe production with higher final L-Phe titers (34 g/L) than the *aroF*^{fbr} strain (28 g/L) by providing higher DAHP synthase activities (Gerigk et al., 2002). If the *aroB* gene was combined as plasmid pF81 (*aroF*^{wt}-*pheA*^{fbr}-*aroL-aroB*), a higher L-Phe titers (up to 38 g/L) could be reached with virtually no by-products from the aromatic biosynthesis pathways and with low acetate formation after 50-h process time (Rüffer et al., 2004). To obtain aromatic amino acid-producing *E. coli* strains including mechanisms for an aromatic amino acid export, engineered *E. coli* strain DV1060 overexpressing *yddG* gene was constructed by Doroshenko and coworkers (Doroshenko et al., 2007). After the fermentation, the accumulation of L-Tyr and L-Phe in the culture medium increased three times, and that of L-Trp elevated 1.5 times.

The potential methods of metabolic engineering applied to the production of L-Phe or other aromatics in *E. coli* are summarized in Figure 1.7.

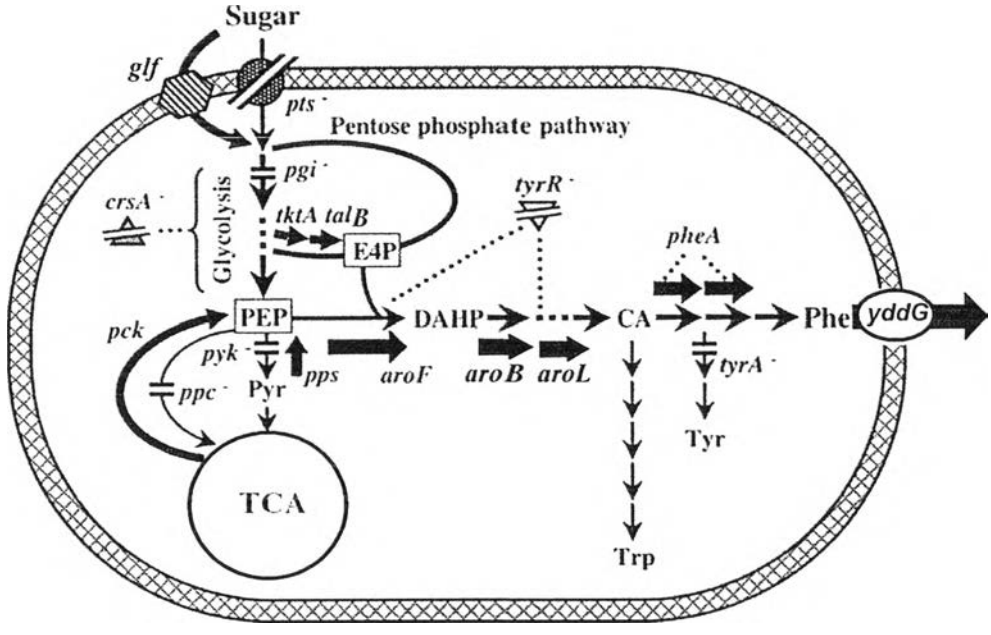

Figure 1.7 Different approaches of metabolic engineering applied to the production of L-Phe or other aromatics in *E. coli*

Abbreviations used: *aroB*, 3-dehydroquinate synthase; *aroF*, L-Tyr-feedback inhibited DAHP synthase; *aroL*, shikimate kinase II; CA, chorismate; *csrA*, carbon storage regulator A; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; E4P, erythrose 4-phosphate; *glf*, glucose facilitator; *pck*, PEP carboxykinase; PEP, phospho*enol*pyruvate; *pgi*, phosphoglucose isomerase; *pheA*, chorismate mutase/prephenate dehydratase; *ppc*, PEP-carboxylase; *pps*, PEP synthetase; *pts*, phospho*enol*pyruvate phosphotransferase system; *pyk*, pyruvate kinase; Pyr, pyruvate; *talB*, transaldolase; *tktA*, transketolase; *tyrA*, chorismate mutase/prephenate dehydrogenase; *tyrR*, tyrosine repressor; *yddG*, aromatic amino acid exporter.

Source: modified from Ikeda, 2006

1.8 Objective of this research

In *E. coli*, phenylpyruvate can be transformed to L-Phe by the activities of three aminotransferases (encoded by *tyrB*, *aspC* and *ilvE* genes) at the last step of L-Phe biosynthesis pathway. Thus, PheDH activity from *B. lentus* is able to link to the activities of the three native aminotransferases through cloning of *phedh* gene into *E. coli*.

Entirely, this work aimed to construct the engineered *E. coli* cells containing *phedh* from *B. lentus* with high efficiency for L-Phe production from glycerol by a process of metabolic engineering. To achieve this goal, *phedh* was overexpressed in combination with some of the following genes:

- *aroB* encoding rate-limiting enzyme, 3-dehydroquinate synthase, in the common aromatic amino acid biosynthesis pathway

- *aroL* encoding rate-limiting enzyme, shikimate kinase II, in the common aromatic amino acid biosynthesis pathway

- *glpF* encoding glycerol facilitator for glycerol uptake

- *glpK* encoding rate-limiting enzyme, glycerol kinase, in glycerol utilization

- *pheA* encoding branch point enzyme, chorismate mutase/prephenate dehydratase, in L-Phe biosynthesis pathway

- *tktA* encoding transketolase for increase in E4P availability

- *yddG* encoding aromatic amino acid exporter for L-Phe excretion

All seven genes are shown in Figure 1.8.

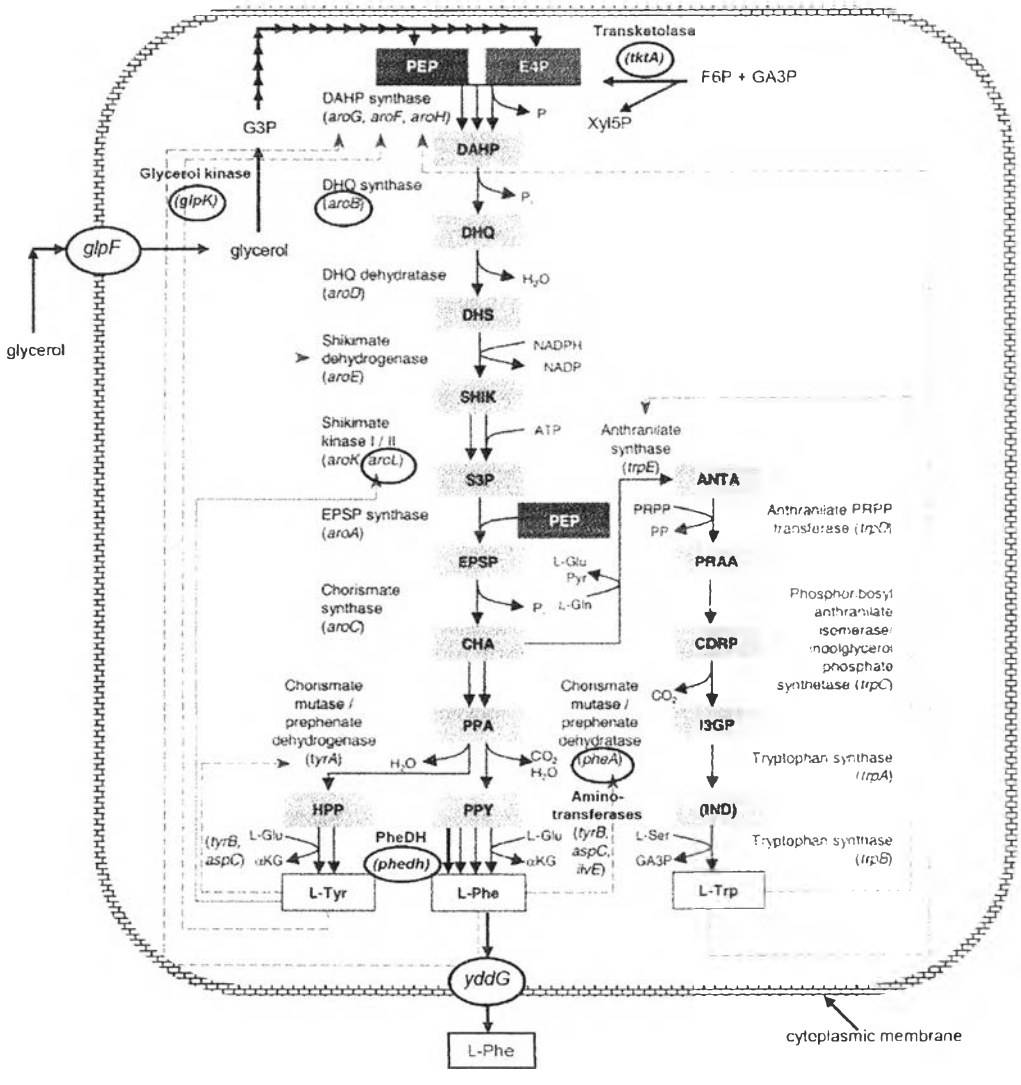

Figure 1.8 All genes that were overexpressed with *phedh* in *E. coli* BL21(DE3) for L-Phe production are shown in oval. Abbreviations used are the same as those described for Figure 1.5. In addition, F6P, fructose 6-phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate

Source: modified from Bongaerts et al., 2001