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review

Biotechnological Production of Vitamins

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Summary

Vitamins are defined as essential micronutrients that are required in trace quantity and cannot be synthesized by mammals. Apart from their *in vivo* nutritional and physiological roles as growth factors for men, animals, plants and microorganisms, vitamins are now being increasingly introduced as food/feed additives, as medical-therapeutic agents, as health aids, and also as technical aids. Production of vitamins by chemical synthesis, or extraction from their known sources has serious disadvantages. This led to an increased interest in substituting these processes with biotechnological processes. For several of these compounds microbiological and algal processes exist, or are rapidly emerging. Different methods like media optimization, mutation and screening, genetic engineering and biocatalyst conversion have been used for improvement of the production of vitamins. The survey describes the current state of vitamin production by biotechnological processes and their significance, as compared to the existing chemical processes.

Key words: vitamins, fermentation, biotechnological production

Introduction

Vitamins, defined as essential micronutrients required in trace quantities that cannot be synthesized by mammals, are essential for metabolism of all living organisms and are synthesized by microorganisms or plants. Apart from their *in vivo* nutritional-physiological roles as growth factors for men, animals, plants and microorganisms, vitamins are now increasingly being introduced as food/feed additives, as medical-therapeutic agents, as health aids, and also as technical aids. Today, many processed foods, feeds, pharmaceuticals, cosmetics, and chemicals contain extraneously added vitamins or vitamin–related compounds.

Most of the vitamins are now industrially produced and widely used in foods, pharmaceuticals and cosmetics. Presently, few of the vitamins are exclusively produced *via* chemical synthesis, while a few others are produced either by chemical synthesis or *via* extraction processes. These processes are energy-intensive, and also suffer from high cost of waste disposal. Furthermore, these processes have growing consumer consciousness with regard to food additive safety. This has led to increased interest in substituting these processes with biotechnological processes. Consequently, biotechnological processes for the production of most of these compounds are rapidly emerging and some are already competing with the existing chemical processes. A list of vitamins produced by biotechnological methods is summarized in Table 1.

Fat-Soluble Vitamins

Of the four fat-soluble vitamins, *viz.* vitamins A, D, E and K, some success has been obtained with biotechnological production of vitamins E and K. These are discussed below:

Vitamin E (α-tocopherol)

Vitamin E comprises a group of lipid-soluble compounds, among which α-tocopherol is the most abundant

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Table 1. Vitamins produced by biotechnological methods

Vitamin	Biotechnological method		Reference
	Enzyme/ microorganism used	Method	
Fat-soluble vitamins			
Vitamin E (α-tocopherol)	Freshwater microalgae *Euglena gracilis*	Fermentative production from glucose	(*1*)
Vitamin K_2	Mutated strain of *Bacillus subtilis*	Fermentation using soybean extract	(*2*)
Water-soluble vitamins			
Ascorbic acid (vitamin C)	2,5-diketo-D-gluconic acid reductase *Cynobacterium* sp.	Fermentative process to 2-keto-L-gulonic acid followed by chemical conversion to L-ascorbic acid	(*3,4*)
Biotin	Fermentation (*Serratia marcescens*)	Fermentative production from glucose by genetically engineered bacterium	(*5*)
	Multiple enzyme system (*Bacillus sphaericus*)	Conversion from diaminopimelic acid using the biotin biosynthetic enzyme system of mutant of (*Bacillus sphaericus*)	
Riboflavin	Fermentation (*Eremothecium ashbyii, Ashbya gossypii, Bacillus* sp., *etc.*)	Fermentative production from glucose	(*6*)
Vitamin B_{12}	Fermentation (*Propionibacterium shermanii, Pseudomonas denitrificans*)	Fermentative production from glucose	(*7*)

and has the highest antioxidant activity *in vivo*. In nature, only photosynthetic organisms are capable of producing α-tocopherol. In humans, α-tocopherol is believed to play a major role in prevention of light induced pathologies of the skin, eyes and degenerative disorders such as atherosclerosis, cardiovascular diseases and cancer. Industrial application of α-tocopherol includes its use in preservation of food, in cosmetics and sunscreens (*8*).

Currently α-tocopherol is obtained by chemical synthesis and by extraction from vegetable oils. Extraction from oils is not efficient, as these typically contain low levels of α-tocopherol. Unlike other vitamins, synthetic α-tocopherol is not identical to the form that occurs in nature. The synthetic form is a mixture of eight stereoisomers collectively known as *all-rac*-alpha-tocopherol, consisting of four 2R- and four 2S-isomers. Studies with animals and humans have demonstrated that the 2R-isomers are preferentially retained; therefore when administered in equal amounts, the bioavailability of natural with respect to synthetic α-tocopherol is 2:1. Furthermore, growing consumer consciousness with regard to food additive safety has led to increased interest in substituting synthetic antioxidant with natural one, which in turn has encouraged research on alternative sources of natural antioxidant including α-tocopherol.

Several strains of freshwater microalgae *Euglena gracilis Z.* (*1*) and marine microalgae *Dunaliella tertiolecta* (*9*) produce α-tocopherol in concentrations higher than conventional foods traditionally considered as rich sources of the vitamin. Optimization studies for α-tocopherol production have been done with *E. gracilis* Z, which involved modification of culture conditions, two-step culture and screening of favourable substrates that enhance not only growth but also production of α-tocopherol. It was observed that high cell density caused a decrease in light penetration to each cell, decreasing photosynthetic activity. This in turn decreased the aerobic conditions and increased the vitamin E content. Furthermore, consumption of oxygen and glucose by respiration would enhance heterotrophic metabolism. The vitamin E content of the high-density photoheterotrophically grown cells by fed-batch culture reached 1.21 mg/g dry cell mass.

Simultaneous production of high amounts of β-carotene, vitamin C and vitamin E has also been successfully demonstrated by *E. gracilis Z.* using two-step culture. In the first step of the batch culture, *E. gracilis Z.* was photoheterotrophically cultivated in modified Oda and modified Hunter media at high light intensity. When the cells reached late exponential phase, they were separated, washed and resuspended in the same volume of Cramer and Mayers (CM) medium for the second step of cultivation. The two-step cultures using high cell densities gave high productivity of antioxidant vitamins (*1,10*).

Carballo-Cardenas *et al.* (*10*) studied α-tocopherol production with *Dunaliella tertiolecta* and *Tetraselmis suecica* in order to assess the effect of light availability. An inverse relation between light availability and α-tocopherol content of *Dunaliella tertiolecta* was observed. In addition, diminished light availability did not limit α-tocopherol in *Dunaliella tertiolecta* and *Tetraselmis suecica.* No correlation between chlorophyll-a (Chl-a) and α-tocopherol production of these species was observed. The addition of nitrate and phosphate to a culture of *Tetraselmis suecica* resulted in an increase of Chl-a and α-tocopherol, demonstrating that nutrient composition can be used as a tool to improve α-tocopherol productivity.

Menaquinone (vitamin K_2)

There are two naturally occurring forms of vitamin K, vitamin K_1 and K_2. Vitamin K_1 (phylloquinone) is produced in plants. Vitamin K_2 (menaquinone, MK) is primarily synthesized by bacteria, and refers to a series of naphthoquinones having a variable side chain between 4 and 13 isoprene units in length. The compounds in the series are referred to as MK-*n*, where *n* denotes the number of isoprene units (Fig. 1) (*11*).

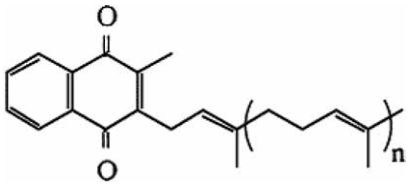

Fig. 1. Structure of menaquinone (MK-*n*)

Vitamin K is an essential cofactor for the post-translational conversion of glutamic acid residues of specific proteins in blood and bone to γ-carboxyglutamic acid. Several comparative studies of the ability of MK homologues to promote the γ-carboxylation of Glu proteins in liver are reported. Homologues MK-4, MK-5 and MK-6 are shown to be highly effective in improving hypoprothrombinaemia induced by an anticoagulant in vitamin K deficient rats (*12*). On the other hand MK-7 has a much higher effect on the decreasing PIVKA-II (protein induced by vitamin K absence or anticoagulant-II) levels than MK-4 (*13*).

Various researchers have extensively studied the mechanism of menaquinone formation in *Bacillus subtilis* (*14*,*15*). Tani and Taguchi (*16*) have reported that as much as 182 mg/L MK was produced using detergent supplement culture and a mutant of *Flavabacterium.* On the other hand, lactic acid bacteria are reported to produce menaquinones with the yield of 29–123 μg/L MK-7, MK-8, MK-9 and MK-10 (*17*). In fermented soybeans, natto, *Bacillus subtilis* produces menaquinones, the major component being MK-7 and the minor one being MK-6. Sato *et al.* (*2*) obtained a diphenylamine-resistant mutant strain D2000-41 from *Bacillus subtilis* strain MH-1 that was isolated from natto. The mutant strain exhibited increased production of MK-7. They also found static cultures to produce higher amounts of MK-7, as compared to agitated and aerated cultures. Sumi (*18*) studied production of vitamin K by the fermentation of okara with seven different natto bacilli. While the MK-4 concentrations remained unchanged during okara fermentation, that of MK-7 markedly increased during the course of fermentation. The highest production rate of 36.6 μg/g was seen in the Chinese natto strain (*Unnan* SL-001) followed by (in μg/g of okara-natto wet mass): 14.2 in *Naruse*, 11.9 in *Asahi*, 6.8 in *Takahashi*, 1.9 in *Miyagino* (natto bacilli for food production), and 5.2 in *Nitto* and 1.9 in *Meguro* (natto bacilli for medicine) after incubation for 4 days at 37 °C. The water-soluble vitamin K was isolated as a dark yellow powder by DEAE Sepharose chromatography and membrane filter fractionations.

Water-Soluble Vitamins

Riboflavin

Riboflavin, or vitamin B_2, is used for human nutrition and therapy and as an animal feed additive. Its deficiency in humans is correlated with loss of hair, inflammation of skin, vision deterioration, and growth failure. This vitamin has also been found to be successful in treatment of migraine (*19*) and malaria (*20*). Riboflavin has been produced commercially by chemical synthesis, by fermentation and by a combination of fermentation and chemical synthesis. Recently, fermentation route has been widely used as it produces the vitamin in a single step, resulting in substantial cost savings. In contrast, chemical processes are multistage, and incur a lot of cost. Most of the producers like BASF, Roche, ADM/Aventis, Hubei Guangji prefer fermentative production of riboflavin over chemical process (*6*).

Although bacteria (*Clostridium* sp.) and yeasts (*Candida* sp.) are good producers, two closely related ascomycete fungi, *Eremothecium ashbyii* and *Ashbya gossypii*, are considered the best riboflavin producers (*21*). *Ashbya gossypii* produces 40 000 times more vitamin than it needs for its own growth. Ferrous ion inhibits riboflavin production by low and moderate overproducers, such as *Clostridia* and *Candida.* The ability of *Eremothecium ashbyii* and *Ashbya gossypii* to repress the effect of iron is responsible for overproduction of riboflavin. In normal microorganisms, it appears that iron represses almost all the biosynthetic enzymes involved in riboflavin production, whereas riboflavin or its derivative inhibit the first enzyme of the pathway, guanosine 5′-triphosphate (GTP) cyclohydrolase II (*22*).

Fermentative production of riboflavin

Fermentative production of riboflavin is carried out in submerged culture. Factors such as the microbial strain, carbon source, minerals, and pH affect the fermentative production of riboflavin. Various researchers have optimized these factors to obtain the maximum yield of riboflavin.

Fermentation using *Ashbya gossypii*. Different carbon sources like palm oil (*23*), corn steep liquor, glucose (*6*), molasses (*24*), whey (*25*) have been tried for riboflavin production. Stahmann *et al*. (*21*) reported riboflavin yields in excess of 15 g/L of culture broth in a sterile aerobic submerged fermentation of *Ashbya gossypii* with a nutrient medium containing molasses or plant oil as major carbon source. Ertrk *et al*. (*25*) studied fermentative production of riboflavin by *Ashbya gossypii* in a medium containing whey. The quantities of riboflavin produced by *Ashbya gossypii* in whey with different supplements like bran, glycine + peptone, sucrose, glycine, yeast extract, peptone, and soybean oil were 389.5, 120, 87.5, 78.3, 68.4, 23.2, and 17.5 mg/L, respectively.

Industrial waste material like activated bleaching earth (waste discharged from an oil refinery plant) (*23*) and agroindustrial byproducts like grape must, beet molasses and peanut seed cake (*26*) have also been used for the production of riboflavin. When waste activated bleaching earth containing 40 % palm oil was used for riboflavin production by *Ashbya gossypii*, the riboflavin concentration reached 2.1 g/L in 10 days. This concentration was almost 1.5 times higher than for cultures grown on pure palm oil (*23*). Riboflavin production in a culture of *Ashbya gossypii* was enhanced by 1.6 times after 4 days (2.5 g/L) with the addition of 1 % mineral support with adsorbed soybean oil to the medium (*27*).

Kalingan and Liao (*28*) determined the effect of various low cost organic wastes as flavinogenic factors and the various concentrations at which they induced flavinogenecity resulting in higher yields of riboflavin. Organic wastes like beef extract, hog casings, blood meal or fish meal supported the production of riboflavin from

Eremothecium ashbyii NRRL 1363. Carbon source at 50 g/L (dextrose equivalents) of molasses and nitrogen source at 50 g/L of peanut seed cake were found to be optimal levels to yield higher riboflavin.

Fermentation using *Bacillus subtilis*. Riboflavin biosynthesis was studied in *Bacillus subtilis* using classical genetics and r-DNA technology. Cloning and DNA nucleotide sequencing indicated that enzymes required for biosynthesis of riboflavin are encoded by gene organization as a single 4.3 kb operon. Gene amplification and substitution of wild type promoters and the regulatory regions with strong constitutive promoter from *Bacillus subtilis* phage SPO 1 have resulted in increased riboflavin production (*29*). The *B. subtilis* riboflavin-producing strain developed by Perkins *et al.* (*30*) contains multiple copies of a modified *B. subtilis* riboflavin biosynthetic operon (*rib* operon) integrated at two different sites in the *B. subtilis* chromosome. The modified *rib* operons are expressed constitutively from strong phage promoters located at the 5′ end and in an internal region of the operon. The engineered strain also contains purine analogue-resistant mutations designed to deregulate the purine pathway and a riboflavin analogue-resistant mutation in *rib*C that deregulates the riboflavin biosynthetic pathway.

One of the proteins encoded by the riboflavin operon of *Bacillus subtilis, Rib*A, is the rate-limiting enzyme in an industrial riboflavin producing strain. Humbelin *et al*. (*31*) introduced an additional single copy of the *rib*A gene into the *sac*B locus of the riboflavin production strain, and expressed constitutively from the medium strength *veg*I promoter. This led to improved riboflavin titers and yields of riboflavin on glucose of up to 25 %.

Recently, Nippon Roche, Japan, has developed and commercialized a single step fermentative riboflavin production using a recombinant *Bacillus subtilis* strain, which effectively produces riboflavin directly from glucose in fed-batch operation (*6*). In order to maintain operational stability and low operating cost, computerized control of the fed-batch cultivation is considered desirable. Horiuchi and Hiraga (*6*) described the industrial application of a fuzzy control system to large-scale recombinant riboflavin production. The system was applied to the on-line control of the feed rate and the pH of the fed-batch culture for the fermentative production of riboflavin. Fuzzy control system has successfully operated a large-scale main fermentor. The operational results showed that the system was able to automate the operation based on the information gained from the operators and to improve both the process productivity and the operational stability in commercial scale production.

Fermentation using *Corynebacterium ammoniagenes*. Improved strains for the production of riboflavin were constructed through metabolic engineering using recombinant DNA techniques in *Corynebacterium ammoniagenes*. In order to increase the expression of biosynthetic genes, DNA fragments that had promoter activities in *C. ammoniagenes* were isolated. When the DNA fragment (P54-6) showing the strongest promoter activity in minimum medium was introduced into the upstream region of the riboflavin biosynthetic genes, the accumulation of riboflavin was elevated 3-fold. In that strain, the activity of GTP cyclohydrolase II, the first enzyme in riboflavin biosynthesis was elevated 2.4-fold, whereas that of riboflavin synthase, the last enzyme in the biosynthesis, was elevated 44.1-fold. Changing the sequence containing the putative ribosome-binding sequence of 3,4-dihydroxy-2-butanone-4-phosphate synthase/GTP cyclohydrolase II gene led to higher GTP cyclohydrolase II activity and strong enhancement of riboflavin production. Throughout the strain improvement, the activity of GTP cyclohydrolase II correlated with the productivity of riboflavin. In the highest producer strain, riboflavin was produced at the level of 15.3 g/L after 72 h in a 5-litre jar fermentor without any end product inhibition (*32*).

Fermentation using *Lactococcus lactis*. Sybesma *et al.* (*33*) developed *Lactococcus lactis* strain using both direct mutagenesis and metabolic engineering for simultaneous overproduction of both folate and riboflavin. The *Lactococcus lactis* MG 1363 was exposed to riboflavin analogue roseoflavin. The roseoflavin resistant strain (*Lactococcus lactis* CB010) showed a deregulated riboflavin biosynthesis resulting in riboflavin production instead of riboflavin consumption. *Lactococcus lactis* CB010 was then transformed with pNZ7010 expressing the gene *folKE* that codes for the bifunctional protein 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine pyrophosphokinase and GTP cyclohydrolase I. This resulted in more than 10-fold increase in extracellular folate level, and more than 2-fold increase in total folate level.

Miscellaneous. Another industrial method for the production of riboflavin is based on microbial production of D-ribose, which then serves as starting material for further chemical synthesis of riboflavin. This chemical conversion process requires multiple steps, and is time consuming resulting in higher costs as compared to direct production by fermentation.

Downstream processing of riboflavin

Riboflavin is recovered from the broth by centrifugation after inactivation of the microorganisms by heat. Pasteurization of the broth ensures that no viable cells of the production organism are present in the final product. After heating, the cell mass is separated from fermentation broth by centrifugation. Differential centrifugation leads to separation of cells and riboflavin crystals because of differences in size and sedimentation behaviour. Riboflavin is then recovered from cell-free broth by using evaporation and vacuum drying.

Vitamin B_{12}

One of the most interesting and fascinating molecules in the world of science and medicine is vitamin B_{12} (cobalamin), which was originally discovered as the anti-pernicious anemia factor in the early 1920s, when two American physicians, Minot and Murphy, demonstrated it to cure pernicious anemia, a disorder first described in 1835, with a diet that included raw liver. In humans, the vitamin is required in trace amounts (approximately 1 μg/day) to assist the action of only two enzymes, methionine synthase and (R)-methylmalonyl-CoA mutase; yet commercially more than 10 t of B_{12} are produced each year from a number of bacterial species.

The term vitamin B_{12} is widely used to describe compounds of the cobalamin group. Natural forms are adenosylcobalamin, methylcobalamin and hydroxocobala-

min. Cyanocobalamin, by definition vitamin B_{12}, is the industrially produced stable cobalamin form which is not found in nature (*34*, Fig. 2). Vitamin B_{12} is obtained exclusively by fermentation process. It is produced by a number of pharmaceutical companies to meet annual demands worldwide. Merck began production of vitamin B_{12} by *Pseudomonas denitrificans* in 1952 and have improved the efficiency of culture more than 30-fold relative to the performance of the original soil isolates by genetic manipulations and microbial screening (*7*). At first, vitamin B_{12} for human therapy and as a food or feed supplement was obtained as a byproduct of *Streptomyces* antibiotic (neomycin, chlortetracycline) fermentation. Good strains were also isolated from manure and sewage sludge. Mutagenic treatments have resulted in improved activity, but in all cases cobalt ions and 5,6--dimethylbenzimidazole (5,6-DMBI) have to be added in addition to the precursors such as glycine, threonine, and aminopropanol (*29*). During the past two to three decades, several microorganisms have been employed for the efficient production of vitamin B_{12}. The list of various microorganisms producing vitamin B_{12} and the respective yields are reported by Martens *et al.* (*35*, Table 2).

Biosynthetic pathway of vitamin B_{12}

Vitamin B_{12} biosynthesis is restricted to microorganisms. Most of the steps in the biosynthesis of vitamin B_{12} have been characterized in *Pseudomonas denitrificans*, *Salmonella typhimurium* and *Propionibacterium freudenreichii*. Roth *et al.* (*36*) reported about the requirement of more than 30 genes for the entire *de novo* biosynthesis of cobalamin, which amounts to about 1 % of a typical bacterial genome. Two different biosynthetic routes for vitamin B_{12} exist in nature:

- aerobic, or more precisely an oxygen-dependent pathway that is found in organisms like *P. denitrificans*, and
- anaerobic, oxygen-independent pathway investigated in organisms like *P. shermanii*, *Salmonella typhimurium* and *Bacillus megaterium*.

Genes encoding enzymes contributing to the oxygen--dependent cobalamin biosynthesis are recognized by the

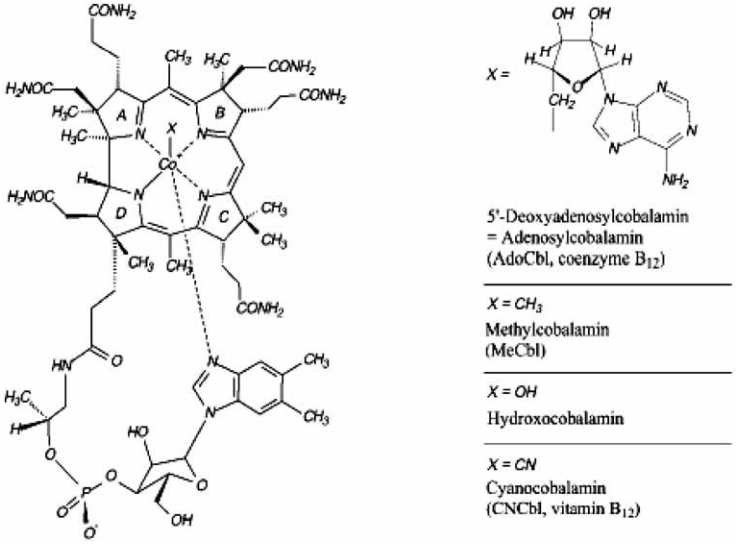

Fig. 2. Structure of vitamin B_{12}

Table 2. Species of microbial producers and microbiological processes recommended for the production of vitamin B_{12}

Species of microorganism	Main component of culture medium	Conditions of fermentation	Vitamin B_{12} production/(mg/L)
Propionibacterium freudenreichii	Glucose	Anaerobiosis, 5,6-dimethyl benzimidazole	206.0
Rhodopseudomonas protamicus	Glucose	5,6-dimethyl benzimidazole	135.0
Propionibacterium shermanii	Glucose	5,6-dimethyl benzimidazole	60.0
Pseudomonas denitrificans	Sucrose	Aerobiosis, betaine	60.0
Nocardia rugosa	Glucose	Aerobiosis	18.0
Rhizobium cobalaminogenum	Sucrose	Aerobiosis	16.5
Micromonospora sp.	Glucose	5,6-dimethyl benzimidazole	11.5
Streptomyces olivaceus	Glucose	5,6-dimethyl benzimidazole	6.0
Nocardia gardneri	Hexadecane	Aerobiosis	4.5
Butyribacterium methylotrophicum	Methanol	Anaerobiosis	3.6
Pseudomonas sp.	Methanol	5,6-dimethyl benzimidazole	3.2
Arthrobacter hyalinus	Isopropanol	5,6-dimethyl benzimidazole	1.1

prefix *cob*, while genes involved in the oxygen-independent pathway are usually named using the prefix *cbi*. A schematic outline of cobalamin biosynthesis and its oxygen-dependent *versus* oxygen-independent differences are shown in Fig. 3.

The biosynthesis of all tetrapyrrole derivatives starts from the C-5 skeleton of glutamate. In the first step, tRNA-bound glutamate is reduced to glutamate-1-semialdehyde by glutamyl-tRNA reductase. The aldehyde is converted in a second step *via* an intramolecular shift to form 5-aminolevulinic acid. Two molecules of 5-aminolevulinic acid are condensed to generate porphobilinogen molecule. Four porphobilinogen molecules are polymerized, rearranged and then cyclized to form uroporphyrinogen III, the first macrocyclic intermediate (*35*). Whilst decarboxylation of uroporphyrinogen III leads to the biosynthesis of hemes and chlorophylls, methylation of uroporphyrinogen III at C-2 and C-7 results in the synthesis of precorrin-2, a dimethylated dipyrrocorphin that is also the last common intermediate in the synthesis of coenzyme F430 and siroheme. The methyl groups added to the tetrapyrrole framework are derived from (S)-adenosyl-L-methionine; and the two methyl groups are added by the action of methyltransferase, which is able to catalyze the addition to both positions (*37*,*38*). At precorrin-2, the two pathways for cobalamin biosynthesis diverge (*39*): in the aerobic pathway, precorrin-2 is methylated at C-20 by a further methyltransferase to give precorrin-3A, while in the anaerobic pathway, precorrin-2 is chelated with cobalt to give cobalt-precorrin-2, a reaction which is catalyzed in *S. enterica* by CbiK (*40*). Thus, the oxygen-dependent and independent pathways for B_{12} synthesis are quite distinct: the oxygen-independent part of the pathway starts with the insertion of cobalt into precorrin-2, while this chelation reaction in the oxygen-dependent part occurs only after nine previous reaction steps. Interestingly, the two cobalt-chelatases employed for these reactions are different in that the oxygen-dependent pathway chelatase requires ATP, in contrast to its anaerobic counterpart, which requires no high-energy equivalents.

A further difference between the two routes is the method employed to promote the ring-contraction process, with the removal of C-20 from the ring. Under aerobic conditions, the C-20 atom of precorrin-3A is oxidized by molecular oxygen, sustained by a Fe_4S_4 cluster containing protein (CobG), with the subsequent release of C-20 as acetate. Under anaerobic conditions, the ring contraction process is likely to be mediated *via* the complexed cobalt ion with its ability to assume different valence states (+1 to +3) to assist in the oxidation, resulting in the release of C-20 as acetaldehyde.

While the B_{12} biosynthetic pathways diverge at precorrin-2, they do join again at the level of adenosylco-

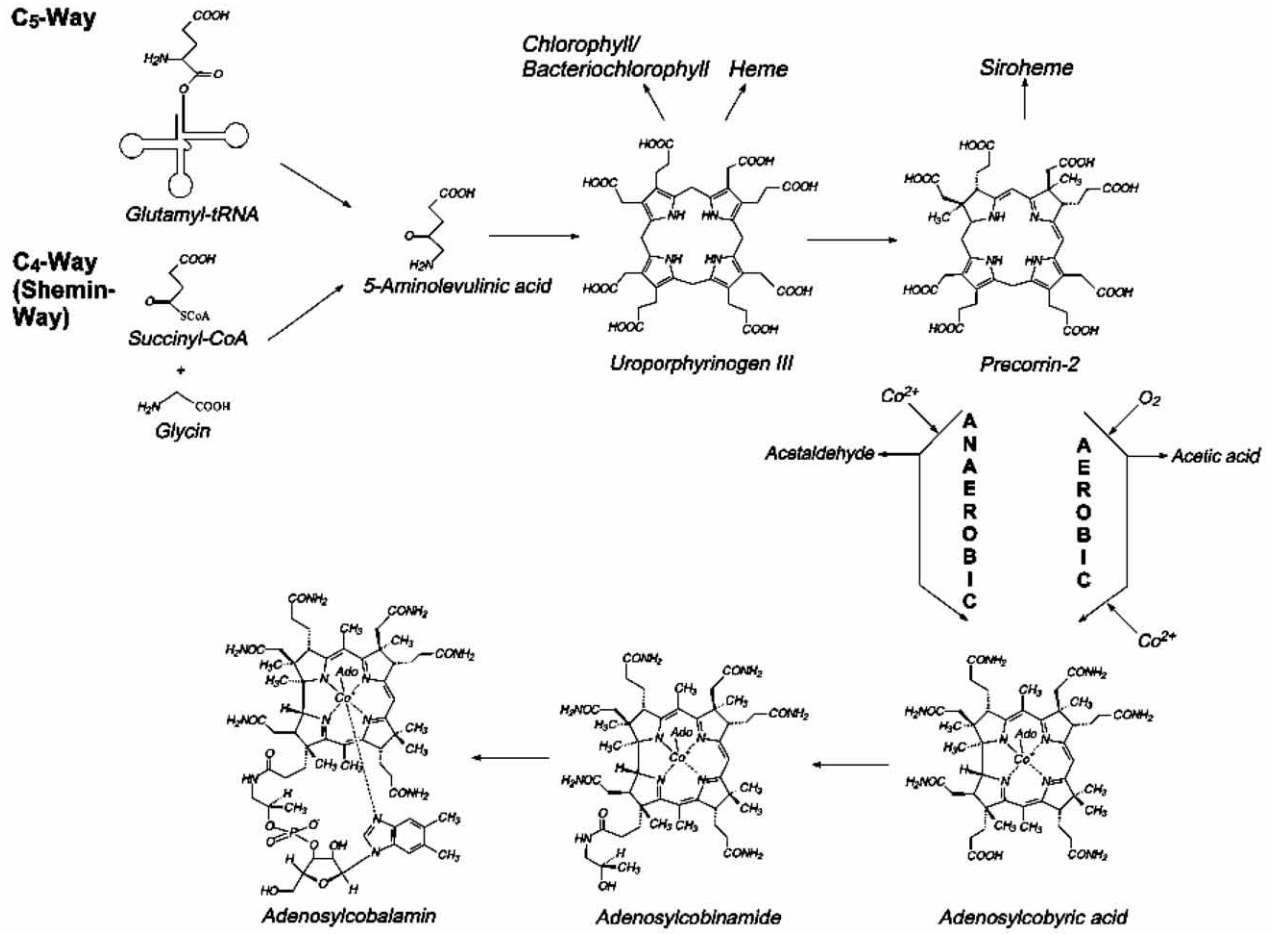

Fig. 3. Schematic representation of the aerobic and anaerobic cobalamin biosynthetic pathways (*35*)

byric acid, which is converted into cobinamide by the attachment of an aminopropanol arm to the propionic acid side-chain of the ring D. The lower nucleotide loop is attached by transferring the phosphoribosyl residue of nicotinic acid mononucleotide to DMBI. The resulting α-ribozole is finally covalently linked to GDP-activated adenosylcobinamide, thereby releasing GMP and giving rise to the completely manufactured coenzyme B_{12} molecule.

Fermentative production of vitamin B_{12} using *Propionibacterium* sp.

It has been demonstrated that *Propionibacteria* species has the highest potential to accumulate vitamin B_{12} intracellularly. *Propionibacterium shermanii* and *Propionibacterium freudenreichii* are most widely used. *Propionibacteria* produce vitamin B_{12} intracellularly and excrete mainly propionic acid and acetic acid extracellularly. All *Propionibacterium* strains employed for vitamin B_{12} production are microaerophilic and produce vitamin B_{12} in high yields only under very low oxygen concentrations. However, the biosynthesis of DMBI requires oxygen. Therefore, the bioprocess of vitamin B_{12} production using *Propionibacterium* strains is divided into two stages. In the first three days of fermentation, the bacteria are grown anaerobically to produce the vitamin B_{12} precursor cobamide, a vitamin B_{12} intermediate missing the DMBI moiety. Subsequently, vitamin B_{12} formation is completed by gentle aeration of the whole culture for 1–3 days, allowing the bacteria to undertake the oxygen dependent synthesis of the DMBI and to link it to cobamide. Furthermore, it is crucial to neutralize the accumulated propionic acid during the whole fermentation process in order to maintain the production culture at pH=7. Propionic acid amounts to 10 % of the fermentation volume (*41*).

Quesada-Chanto *et al*. (*42*) optimized the production of propionic acid and vitamin B_{12} by using *Propionibacterium acidipropionici* NRRL B3569 in continuous culture. Their investigations showed that within a concentration range of 30–170 g/L of sucrose in the fermentation medium, no significant substrate inhibition occurred. In the optimization of the pH value, temperature, and aeration, it was established that the conditions for propionic acid production and vitamin B_{12} production are different. Whereas the optimal production of propionic acid took place under completely anaerobic conditions with a pH value of 6.5 and temperature of 37 °C, optimal vitamin B_{12} production required a temperature of 40 °C and aerobic conditions (0.5 vvm aeration at 100 rpm) with a pH value of 6.5.

Marwaha *et al*. (*7*) have studied the role of amino acids, betaine and choline in vitamin B_{12} biosynthesis by the three strains of *Propionibacterium*, *viz. P. shermanii* 566, *P. shermanii* and *Propionibacterium* arl AKU 1251. They supplemented whey permeate medium with eleven amino acids (0.05 %, by mass per volume). Betaine hydrochloride and choline chloride at mass per volume ratios of 0.25, 0.50 and 0.75 % were evaluated. Of the eleven amino acids tested, L-glutamic acid promoted both growth and product formation to a maximum in all three strains. Betaine was found to be better stimulator than choline. The stimulatory effect of betaine and choline on vitamin B_{12} synthesis in strains of *Propionibacterium* sp. may be explained similarly to the case of *Pseudomonas denitrificans*, *i.e.* the compounds do not need to be metabolized to be stimulatory and may not even need to enter the cell to exert their influence on product formation.

Marwaha and Sethi (*43*) utilized dairy waste for the production of vitamin B_{12}. *Propionibacterium shermanii* 566 synthesized 5.76 mg of vitamin B_{12} per litre of whey containing 4 % lactose supplemented with 0.5 % $(NH_4)_2HPO_4$ when fermentation was carried out at 30 °C under anaerobiosis for the first half (84 h) followed by aerobiosis for the second half of the fermentation (84 h). The metabolite started accumulating at the end of the maximum growth phase (4th day) and lasted until the curve reached a plateau (7th day). Growth of *P. shermanii* 566 also reduced the biochemical oxygen demand (BOD) of the whey by >90 %, thereby decreasing the BOD of the milk plant effluents. The vitamin B_{12}-enriched fermented whey was found to replace the animal protein factor (APF) as a vitamin B_{12} source in poultry rations. Therefore, the technology developed is a worthwhile attempt to utilize whey for vitamin B_{12} production and, in turn, reduce the water pollution problem. The same authors (*44*) simulated the fermentation conditions for vitamin B_{12} biosynthesis from whey. The observed results and simulated expected values obtained by fitting statistical equations to the recorded data showed that 24-hour-old inoculum, *c*(iron)=5 mg/L and 4 % whey lactose were optimal for vitamin B_{12} biosynthesis when fermentation was carried out under anaerobic (84 h) and aerobic (84 h) conditions at 30 °C. The supplementation of whey medium with 0.5 % $(NH_4)_2PO_4$ further enhanced the metabolite yield. *P. shermanii* under optimal cultural conditions was found to be better than *Propionibacterium* arl AKU 1251 in fermenting whey lactose for product formation.

Yongsmith and Chutima (*45*) studied the production of vitamin B_{12} by living bacterial cells immobilized in calcium alginate gels. Whole viable cells of *Propionibacterium* sp. strain arl AKU 1251 were immobilized in calcium alginate gels. The vitamin B_{12} production and growth of immobilized cells could be increased by incubation of the entrapped cells in a medium containing high concentrations of carbon and nitrogen sources. The presence of precursors of vitamin B_{12}, namely cobaltous sulphate and 5,6-dimethyl benzimidazole, together with surfactant Tween 80, at optimum concentrations markedly enhanced the vitamin B_{12} production, the maximum yield reaching as high as 20 mg/L of medium.

For enhanced production of vitamin B_{12}, random mutagenesis has been used to generate the mutant strains producing vitamin B_{12} in high yield. In general, a high yield of vitamin B_{12} has been achieved by treating the microorganisms with mutagenic agents such as UV light or chemical reagents and selecting the strains with practical advantages, such as genetic stability, reasonable growth rates and resistance to high concentrations of toxic intermediates present in the growth medium (*35*). Advances in the molecular biology and biochemistry related to the biosynthesis of vitamin B_{12} have led to the isolation of several enzymes responsible for the synthesis of vitamin B_{12}. Piao *et al*. (*46*) studied vitamin B_{12} production in genetically engineered *Propionibacterium*

freudenreichii. They described the expression of various genes that are required for the biosynthesis of vitamin B_{12} from δ-amino-levulinic acid (ALA). The results showed increased production of vitamin B_{12} in genetically engineered *Propionibacterium freudenreichii.*

The production of tetrapyrrole compounds and vitamin B_{12} by using genetically engineered *Propionibacterium freudenreichii* has been overviewed by Murooka *et al.* (*47*). They have reviewed the advancement of genetic engineering in *P. freudenreichii* in recent years, covering the molecular aspects of the formation of tetrapyrrole compounds and vitamin B_{12}.

Problems associated with the production of vitamin B_{12}. The major problem in vitamin B_{12} production using *Propionibacterium* is the growth inhibition of the cell due to the accumulation of inhibitory metabolites such as propionic acid and acetic acid (*48*). Ken-ichiro *et al.* (*49*) studied various approaches of controlling the concentrations of propionic acid at low level as follows:

a) the periodic cultivation of *Propionibacterium* where dissolved oxygen (DO) concentration was alternatively changed between 0–1 ppm,
b) cell recycle system using hollow fiber module,
c) mixed culture using *Propionibacterium* and *Ralstonia eutropha* where the latter microorganism assimilates the propionic acid produced by the former.

It was found that the productivity of vitamin B_{12} was highest using the cell recycle system, while if the performance was evaluated based on the amount of vitamin B_{12} produced per unit volume of the medium used, the mixed culture system gave the highest value.

Performance of *Propionibacterium freudenreichii* in anaerobic, aerobic and periodic fermentations was studied. It was found that oxygen is a key to the metabolic regulation. Cells could grow faster during a shorter period (6 h) after a DO-shift. However, long time aerobic fermentation (over 6 h) is disadvantageous in cell growth due to the inhibititory effect of oxygen on the synthesis of cytochromes. The propionate was decomposed immediately and pyruvate was accumulated after the DO-shift. Low DO was found to be advantageous in cell growth, in decomposition of propionate, and in lowering the production of acetate. A novel cyclic operation where anaerobic and aerobic conditions were alternatively implemented was developed advantageously in order to enhance the production of vitamin B_{12} (*50*).

Fermentative production of vitamin B_{12} using *Pseudomonas dinitrificans*

Pseudomonas dinitrificans growth parallels cobalamin synthesis under aerobic conditions, if the culture is directly supplemented with 5,6-DBIM and cobalt salts. Maintaining low DO has a favourable effect. Several vitamin B_{12} derivatives can be produced either by direct fermentation or by chemical conversion of cyanocobalamin. The culture is aerated during the whole fermentation process of about 2–3 days at 30 °C and pH values are maintained at 6–7.

The amplification of the eight genes in the *cobF–cobM* operon in *P. denitrificans* and of the *cobA* and *cobE* genes resulted in the increased production of vitamin B_{12}. The term 'amplification' is used to describe an increase in gene copy number by the use of multicopy plasmids. In *P. denitrificans*, a 30 % increase in cobalamin production was detected, caused by amplification of the *cobF–cobM* gene cluster. An additional productivity enhancement of 20 % was achieved by increasing the *cobA* and *cobE* copy number. The heterologous expression of the *corA* gene from *Methanobacterium ivanovii*, encoding an enzyme devoid of substrate inhibition and *bluB*, *bluE* and *bluF* genes from *Rhodobacter capsulatus*, is suggested to overcome the substrate inhibition of the *cobA*-encoded methyltransferase, which catalyzes the first dedicated steps of vitamin B_{12} biosynthesis (*51*). Cellular DMBI biosynthesis was significantly enhanced by *trans*-expression of the *bluB* gene of *R. capsulatus* (*52*). Stimulation of vitamin B_{12} production by (R)-1-amino-2-propanol and *O*-phospho-L-threonine, a new intermediate assumed to take the place of (R)-1-amino-2-propanol in the formation of the nucleotide loop of vitamin B_{12}, was also detected. The positive effects of *O*-phospho-L-threonine on vitamin B_{12} production were also achieved by *trans*-expression of the *bluE* and *bluF* genes of *R. capsulatus* (*52*).

Miscellaneous

The formation of B vitamins (nicotinic acid and nicotinamide, thiamine, vitamin B_6 and vitamin B_{12}) during the soaking of soybeans by bacteria, isolated from tempeh, was investigated (*53*). Among the isolates examined, no vitamin B_6 producer was found. After inoculation of the soaking soybeans with *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomas fluorescens* and *Streptococcus* sp. the concentrations of vitamin B_{12} increased significantly. For the first time it was found that soakings inoculated with *C. freundii* showed an increased vitamin B_{12} content. Nicotinic acid and nicotinamide were produced by *Lactobacillus* spp. and *C. freundii.* The latter synthesized thiamine as well.

Methanogens are also reported to be used for the production of vitamin B_{12} (*54*,*55*). Vitamin B_{12} production by methanogens may have following advantages over conventional vitamin B_{12} producers:

- the concentration of the vitamin B_{12} in the broth is ten times greater than that using propionic acid utilizing microbes;
- the main product, methane, does not inhibit growth of methanogens and could provide a high cell density culture system;
- methanol, CO_2 and acetic acid used as substrates are inexpensive, relatively stable and renewable.

However, long residence times are required for the substrate because methanogens grow more slowly than aerobic microbes, and this makes the fermentation facility larger. Zhang *et al.* (*55*) studied biogas fermentation from alcohol waste fluid to evaluate the anaerobic digestion process and the production of vitamin B_{12} as a by-product. Anaerobic digestion using acclimated methanogens was performed using the continuously stirred tank reactor (CSTR) and fixed-bed reactor packed with rock wool as carrier material at 55 °C. They also studied the effects of metal ions added to the culture broth on methane and vitamin B_{12} formation. Vitamin B_{12} production was 2.92 mg/L in the broth of the fixed-bed reactor, and twice that in the CSTR. Yang *et al.* (*54*) studied continuous methane fermentation and the production of vita-

min B_{12} in a fixed reactor packed with loofah. Loofah carrier immobilized almost 95 % of the methanogens, which led to the more effective bioreaction. Fermentation using CO_2/H_2 acclimated methanogens was conducted in a jar fermentor with hydraulic reaction times (HRTs) of three and six days. With an HRT of three days, methane production rate and vitamin B_{12} concentration in the culture broth and liquid were 6.18 L/L/h and 2.88 mg/L, respectively; these values were 11.96 L/L/h and 37.54 mg/L in the HRT of six days.

A continuous fermentation of extracellular vitamin B_{12} compounds was attempted by Mazumder *et al*. (*56*) using a diatomaceous clay fixed–bed reactor. They used *Methanosarcina bakeri* for the production of vitamin B_{12} using methanol as the substrate.

Inoue *et al*. (*57*) reported the production of vitamin B_{12} by *Acetobacterium* sp. and its tetrachloromethane resistant strains. Of the 800 isolates, a strictly anaerobic bacterium, tentatively designated as 69, isolated from sea sediment was selected and used for the studies. The isolate grown on methanol produced 11 mg of cyanocobalamin per gram of dry cells after 7-day cultivation. Tetrachloromethane resistant strains were obtained by ethylmethanesulphonate treatment, and they produced 23 mg of cyanocobalamin per gram of dry cells in the presence of 10 μM tetrachloromethane.

Bainotti *et al*. (*58*) studied the kinetics of the inhibition of substrate and product on the growth of the acetogen (*Acetobacterium* sp.) in a defined medium with methanol and CO_2. They also modified the medium composition in order to achieve the best growth yield for *Acetobacterium* sp. in batch culture using different concentrations of nutrients in the basal medium. They reported the highest production of 4.84 mg/L of cobalamin with the modified medium (supplemented with 2 g/L of yeast extract) as compared to 2.21 mg/L with basal medium (supplemented with 0.4 g/L of yeast extract) and 3.19 mg/L with synthetic medium (without yeast extract).

Recovery of vitamin B_{12}

The study of separation and purification processes of the fermentation products is most important for their commercial success. Recovery and purification of high value bioproducts from their crude sources involve various steps (extraction, membrane filtration, and sorption), which lead to low overall yields. Conventional chromatographic and chemical processes are capital and energy consuming due to a number of post- and pretreatment steps involved in a processing scheme. A rapid and selective mode of recovery of the target molecule from the crude feedstock can prove highly advantageous in improving the product yields and thus reducing the overall cost of downstream processing. Adsorptive separations are often used in the downstream processing using various interactions, *e.g.* ionic, hydrophobic, affinity, *etc*. for the recovery of biomolecules, namely antibiotics and vitamins.

The steps in the downstream processing for the recovery of vitamin B_{12} are summarized in Fig. 4. The biomass is separated by centrifugation to obtain a cell mass concentrate, which is then dried. Alternatively, the entire contents of the fermentor can be concentrated or spray dried. Cell lysis by heating the centrifuged cell mass in an aqueous solution, or by other methods to get corrinoids, can be used. Corrinoids are converted to vitamin B_{12} or cyanocobalamin by the addition of potassium cyanide, usually in the presence of sodium nitrite and heat. The vitamin solution is clarified subsequently by filtration, treatment with zinc chloride, and then precipitated out by the addition of tannic acid or cresol to give the product of 80 % purity, which is suitable for use as animal food additive. For greater purity, which is required for pharmaceutical use, the clarified solution is extracted with organic solvents, such as carbon tetrachloride, and then with water and butanol, followed again by organic solvents. In addition, adsorption processes such as on ion exchangers, aluminium oxide, or activated carbon can be used. Pure vitamin B_{12} can be obtained by crystallization after the addition of organic solvents, such as phenol and water.

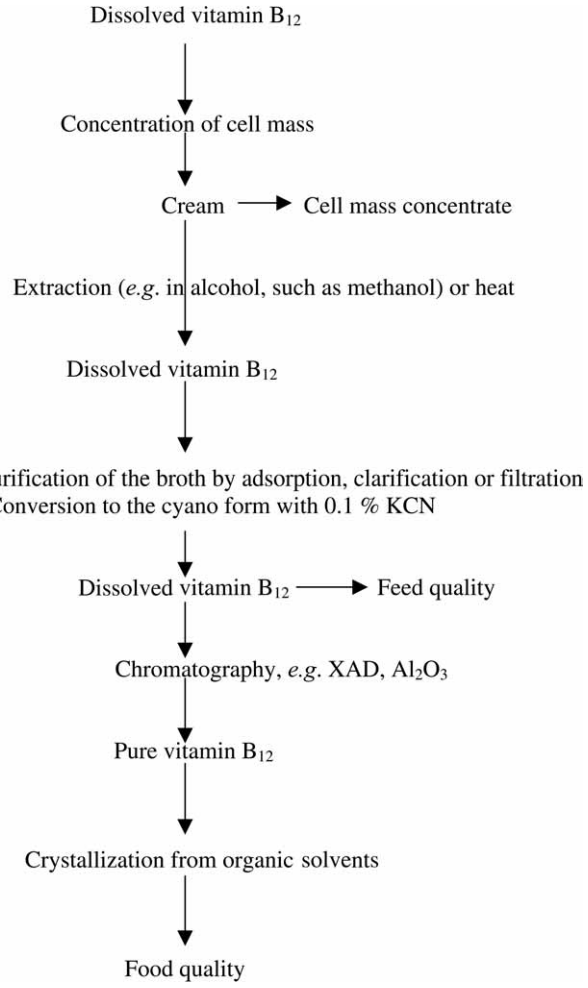

Fig. 4. Downstream processing of vitamin B_{12}

Ramos *et al*. (*59*) studied the adsorption of vitamin B_{12} from aqueous solution on non-ionic polymeric adsorbents from the point of view of modelling and experiments. Adsorption equilibrium isotherms and intraparticle effective diffusivities were measured in batch and continuous stirred tank adsorbers, respectively. The parameters obtained were introduced in a model to predict the breakthrough curves in fixed bed experiments.

Ascorbic acid

L-ascorbic acid is an important metabolite for most living organisms. In humans, it is necessary for different physiological functions and thus is an essential nutrient (*60*). Deficiency in vitamin C leads to the disease scurvy due to the role of the vitamin in the post-translational modification of collagens. The most important function of ascorbic acid is to protect tissue from harmful oxidative products (*61*).

L-ascorbic acid finds its use mainly in food industry, being a vitamin as well as an antioxidant. Approximately 50 % of synthetic ascorbic acid is used in vitamin supplements and pharmaceutical preparations. Because of its antioxidant properties and its potential to stimulate collagen production, it is also widely used as an additive to cosmetics (*62*). The current global market of L-ascorbic acid is in excess of US$ 585 million with an annual growth rate of 3 % (*63*).

At present the majority of commercially manufactured L-ascorbic acid is synthesized *via* Reichstein process using D-glucose as a starting material (Fig. 5). Some processes using bioconversions have been described, but until now, due to low yields obtained, they have not been developed (*64*). Reichstein process involves six chemical steps and one fermentation step for oxidation of D-sorbitol to L-sorbose. Overall yield of L-ascorbic acid from D-glucose obtained by Reichstein process is ~50 %. It is highly energy consuming since it requires high temperature and/or pressure for many steps. In addition, most of the chemical transformations involve considerable quantities of organic and inorganic solvents and reagents like acetone, sulphuric acid and sodium hydroxide. Due to these aspects, straight environmental control of the process is required, resulting in significant waste disposal cost. These economical factors have generated substantial interest in exploitation of microbial biotransformation in the manufacturing of L-ascorbic acid (*3*,*29*). Recent innovations in fermentation processes and advances in biochemistry and recombinant DNA technology widen the options available for exploitation of biotechnology in L-ascorbic acid production (*65*).

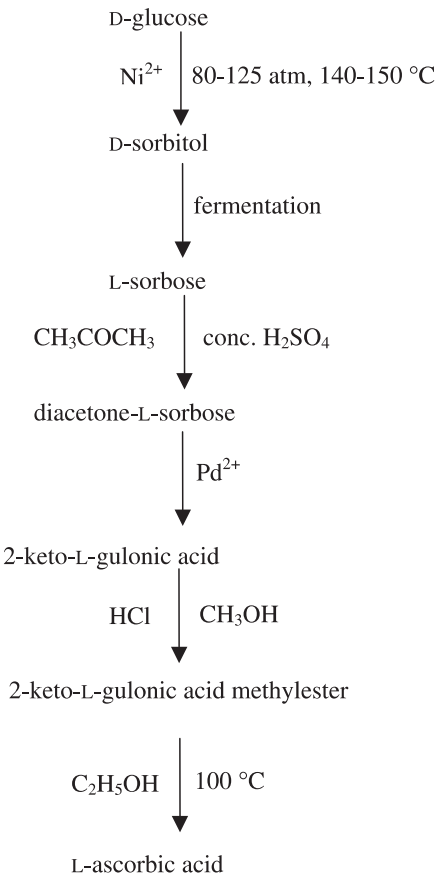

Fig. 5. The Reichstein process for L-ascorbic acid production

Bacterial fermentation processes

At present there are six bacterial fermentation processes for the production of 2-keto-L-gulonic acid, a direct precursor of L-ascorbic acid (*3*). Different pathways, named after one of the metabolic intermediates are sorbitol pathway, L-idonic acid pathway, L-gulonic acid pathway, 2-keto-D-gluconic acid pathway, 2,5-diketo-D-gluconic acid pathway, and 2-keto-L-gulonic acid pathway. These are all summarized in Fig. 6. The most commercially advanced methods are the oxidation of D-sorbitol or L-sorbose to 2-keto-L-gulonic acid (2-KLG) *via* the intermediate L-sorbosone (sorbitol pathway), and the oxidation of D-glucose to 2-keto-L-gulonate *via* D-gluconic acid, 2-keto-D-gluconic acid and 2,5 diketo-D-gluconic acid (2-keto-D-gluconic acid pathway) (*4*).

Sorbitol pathway. Sorbitol is transformed by fermentation to 2-KLG *via* the intermediate L-sorbosone. Transformation is performed by several strains of genera *Pseudomonas* and *Acetobacter,* which catalyse the oxidation of L-sorbose (and/or D-sorbitol) to 2-KLG *via* a series of membrane-bound dehydrogenases, leading to the formation of L-sorbosone (*66*). The final oxidation to 2-KLG is catalyzed by either membrane-bound (*67*) or cytosolic sorbosone dehydrogenases (*68*), depending on the strain. Sugisawa *et al.* (*69*) isolated cultures of *G. oxydans* that produced up to 60 g/L of 2-KLG from L-sorbose or D-sorbitol, with 60 % conversion.

Genetic engineering has also been used in strain improvement to enhance yield *via* D-sorbitol pathway (*66*). *Glucanobacter oxydans* is the species of choice for this purpose but the subcellular (cytosolic or membrane-bound) location of dehydrogenase required for the conversion of D-sorbitol to 2-KLG varies from strain to strain. Transfer of D-sorbitol pathway intermediates into the cytoplasm of these strains is detrimental owing to the presence of cytosolic reductase, which channels intermediates in the pentose phosphate cycle. To overcome this problem, membrane-bound dehydrogenases from alternative sources have been expressed in recombinant *Glucanobacter oxydans* to complement or replace cytosolic enzymes. For example, from IFO 12258 of *Acetobacter liquefaciens* strain membrane-bound sorbosone dehydrogenase was expressed in the OX4 strains of *Glucanobacter oxydans,* which had membrane-bound sorbitol dehydrogenase and sorbose dehydrogenase but not cytosolic dehydrogenase. Significant yield improvements of 2-KLG from both L-sorbose (68 to 81 %) and L-sorbosone (23 to 83 %) were observed in resulting strains but there were no yield improvements under fermentation conditions (*68*).

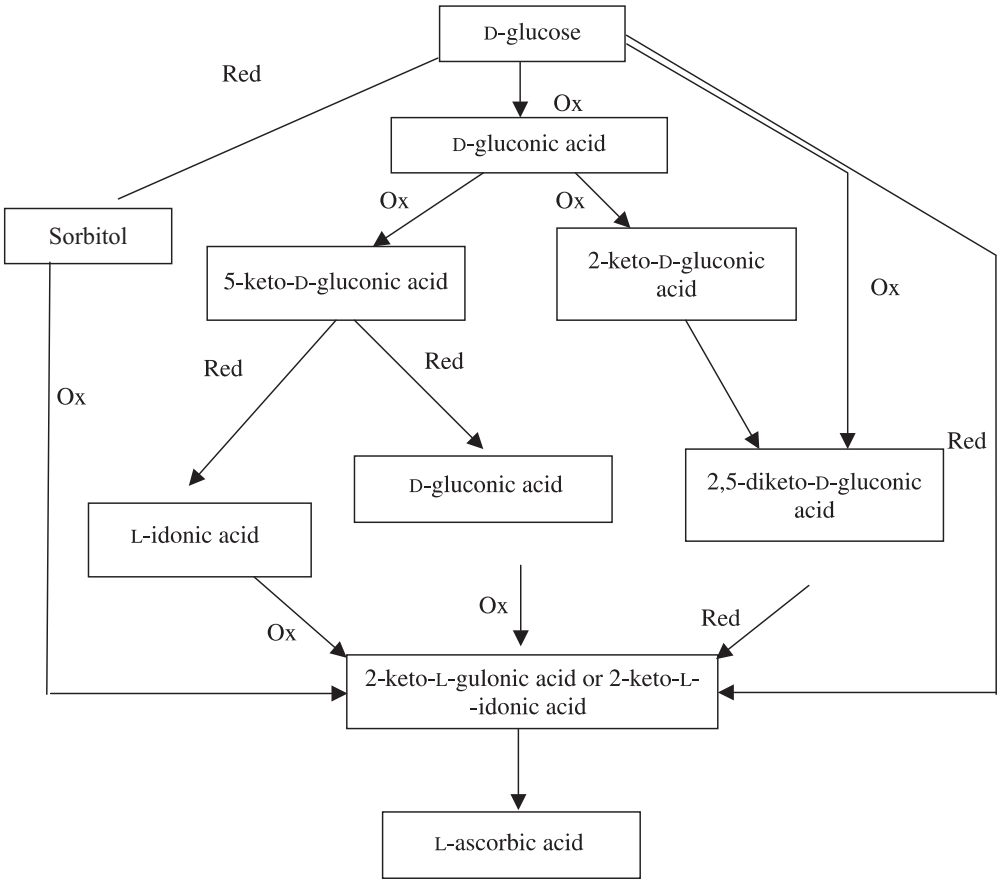

Fig. 6. Different pathways for transformation of glucose to 2-keto-L-gulonic acid

Saito *et al*. (*70*) isolated *Glucanobacter oxydans* G624, which could convert D-sorbitol to L-sorbose almost quantitatively *via* a membrane-bound sorbitol dehydrogenase, but was unable to synthesize 2-KLG. Membrane-bound sorbose dehydrogenase and cytosolic sorbosone dehydrogenase were cloned from *Glucanobacter oxydans* T-100, a strain that can produce 2-KLG, and expressed in *Glucanobacter oxydans* G624. After optimization of the expression system, a chemical mutagenesis to block further 2-KLG metabolism resulted in a *Glucanobacter oxydans* strain which provided more than 85 % yield of 2-KLG.

2-keto-D-gluconic acid pathway. In this pathway, D-glucose is transformed to 2-KLG *via* D-gluconic acid, 2-keto-D-gluconic acid and 2,5-diketo-D-gluconic acid (2,5-DKG). Until now no bacterial strains capable of efficiently catalyzing the complete conversion of D-glc to 2-KLG have been isolated. This is carried out in three main steps; each step is carried out by using different microorganisms (*3*): (*i*) transformation of D-glucose to 2-keto-D-gluconic acid: the transformation of glucose to 2-keto-D-gluconic acid is carried out by *Acetobacter melanogenus* (*71*) and *Pseudomonas albosesamae* (*72*). Some *Acetobacter* strains also synthesize 2-keto-D-gluconic acid; (*ii*) oxidation of 2-keto-D-gluconic acid: this oxidation is carried out by *Bacterium hoshigaki* and *Bacterium gluconicum* with 2,5-DKG as a product. In addition, *Acetomonas albosesamae* can directly transform D-glucose to 2,5-DKG; (*iii*) oxidation of 2,5-DKG acid: Sonoyama *et al*. (*73*) have described the process for conversion of 2,5-DKG into 2-KLG. The mentioned strains were of the genera *Brevibacterium* and *Pseudomonas*, and maximum yield was obtained from *Brevibacterium ketosoreductum*. The use of *Corynebacterium* has also been suggested.

The use of mixed or sequential fermentations for conversion of D-glucose to 2-KLG was originally exploited by Sonoyama *et al*. (*74*) who used a mutant *Erwinia* strain to convert D-glucose into 2,5-DKG. The whole culture broth was then treated with sodium dodecyl sulphate to reduce cell viability, mixed with glucose and then supplied to a mutant strain of *Corynebacterium* for conversion to 2-KLG. The whole process achieved yields of 2-KLG of up to 85 % from D-glucose with final 2-KLG concentrations of 10.5 g/L. This approach improved previous methods based on the harvesting of 2,5-DKG from the first culture broth, as up to 80 % of this unstable compound can be lost during such a process (*71*).

These efforts were later superseded by the extension of the metabolic capacity of the bacterial strain *via* genetic engineering. Anderson *et al*. (*75*) cloned a cytosolic 2,5-DKG reductase gene from *Corynebacterium* sp. and expressed it in *Erwinia herbicola*. The recombinant organism was capable of synthesizing 2-KLG from D-glucose but the yields attained were very poor (1 g/L of 2-KLG from 20 g/L of D-glucose). This was attributed to the requirement for transport of intermediates between the periplasmic space containing the three enzymes necessary for the conversion of D-glucose into 2,5-DKG and the cytoplasm containing 2,5-DKG reductase required

for 2-KLG synthesis. However, Grindley *et al.* (*76*) showed that recombinant *E. citreus* expressing cytoplasmic 2,5--DKG reductase from *Corynebacterium* sp. could accumulate 19.8 g/L of 2-KLG with a 49 % conversion efficiency from glucose. Such improvements in yield were attained by optimization of the fermentation conditions, careful selection of the promoter controlling the expression of the 2,5-DKG reductase gene and by the use of mutant strains of *E. citreus* which were unable to use 2,5-DKG or 2-KLG for growth. Recently, a highly efficient *in vitro* process for the production of 2-KLG *via* this route has been established. Bioreactors were described based on the use of an NADPH-dependent glucose dehydrogenase. The product of this reaction was converted to 2,5-DKG using permeabilised cells of *Pseudomonas citrea* and further transformed to 2-KLG by a soluble 2,5-DKG reductase. The glucose dehydrogenase and 2-DKG reductase formed a redox couple resulting in the *in vitro* regeneration of the nicotinamide cofactor. Glucose could be converted to 2-KLG with >60 % conversion, with an overall productivity of 2 g(2-KLG)/L/h (*77*).

Bioconversion of 2-KLG to L-ascorbic acid

In the conventional process, 2-KLG is converted to L-ascorbic acid chemically *via* two routes. The first involves multiple steps including:

- esterification of a 2-KLG derivative under strongly acidic conditions to produce methyl 2-keto-L-gulonate (MeKLG);
- reaction of MeKLG with a base to produce a metal ascorbate salt;
- treatment of metal ascorbate salt with an acidulant to obtain ascorbic acid.

The second route is a one-step method comprising acid-catalyzed cyclization of KLG.

Both methods are commercially undesirable due to the requirement for multiple chemical steps (first route), or the use of large amounts of gaseous hydrogen chloride or requirement for very expensive process equipment (second route) (*65*). To overcome this problem, several methods were discovered for the conversion of 2-KLG to L-ascorbic acid. Hubbs (*78*) used hydrolase enzyme to convert esters of 2-KLG to L-ascorbic acid. Similarly, lactonases isolated from *Zymomonas mobilis*, *Escherichia coli* and *Fusarium oxysporium* were reported to convert 2-KLG to L-ascorbic acid. However, the possibility of process improvement through better reactor design and enzyme improvement methods can be used to increase the yields.

Some yeast strains like *Candida blankii* and *Cryptococcus dimennae* can also convert 2-KLG to L-ascorbic acid. However, it gives very low yields with accumulation of only 25 μg/mL of L-ascorbic acid in the medium with 5 mg/mL of 2-KLG after 48 h of incubation. In both species, mutagenesis of the catabolic pathway and further investigation into optimum culture conditions can improve yields (*79*).

Yeast based fermentation process for production of ascorbic acid

Earlier studies had suggested yeast cells to contain L-ascorbic acid. However, reassessment of these claims in recent times using improved methodology has suggested that yeasts do not possess an endogenous biochemical pathway for the synthesis of vitamin C, but can synthesize D-erythroascorbic acid. This compound fulfills similar antioxidant functions in yeast but has no antiscorbutic activity (*80*). Yeast cells are, however, known to accumulate L-ascorbic acid when incubated with L-galactose, L-galactono-1,4-lactone or L-galactono-1,4-lactone intermediates from plants or animal pathway (*81*). Mounting evidence suggests that biosynthesis of L-ascorbic acid from this substrate in yeast occurs *via* the activity of enzyme of the D-erythroascorbic acid pathway (*82*). Moreover, expression of D-arabinono-1,4-lactone oxidase from *Saccharomyces cerevisiae* in *Escherichia coli* can overproduce D-erythroascorbic acid and L-ascorbic acid when supplied with D-arabinono-1,4-lactone and D-galactono-1,4-lactone, respectively (*83*).

Saccharomyces cerevisiae and *Zygosaccharomyces bailii* accumulate L-ascorbic acid intracellularly when incubated with L-galactose. Overexpression of the D-arabinose dehydrogenase and D-arabinono-1,4-lactone oxidase in *Saccharomyces cerevisiae* enhances this ability significantly. In fact, the recombinant strain even gains ability to accumulate L-ascorbic acid in culture media. Even better results can be obtained by overexpression of the plant enzyme L-galactose dehydrogenase from *Arabidopsis thaliana* (*84*).

Production of L-ascorbic acid using algae

Skatrud and Huss (*85*) described the method for the efficient production of L-ascorbic acid in algae. The method involved initial growth of *Chlorella pyrenoidosa* ATCC 53170 in a fermentor with a carbon source that is sufficient for the cells to grow to an intermediate density. At the depleted stage, additional carbon source was added sequentially or continuously to maintain the carbon source concentration below a predetermined level until the addition is terminated. This resulted in the production of 1.45 g/L of L-ascorbic acid. *Euglena gracilis Z.* is one of the few microorganisms which simultaneously produce antioxidant vitamins such as β-carotene (71 mg/L), vitamin C (86.5 mg/L) and vitamin E (30.1 mg/L) (*1*).

Biotin

Biotin (vitamin H) is one of the most fascinating cofactors involved in central pathways in pro- and eukaryotic cell metabolism. Since its original discovery in 1901, research has led to the discovery of the complete biotin biosynthesis pathways in many different microbes and much work has been done on the highly intriguing and complex biochemistry of biotin biosynthesis. While humans and animals require several hundred micrograms of biotin per day, most microbes, plants and fungi appear to be able to synthesize the cofactor themselves. Biotin is added to many food, feed and cosmetic products, creating a world market of 10–30 t/year. However, the majority of the biotin sold is synthesized chemically (Goldberg and Sternbach synthesis). Since the chemical synthesis is linked with a high environmental burden, much effort has been put into the development of biotin-overproducing microbes. Fermentative production of biotin has received much attention because of its potential to lower the production cost.

Biosynthetic pathway for biotin

Biosynthetic pathway of biotin in different bacterial strains such as *Escherichia coli* (*86*), *Bacillus sphaericus* (*87*), *Bacillus subtilis* (*88*) and *Sphingomonas* sp. (*89*) has been reported. Only the last step, the conversion of dethiobiotin (DTB) into biotin, has not been enzymatically resolved, although *Bacillus sphaericus* could convert dethiobiotin into (+)-biotin. A major contribution to the understanding of metabolic pathway for biotin synthesis from pimelyl-CoA was originally described for *E. coli*. The pathway of biotin passes through three intermediates, 7-keto-8-aminopelargonic acid (KAPA), 7,8-diaminopelargonic acid (DAPA) and DTB. These intermediates are individually or collectively referred to as vitamers or total vitamers. Biosynthetic pathway for biotin is shown in Fig. 7. The main problem in biotin synthesis is the repressive action of biotin on all biotin-synthesizing enzymes.

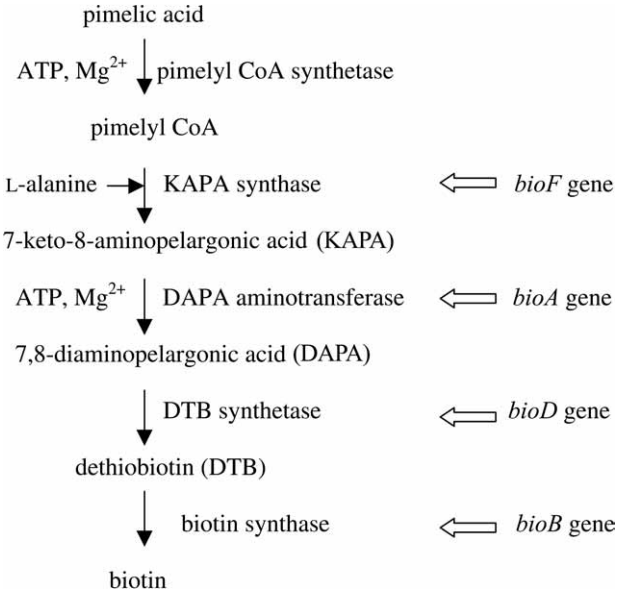

Fig. 7. Biosynthetic pathway of biotin (*29,89*)

Ogata *et al*. (*90*) screened microorganisms and demonstrated that the bacterium *B. sphaericus*, in contrast to *E. coli*, can excrete significant quantities of biotin synthetic pathway intermediates from precursor, pimelic acid. Ohsawa *et al*. (*91*) and Gloeckler *et al*. (*5*) described how *B. sphaericus* biotin (*bio*) genes have been cloned and expressed in *E. coli* and bacilli. But, *B. sphaericus* is difficult to culture, since it seems unable to efficiently utilize glucose as the carbon source, and ammonia as the nitrogen source; it rather uses complex organic molecules.

Brown *et al*. (*92*) studied the production of biotin by recombinant strains of *E. coli*. They have constructed, by means of genetic engineering, recombinant strains of *E. coli*, which overproduced the vitamin. For further studies the strain used was *E. coli* C268 having a *bioA* genotype and plasmid pTG3410 with two expression cassettes. They compared biotin and vitamers produced in tubes (5 mL), Ehrlenmeyer flasks (200 mL), and 2- and 20-litre fermentors. Various parameters influencing vitamers and biotin formation were evaluated. The parameters were culture scale, culture type, inoculation step, pH, temperature, dissolved oxygen, medium constituent variation, and precursor addition. They concluded that 20-litre fermentor is the best for the production. Batch culture with carbon source feed (glycerol) must be used. Casamino acids influenced the production of vitamin, while proteose peptone influenced plasmid viability. The optimum pH and temperature conditions were 7 and 37 °C, respectively. The precursor pimelic acid (300 mg/L) is not limiting for the biotin production. However, a higher concentration results in the formation of more vitamers, but not in extra biotin. Fed-batch fermentation enabled the cell dry mass to be increased by a factor of 18–50 g/L and biotin by a factor of 30–45 mg/L, a significant gain over the values found in 2-litre fermentor.

Biotin production under limiting growth conditions by *Agrobacterium/Rhizobium* HK4 transformed with a modified *Escherichia coli bio* operon was studied by Shaw *et al*. (*93*). The *E. coli bio* operon was modified to improve biotin production by host cells as follows:

- the divergently transcribed wild-type *bio* operon was reorganized into one transcriptional unit;
- the wild-type *bio* promoter was replaced with a strong artificial (*tac*) promoter;
- a potential stem loop structure between *bio*D and *bio*A was removed; and
- the wild-type *bio*B ribosomal binding site (RBS) was replaced with an artificial RBS that resulted in improved *bio*B expression.

The effects of the modifications on the *bio* operon were studied in *E. coli* by measuring biotin and dethiobiotin production, and *bio* gene expression with mini-cells and two-dimensional polyacrylamide gel electrophoresis. The modified *E. coli bio* operon was introduced into a broad host-range plasmid and used to transform *Agrobacterium/Rhizobium* HK4, which then produced 110 mg/L of biotin in a 2-litre fermentor, growing on a defined medium with diaminononanoic acid as the starting material. Biotin production was not growth phase dependent in this strain, and the rate of production remained high under limiting (maintenance) and zero growth conditions.

Saito *et al*. (*89*) compared biotin production by recombinant *Sphingomonas* sp. pSP304 under various agitation conditions. A complex medium containing (in g/L): glycerol 60.0, yeast extract 30.0, casamino acids 5.0, K_2HPO_4 1.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4$ 0.22, and tetracycline 0.01 was used, and biotin was produced in the stationary phase after glycerol starvation. Different impellers used were turbine blade, anchor, Maxblend®, EGSTAR®, turbolift and screw. The maximum production (66 mg/L) was obtained with a jar-fermentor with turbine-blade impeller and the sintered sparger at 400 rpm. It was observed that cell morphology changed with differing agitation conditions. Biotin production was relatively high in slightly long rod-shaped cells but low in elongated cells.

Conclusions

Microbial or microalgal processes for vitamin production have many advantages compared to chemical synthesis processes (if at all available). The products

from chemical processes are often racemic mixtures, while fermentation or bioconversion reactions yield the desired enantiomeric compound. In addition, advances in biochemistry and DNA technology together with genomic revolution have widened the options available for the exploitation of biotechnology in vitamin production. Furthermore, biotechnological processes and products generally have a positive environmental impact and a positive appeal to people.

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