11 Vitamins and Related Compounds: Microbial Production

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1 Introduction

Vitamins are defined as essential micronutrients that are not synthesized by mammals. Most vitamins are essential for the metabolism of all living organisms, and they are synthesized by microorganisms and plants. Coenzymes (and/or prosthetic groups) are defined as organic compounds with low molecular weight that are required to show enzyme activity by binding with their apoenzymes. Many coenzymes are biosynthesized from vitamins and contain a nucleotide (or nucleoside) moiety in their molecules. Besides their functions as vitamins and coenzymes, most of vitamins and coenzymes have been shown to have various other biofunctions. Accordingly, it is more appropriate to understand both as effective biofactors (see FRIEDRICH, 1988 for basic information).

Most vitamins and related compounds are now industrially produced and widely used as food or feed additives, medical or therapeutic agents, health aids, cosmetic and technical aids, and so on. Thus, vitamins and related compounds are important products for which many biotechnological production processes (i.e., fermentation and microbial/enzymatic transformation) as well as organic chemical synthetic ones have been reported; some of them are now applied for large-scale production. Industrial production methodology, annual production amounts, and fields of application for these vitamins and related compounds are summarized in Tabs. 1–3.

In this chapter, some of the vitamins and related compounds are described from the viewpoint of their microbial production. Previous reviews, from a similar viewpoint, may be useful for further information (DE BAETS et al., 2000; EGGERSDORFER et al., 1996; FLORENT, 1986; SHIMIZU and YAMADA, 1986; VANDAMME, 1989).

2 Water-Soluble Vitamins

2.1 Riboflavin (Vitamin B_2) and Related Coenzymes

Riboflavin is used for human nutrition and therapy and as an animal feed additive. The crude concentrated form is also used for feed. It is produced by both synthetic and fermentation processes [major producers, Hoffmann-La Roche (Switzerland), BASF (Germany), ADM (USA), Takeda (Japan)]. The current world production of riboflavin is about 2,400 t a^{-1}, of which 75% is for feed additive and the remaining for food and pharmaceuticals. Two closely related ascomycete fungi, *Eremothecium ashbyii* and *Ashbya gossypii*, are mainly used for the industrial production (OZBAS and KUTSAL, 1986; STAHMANN et al., 2000). Yields much higher than 10 g of riboflavin per liter of culture broth are obtained in a sterile aerobic submerged fermentation with a nutrient medium containing molasses or plant oil as a major carbon source. Yeasts (*Candida flaeri*, *C. famata*, etc.) and bacteria can also be used for the practical production. Riboflavin production by genetically engineered *Bacillus subtilis* and *Corynebacterium ammoniagenes* which overexpress genes of the enzymes involved in riboflavin biosynthesis reach 4.5 g L^{-1} and 17.4 g L^{-1}, respectively (KOIZUMI et al., 1996; PERKINS et al., 1999). D-Ribose is used as the starting material in the chemical production processes, in which it is transformed to riboflavin in three steps. D-Ribose is obtained directly from glucose by fermentation with a genetically engineered *Bacillus* strain which is transketolase-defective and overexpresses the gluconate operon (DE WULF and VANDAMME, 1997).

Flavin mononucleotide (FMN), a coenzyme form of riboflavin, is synthesized from riboflavin by chemical phosphorylation, after which FMN is crystallized as the diethanolamine salt to separate isomeric riboflavin phosphates and unreacted riboflavin.

The other coenzyme form of riboflavin, flavin adenine dinucleotide (FAD), is used in pharmaceutical and neutraceutical applications. Several tons of FAD are annually pro-

Tab. 1. Industrial Production of Vitamins and Coenzymes

Compound	Production Method			World Production [t a^{-1}]		Use
	Biotechnological	Chemical	Extraction	1980s[a]	1990s[b]	
Thiamin (B_1)		+		1,700	4,200	food, pharmaceutical
Riboflavin (B_2)	+			2,000	2,400	feed, pharmaceutical
FAD	+	+			10	pharmaceutical
Nicotinic acid, nicotinamide	+	+		8,500	22,000	feed, food, pharmaceutical
NAD, NADP	+					technical
Pantothenic acid	+[c]	+		5,000	7,000	feed, food, pharmaceutical
Coenzyme A	+					technical, neutraceutical
Pyridoxine (B_6)		+		1,600	2,550	feed, food, pharmaceutical
Biotin	(+)[d]	+		2.7	25	feed, pharmaceutical
Folic acid		+		100	400	feed, food, pharmaceutical
Vitamin B_{12}	+			12	10	feed, food, pharmaceutical
Vitamin C	+[c]			40,000	60,000	feed, food, pharmaceutical
ATP	+					pharmaceutical, technical
S-Adenosyl-methionine	+					pharmaceutical, nutraceutical
Lipoic acid		+				pharmaceutical
Pyrroloquinoline quinone	+	+				technical
Vitamin A		+		2,500	2,700	feed, food, pharmaceutical
β-Carotene	+		+	100	400	feed, food
Ergosterol	+			25	38	feed, food
Vitamin D_3		+	+		5,000	feed, food
α-Tocopherol (E)	(+)[d]	+	+	6,800	22,000	feed, food, pharmaceutical, nutraceutical
PUFAs[e]	+		+			feed, food, pharmaceutical, nutraceutical
Phylloquinone (K_1)		+			3.5	pharmaceutical
Menaquinone (K_2)		+			500	pharmaceutical
Ubiquinone-10	+					feed, food, pharmaceutical

[a] Values were taken from FLORENT (1986).
[b] Values were taken from EGGERSDORFER et al. (1996).
[c] Hybrid of microbial and chemical reactions.
[d] Parentheses indicate pilot scale process.
[e] PUFAs, polyunsaturated fatty acids.

duced by chemical synthesis or by microbial transformation. The latter uses FMN and adenosine 5′-triphosphate (ATP) as the substrates and *C. ammoniagenes* cells as a source of FMN adenylyltransferase. In this transformation, ATP is generated from adenine and phosphoribosyl pyrophosphate is *de novo* synthesized from glucose by the same organism (see Sect. 2.8). In a similar fashion using the *C. ammoniagenes* ATP generating system, genetically engineered strains of *Escherichia coli* which overexpress flavokinase, and FMN adenylyltransferase can be used as the catalyst in the transformation from riboflavin (KITATSUJI et al., 1992) (Fig. 1).

Tab. 2. Microbial and Enzymatic Processes for the Production of Water-Soluble Vitamins and Coenzymes

Vitamin, Coenzyme	Enzyme (Microorganism)	Method
Vitamin C (2-Keto-L-gulonic acid)	2,5-diketo-D-gulonic acid reductase (*Corynebacterium* sp.)	enzymatic conversion of 2,5-diketo-D-gluconate obtained through fermentative process to 2-keto-L-gulonic, followed by chemical conversion to L-ascorbic acid
Biotin	fermentation (*Serratia marcescens*)	fermentative production from glucose by a genetically engineered bacterium
	multiple enzyme system (*Bacillus sphaericus*)	conversion from diaminopimelic acid using the biotin biosynthesis enzyme system of a mutant of *B. sphaericus*
Pantothenic acid (D-Pantoic acid)	lactonohydrolase (*Fusarium oxysporum*)	resolution of D,L-pantolactone to D-pantoic acid and L-pantolactone by stereoselective hydrolysis
Coenzyme A	multiple enzyme system (*Brevibacterium ammoniagenes*)	conversion by enzymatic coupling of ATP-generating system and coenzyme A biosynthesis system of *B. ammoniagenes* (parent strain or mutant) with D-pantothenic acid, L-cysteine, and AMP (or adenosine, adenine, etc.) as substrates
Nicotinamide	nitrile hydratase (*Rhodococcus rhodochrous*)	hydration of 3-cyanopyridine
Nicotinic acid	nitrilase (*Rhodococcus rhodochrous*)	hydrolysis of 3-cyanopyridine to form corresponding acid (nicotinic acid) and ammonia
NAD	multiple enzyme system (*Corynebacterium ammoniagenes*)	conversion by enzymatic coupling of ATP-generating system and NAD biosynthesis enzymes of *B. ammoniagenes* with adenine and nicotinamide as substrates
NADP	NAD kinase (*Brevibacterium* sp., *Corynebacterium* sp., etc.)	phosphorylation of NAD with ATP as the phosphate group donor
NADH	formic acid dehydrogenase (*Arthrobacter* sp., *Candida boidinii*, etc.)	reduction of NAD with formic acid as the hydrogen donor
NADPH	glucose dehydrogenase (*Bacillus* sp., *Gluconobacter* sp., etc.)	reduction of NADP with glucvose as the hydrogen donor
Riboflavin	fermentation (*Eremothecium ashbyii*, *Ashbya gossypii*, *Bacillus* sp., etc.)	fermentative production from glucose

Tab. 2. Continued

Vitamin, Coenzyme	Enzyme (Microorganism)	Method
FAD	FAD synthetase (*Corynebacterium* sp., *Arthrobacter* sp., etc.)	enzymatic pyrophosphorylation of ATP and flavin mononucleotide synthesized chemically
ATP	multiple enzyme system (baker's yeast, methylotrophic yeasts, *Corynebacterium ammoniagenes*, etc.)	ribotidization of adenine (or adenosine) under coupling of the glycolysis system or methanol oxidation system
S-Adenosylmethionine	S-adenosylmethionine synthetase (*Saccharomyces saké*)	conversion of L-methionine by *S. saké* mutant
S-Adenosylhomocysteine	S-adenosylhomocysteine hydrolase (*Alcaligenes faecalis*)	condensation of adenosine and homocysteine
L-Carnitine	β-oxidation-like enzymes (*Agrobacterium* sp.) aldehyde reductase (*Sporobolomyces salmonicolor*)	conversion of butyrobetaine to L-carnitine enzymatic asymmetric reduction of 4-chloroacetoacetic acid ester to *R*-(−)-3-hydroxy-4-chlorobutanoic acid ester, followed by its chemical conversion to L-carnitine
Pyridoxal-5′-phosphate	pyridoxamine oxidase (*Pseudomonas* sp.)	oxidation of chemically synthesized pyridoxine-5′-phosphate
CDP-choline, GDP-glucose, etc.	CDP-choline pyrophosphorylase, NDP-glucose pyrophosphorylase, etc. (yeasts, etc.)	pyrophosphoric acid condensation of choline (or glucose, etc.) and nucleotide triphosphate (or the corresponding nucleoside)
Vitamin B_{12}	fermentation (*Propionibacterium shermanii*, *Pseudomonas denitrificans*, etc.)	fermentative production from glucose
Pyrroloquinoline quinone (PQQ)	fermentation (methanol-utilizing bacterium)	fermentative production from methanol

2.2 Nicotinic Acid, Nicotinamide, and Related Coenzymes

The world production of nicotinic acid and nicotinamide is estimated to be 22,000 t a^{-1} [major producers, BASF, Lonza (Switzerland) and Degussa (Germany)]. The major use (ca. 75%) is for animal nutrition and the remaining for food enrichment and pharmaceutical application. Chemical processes involving oxidation of 5-ethyl-2-methylpyridine or total hydrolysis of 3-cyanopyridine are used for nicotinic acid production. Bacterial nitrilase has been shown to be useful for the same purpose (Fig. 2a). For example, 3-cyanopyridine is almost stoichiometrically converted to nicotinic acid (172 g L^{-1}) on incubation with the nitrilase-overexpressed *Rhodococcus rhodochrous* J1 cells (NAGASAWA and YAMADA, 1989). The same *R. rhodochrous* enzyme can be used for the production of *p*-aminobenzoic acid from *p*-aminobenzonitrile.

Tab. 3. Microbial and Enzymatic Processes for the Production of Fat-Soluble Vitamins

Vitamin	Enzyme (Microorganism)	Method
Vitamin E and K_1 side chains	multiple enzyme system (*Geotrichum candidum*)	enzymatic conversion from (*E*)-3-(1′,3′-dioxolane-2′-yl)-2-butene-1-ol
[(*S*)-2-methyl-γ-butyrolactone]	reductase bakers' yeast, (*Geotrichum* sp., etc.)	asymmetric reduction of ethyl-4,4-dimethoxy-3-methylcrotonate
[(*S*)-3-methyl-γ-butyrolactone]	multiple enzyme system (*Candida* sp., etc.)	stereoselective oxidation of isobutyric acid
[(*S*)- or (*R*)-β-hydroxy-isobutyric acid]		
Vitamin K_2	multiple enzyme system (*Flavobacterium* sp.)	conversion of quinone- and side chain-precursors to the vitamin
Arachidonic acid	fermentation (*Mortierella alpina*)	fermentative production from glucose
Dihomo-γ-linolenic acid	fermentation (*Mortierella alpina*)	fermentative production from glucose by a Δ5-desaturase-defective mutant
Mead acid	fermentation (*Mortierella alpina*)	fermentative production from glucose by a Δ12-desaturase-defective mutant
Eicosapentaenoic acid	multiple enzyme system (*Mortierella alpina*)	Δ17-desaturation of arachidonic acid or conversion from α-linolenic acid

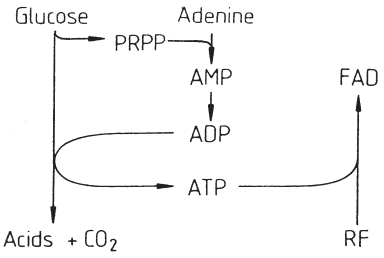

Fig. 1. Schematic representation for the FAD production from riboflavin (RF) coupled with bacterial ATP-generating system (see also Fig. 8).

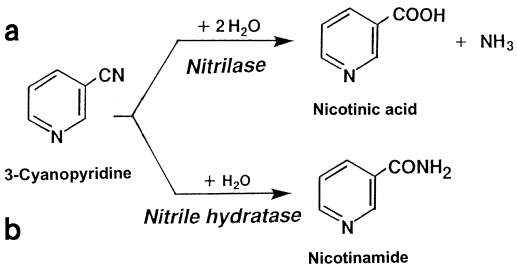

Fig. 2. Transformation of 3-cyanopyridine to nicotinic acid by nitrilase **a** and nicotinamide by nitrile hydratase **b**.

Nicotinamide is available from partial hydrolysis of 3-cyanopyridine, which is performed by both chemical and enzymatic processes. The enzymatic process uses nitrile hydratase as the catalyst (Fig. 2b). This novel enzyme catalyzing a simple hydration reaction was discovered as one of the responsible enzymes for the two-step transformation of nitriles to acids via amides (ASANO et al., 1980). Extensive studies of this enzyme as well as screening of the enzyme from a variety of microbial strains revealed the presence of several different types of nitrile hydratases, especially Co- and Fe-containing enzymes, in various bacteria (KOBAYASHI et al., in press). The Co-containing enzyme from *R. rhodochrous* J1 hydrates various kinds of aliphatic and aromatic nitriles to the corresponding amides and has been shown to be useful for the production of useful amides (YAMADA and KOBAYASHI, 1996). For example, using the bacterial cells containing highly elevated amounts of this enzyme exceeding 50% of the total cellular proteins, 1.23 kg of 3-cyanopyridine suspended in 1 liter of water are

stoichiometrically converted to 1.46 kg of nicotinamide crystals (NAGASAWA and YAMADA, 1989). Based on these studies, Lonza (Switzerland) has constructed a plant for the commercial production of nicotinamide in China in 1997. This enzymatic process surpasses the chemical process in regard to several points such as stoichiometric conversion of high concentration of the substrate and the quality of the product actually with zero contents of byproducts. The same enzyme has been used for the industrial production of acrylamide from acrylonitrile by Nitto (Japan) since 1991.

Nicotinamide adenine dinucleotide (NAD) is used in pharmaceutical application and as a reagent for clinical analysis. Nicotinamide adenine dinucleotide phosphate (NADP) is also used for analysis. Practical production of NAD is carried out by extraction. Yeasts such as *Saccharomyces cerevisiae* are favorable sources of NAD. It is also produced by microbial transformation utilizing the salvage pathway for the biosynthesis of NAD from nicotinamide (or nicotinic acid) and ATP. On cultivation of *Corynebacterium ammoniagenes* with the precursors, nicotinamide and adenine, the amount of NAD in the medium reaches 2.3 mg mL^{-1} (NAKAYAMA et al., 1968). For the mechanism involved in the transformation it has been suggested that both precursors are first ribotidated to nicotinamide monophosphate and ATP, respectively, which are then converted to NAD by pyrophosphorylation (for ATP generation, see Sect. 2.8). NADP can be prepared by enzymatic phosphorylation. Reduced forms of these coenzymes, NADH and NADPH, can be obtained by both chemical and enzymatic methods. The latter uses formate dehydrogenase from methanol-utilizing yeasts for NADH. Glucose dehydrogenase from *Bacillus* sp. is also used for both NADH and NADPH. *In situ* regeneration of these coenzymes is currently attracting more attention for the production of chiral alcohols from prochiral carbonyl compounds with carbonyl reductases (Fig. 3). For example, genetically engineered *E. coli* cells overexpressing glucose dehydrogenase from *Bacillus* sp. and aldehyde reductase from *Sporobolomyces salmonicolor* effectively catalyze stereospecific reduction of ethyl 4-chloro-3-oxobutanoate to ethyl *R*(−)-4-chloro-3-hydroxybutanoate in the presence of glucose and a catalytic amount of NADP (SHIMIZU and KATAOKA, 1999a; SHIMIZU et al., 1997).

2.3 Pantothenic Acid and Coenzyme A

About 6,000 t of calcium D-pantothenate are produced annually. It is mainly used as an animal feed additive (80%). It is also used for pharmaceutical, health care and food products. D-Pantothenyl alcohol (1,000 t a^{-1}) is also used for the same purposes. The commercial production process involves reactions yielding racemic pantolactone from isobutyraldehyde, formaldehyde, and cyanide, optical resolution of the racemic pantolactone to D-pantolactone, and condensation of D-pantolactone with β-alanine to form D-pantothenic acid. 3-Aminopropanol is used for D-pantothenyl alcohol [major producers, Hoffmann-La Roche, Fuji (Japan), and BASF]. The conventional optical resolution which requires expensive alkaloids as resolving agents is troublesome. Recently, an efficient enzymatic method has been introduced into this optical resolution step (SHIMIZU et al., 1997). This enzymatic resolution uses a novel fungal enzyme, lactonohydrolase, as the catalyst. The enzyme catalyzes stereospecific hydrolysis of various kinds of lactones. D-Pantolactone is a favorable substrate of this enzyme, but the L-enantiomer is not hydrolyzed

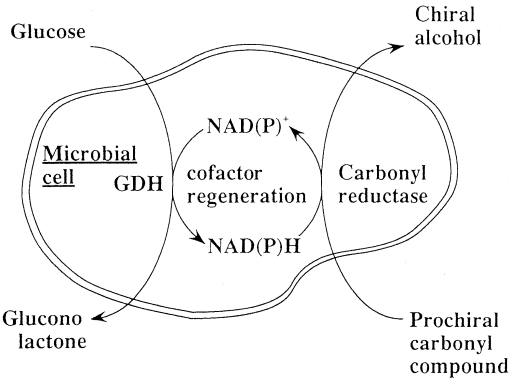

Fig. 3. *In situ* NAD(P)H regeneration with glucose dehydrogenase (GDH) for the stereospecific reduction of prochiral carbonyl compounds to chiral alcohols.

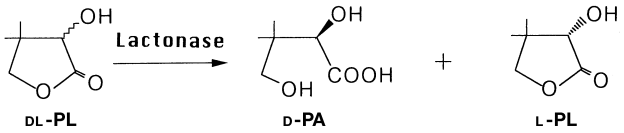

Fig. 4. Principle of the optical resolution of D,L-pantolactone by fungal lactonase. PL, pantolactone; PA, pantoic acid.

at all (SHIMIZU et al., 1992). Thus, the racemic mixture can be separated into D-pantoic acid and L-pantolactone (Fig. 4). As this lactonase reaction is an intermolecular ester bond hydrolysis, the pantolactone as the substrate needs not to be modified for resolution, which is one of the practical advantages of the use of this enzyme. Several filamentous fungi of the genera *Fusarium*, *Gibberella*, and *Cylindrocarpon* show high activity of this enzyme. On incubation with *Fusarium oxysporum* cells for 24 h at pH 7.0, D-pantolactone in a racemic mixture (700 g L^{-1}) is almost completely hydrolyzed to D-pantoic acid (96% ee) (KATAOKA et al., 1995a, b). Practically, this stereospecific hydrolysis is carried out by *F. oxysporum* cells immobilized with calcium alginate gels. When the immobilized cells were incubated in a racemic mixture (350 g L^{-1}) for 21 h at 30 °C, 90–95% of the D-pantolactone was hydrolyzed to D-pantoic acid (90–97% ee). After repeated reaction for 180 times (i.e., 180 d), the immobilized cells retained about 90% of their initial activity (SHIMIZU and KATAOKA, 1996, 1999b; SHIMIZU et al., 1997). The overall process for this enzymatic resolution is compared with the conventional chemical process in Fig. 5. The enzymatic process can skip several tedious steps which are necessary in the chemical resolution. Based on these studies, Fuji (Japan) changed over their chemical resolution with this enzymatic resolution in 1999.

Several enzymatic methods to skip this resolution step have also been reported. The two-step chemicoenzymatic method, which involves a one-pot synthesis of ketopantolactone and its stereospecific reduction to D-pantolactone (SHIMIZU and YAMADA, 1989b; SHIMIZU et al., 1997) is practically promising. The chemical synthesis is performed in one step from isobutyraldehyde, sodium methoxide, diethyl oxalate, and formalin at room temperature

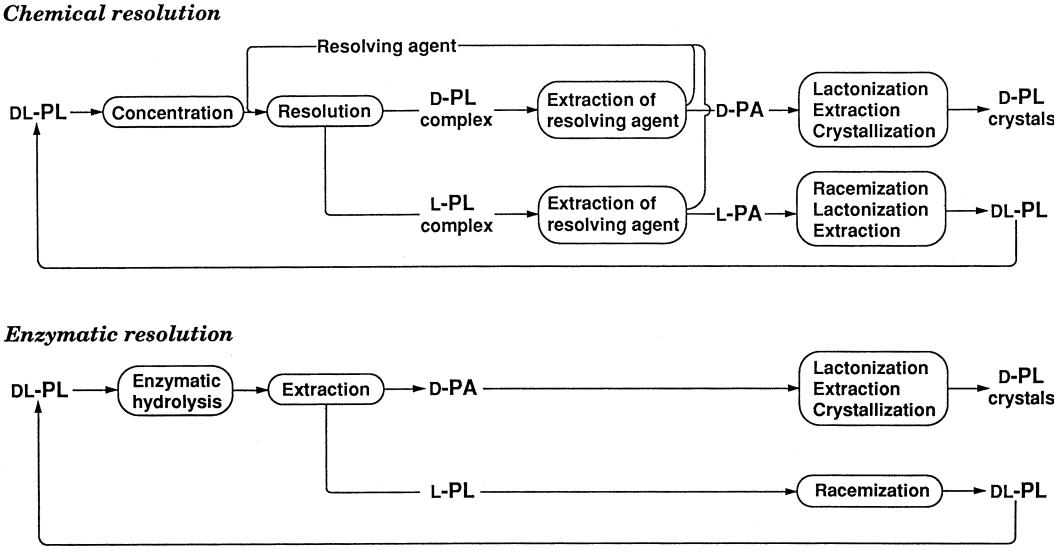

Fig. 5. Comparison of chemical and enzymatic methods for the optical resolution of D,L-pantolactone. For abbreviations, see Fig. 4.

with a yield of 81%. The bioreduction is performed in the presence of glucose as an energy source for the reduction and *Candida parapsilosis* cells with high carbonyl reductase activity as the catalyst. In this bioreduction, ketopantolactone is stoichiometrically converted to D-pantolactone (90 g L^{-1}, 94% ee) with a molar yield of 100% (HATA et al., 1987). Alternatively, ketopantoic acid, which is easily obtained from ketopantolactone by spontaneous hydrolysis under mild alkaline conditions, can be used as the substrate for the stereospecific bioreduction. In this case, *Agrobacterium* sp. cells with high activity of ketopantoic acid reductase are used as the catalyst. The yield of D-pantoic acid was 119 g L^{-1} (molar yield, 90%; optical purity, 98% ee) (KATAOKA et al., 1990). The chemical step can be replaced by an enzymatic one using L-pantolactone dehydrogenase of *Nocardia asteroides*. The enzyme specifically oxidizes the L-isomer in a racemic pantolactone mixture to ketopantolactone, which is then converted to D-pantolactone or D-pantoic acid by the above mentioned reduction with *C. parapsilosis* or *Agrobacterium* sp., respectively (KATAOKA et al., 1991a, b; SHIMIZU et al., 1987) (Fig. 6).

A direct fermentation process for D-pantoic acid and/or D-pantothenic acid is also promising. A genetically engineered strain of *E. coli* overexpressing pantothenic acid biosynthesis enzymes produced 65 g L^{-1} D-pantothenic acid from glucose upon addition of β-alanine as a precursor (HIKICHI et al., 1993).

Coenzyme A (CoA) is used as an analytical reagent and for pharmaceutical, neutraceutical, and cosmetic applications. A successful microbial transformation method uses *Brevibacterium ammoniagenes* cells, in which all five enzymes necessary for the biosynthesis of CoA from D-pantothenic acid, L-cysteine, and ATP abundantly occur, as the catalyst, and these three precursors as the substrates (SHIMIZU and YAMADA, 1986, 1989b). On cultivation of the bacterium in a medium containing glucose (10%), D-pantothenic acid, L-cysteine and AMP (or adenine), from which ATP is effectively generated by the same bacterium (see Sect. 2.8), the yield of CoA was 3–6 g L^{-1}. Higher yields (ca. 20 g L^{-1}) were obtained when 4′-phosphopantothenic acid was used in place of D-pantothenic acid or oxypantetheine-resistant mutants which were free from the feedback inhibition of pantothenate kinase by CoA were used as the catalyst (SHIMIZU et al., 1984). In a similar manner, all the intermediates involved in the biosynthesis of CoA from D-pantothenic acid, i.e., 4′-phosphopantothenic acid, 4′-phosphopantothenoylcysteine, 4′-phosphopantetheine, and 3′-dephospho-CoA, can be prepared (SHIMIZU and YAMADA, 1986, 1989b).

2.4 Pyridoxine (Vitamin B_6)

Vitamin B_6 compounds, mainly pyridoxine and pyridoxal 5′-phosphate, are exclusively produced by chemical synthesis [ca. 2,500 t a^{-1}; major producers, Takeda, Hoffmann-La Roche, Fuji/Daiichi (Japan)]. They have many pharmaceutical and feed/food applications. Recent chemical and molecular biology studies revealed that 1-deoxy-D-xylulose and 4-hydroxy-L-threonine are the precursors for the biosynthesis of pyridoxine (TAZOE et al., 2000), but its complete biosynthetic pathway is not known in detail. Screening for vitamin B_6 producers among microorganisms found several potential strains, such as *Klebsiella* sp., *Flavobacterium* sp., *Pichia guilliermondii*, *Bacillus subtilis*, *Rhizobium meliloti* and so on.

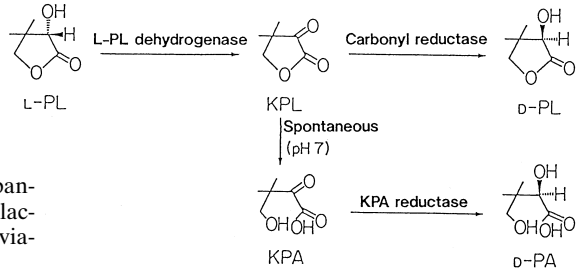

Fig. 6. Enzymatic routes for the synthesis of D-pantolactone via ketopantolactone. KPL, ketopantolactone; KPA, ketopantoic acid. For other abbreviations, see Fig. 4.