27 Microbial Production of Vitamins

Vitamins are organic compounds that perform specific biological functions for normal maintenance and optimal growth of an organism. These ***vitamins cannot be synthesized by the higher organisms***, including man, and therefore they have to be supplied in small amounts in the diet.

Microorganisms are capable of synthesizing the vitamins. In fact, the bacteria in the gut of humans can produce some of the vitamins, which if appropriately absorbed can partially meet the body's requirements. It is an accepted fact that after administration of strong antibiotics to humans (which kill bacteria in gut), additional consumption of vitamins is recommended.

Microorganisms can be successfully used for the commercial production of many of the vitamins e.g. thiamine, riboflavin, pyridoxine, folic acid, pantothenic acid, biotin, vitamin B_{12}, ascorbic acid, β-carotene (provitamin A), ergosterol (provitamin D). However, from economic point of view, it is feasible to produce vitamin B_{12}, riboflavin, ascorbic acid and β-carotene by microorganisms. ***For the production of ascorbic acid (vitamin C), the reader must refer Chapter 24.***

VITAMIN B_{12}

The disease, ***pernicious anemia***, characterized by low levels of hemoglobin, decreased number of erythrocytes and neurological manifestations, has been known for several decades. It was in 1926 some workers reported the liver extracts could cure pernicious anemia. The active principle was later identified as vitamin B_{12}, a water soluble B-complex vitamin.

Occurrence

Vitamin B_{12} is present in animal tissue at a very low concentration (e.g. 1 ppm in the liver). It occurs mostly in the coenzyme forms-methylcobalamin and deoxyadenosylcobalamin. Isolation of vitamin B_{12} from animal tissues is very expensive and tedious.

Chemistry

Vitamin B_{12} (cyanocobalamin) is a water soluble vitamin with complex structure. The empirical formula of cyanocobalamin is $C_{63}H_{90}N_{14}O_{14}PCO$. The structure of vitamin B_{12} consists of a corrin ring with a central cobalt atom. The corrin ring is almost similar to the tetrapyrrole ring structure found in other porphyrin compounds e.g. heme (with Fe) and chlorophyll (with Mg).

The corrin ring has four pyrrole units. Cobalt present at the centre of the corrin ring is bonded to the four pyrrole nitrogens. Cobalt also binds to dimethylbenzimidazole and aminoisopropanol. Thus, cobalt atom present in vitamin B_{12} is in a coordination state of six.

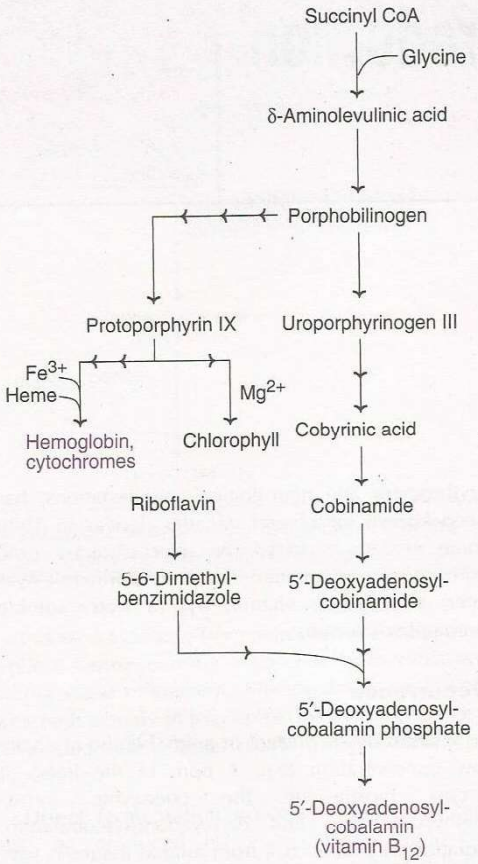

Fig. 27.1 : An outline of the biosynthesis of vitamin B_{12}.

Biosynthesis

Vitamin B_{12} is ***exclusively synthesized*** in nature ***by microorganisms***. An outline of the pathway is depicted in ***Fig. 27.1***. The biosynthesis of B_{12} is comparable with that of chlorophyll and hemoglobin. Many of the reactions in the synthesis of vitamin B_{12} are not yet fully understood.

COMMERCIAL PRODUCTION OF VITAMIN B_{12}

Vitamin B_{12} is commercially produced by fermentation. It was first obtained as a byproduct of *Streptomyces* fermentation in the production of certain antibiotics (streptomycin, chloramphenicol, or neomycin). But the yield was very low. Later, high-yielding strains were developed. And at present, vitamin B_{12} is entirely produced by fermentation. It is estimated that the world's annual production of vitamin B_{12} is around 15,000 kg.

High concentrations of vitamin B_{12} are detected in sewage-sludge solids. This is produced by microorganisms. Recovery of vitamin B_{12} from sewage-sludge was carried out in some parts of United States.

Unlike most other vitamins, the chemical synthesis of vitamin B_{12} is not practicable, since about 20 complicated reaction steps need to be carried out. ***Fermentation of vitamin B_{12} is the only choice.***

Microorganisms and yields of vitamin B_{12}

Several microorganisms can be employed for the production of vitamin B_{12}, with varying yields. Glucose is the most commonly used carbon source. Some examples of microbes and their corresponding yields are given in ***Table 27.1***. The most commonly used microorganisms are — *Propionibacterium freudenreichii, Pseudomonas denitrificans, Bacillus megaterium* and *Streptomyces olivaceus.*

Genetically engineered strains for vitamin B_{12} production : By employing modern techniques of genetic engineering, vitamin B_{12} production can be enhanced. A protoplast fusion technique between *Protaminobacter rubber* and *Rhodopseudomonas spheroides* resulted in a hybrid strain called *Rhodopseudomonas protamicus.* This new strain can produce as high as 135 mg/l of vitamin B_{12} utilizing carbon source.

Table 27.1 Microorganisms with corresponding yields of vitamin B_{12}

Microorganism	*Yield (mg/l)*
Bacillus megaterium	0.51
Streptomyces olivaceus	3.31
Butyribacterium rettgeri	5.0
Micromonospora sp	11.5
Propionibacterium freudenreichii	19.0
Propionibacterium shermanii	35.0
Pseudomonas denitrificans	60.0
Hybrid strain	
Rhodopseudomonas protamicus	135.0

Production of vitamin B_{12} using *Propionibacterium* sp

Propionibacterium freudenreichii and *P. shermanii*, and their mutant strains are commonly used for vitamin B_{12} production. The process is carried out by adding cobalt in two phases.

Anaerobic phase : This is a preliminary phase that may take 2-4 days. In the anaerobic phase 5'-deoxyadenosylcobinamide is predominantly produced.

Aerobic phase : In this phase, 5, 6-dimethyl-benzimidazole is produced from riboflavin which gets incorporated to finally form coenzyme of vitamin B_{12} namely 5'-deoxyadenosylcobalamin.

In recent years, some fermentation technologists have successfully clubbed both an anaerobic and aerobic phases to carry out the operation continuously in two reaction tanks.

The ***bulk production of vitamin B_{12}*** is mostly done ***by submerged bacterial fermentation with beet molasses medium supplemented with cobalt chloride***. The specific details of the process are kept as a guarded secret by the companies.

Recovery of vitamin B_{12} : The cobalamins produced by fermentation are mostly bound to the cells. They can be solubilized by heat treatment at 80–120°C for about 30 minutes at pH 6.5-8.5. The solids and mycelium are filtered or centrifiged and the fermentation broth collected. The cobalamins can be converted to more stable cyanocobalamins. This vitamin B_{12} is around 80% purity and can be directly used as a feed additive. However, for medical use (particularly for treatment of pernicious anemia), vitamin B_{12} should be further purified (95–98% purity).

Production of vitamin B_{12} using *Pseudomonas* sp

Pseudomonas denitrificans is also used for large scale production of vitamin B_{12} in a cost-effective manner. Starting with a low yield (0.6 mg/l) two decades ago, several improvements have been made in the strains of *P. denitrificans* for a tremendous improvement in the yield (60 mg/l).

Addition of cobalt and 5, 6-dimethyl benzimidazole to the medium is essential. The yield of vitamin B_{12} increases when the medium is supplemented with betaine (usual source being sugar beet molasses).

Carbon sources for vitamin B_{12} production

Glucose is the most commonly used carbon source for large scale manufacture of vitamin B_{12}. Other carbon sources like ***alcohols*** (methanol, ethanol, isopropanol) and ***hydrocarbons*** (alkanes, decane, hexadecane) with varying yields can also be used.

A yield of 42 mg/l of vitamin B_{12} was reported using methanol as the carbon source by the microorganism *Methanosarcina barkeri*, in fed-batch culture system.

RIBOFLAVIN

Riboflavin (vitamin B_{12}) is a water soluble vitamin, essential for growth and reproduction in man and animals. Deficiency of riboflavin in rats causes growth retardation, dermatitis and eye lesions. In humans, ***vitamin B_2 deficiency*** results in ***cheilosis*** (fissures at the corner of mouth), ***glossitis*** (purplish tounge) and ***dermatitis***. Riboflavin exerts its biochemical functions through the coenzymes namely ***flavin adenine dinucleotide (FAD)*** and ***flavin mononucleotide (FMN)***.

Occurrence

Riboflavin occurs in milk and milk products, meat, eggs, liver and kidney. While in milk and eggs, it is present in free form, in other foods it is found in the form of flavoproteins (i.e. coenzymes of riboflavin bound to proteins).

Chemistry

Riboflavin contains ***6, 7-dimethyl isoalloxazine*** (a heterocyclic 3 ring structure) attached to ***D-ribitol*** by a nitrogen atom. The isoalloxazine ring participates in the oxidation-reduction reactions brought out by the coenzymes (FAD and FMN).

Biosynthesis

The biosynthetic pathway of riboflavin, elucidated for the microorganisms *Ashbya gossypii* and *Eremothecium ashbyii* is depicted in ***Fig. 27.2***. The overproduction of riboflavin in these organisms takes place mainly due to the constitutive nature of the riboflavin synthesizing enzymes. Iron which inhibits the production of vitamin B_{12} in clostridia and yeasts, has no effect on *A. gossypii* and *E. ashbyii*.

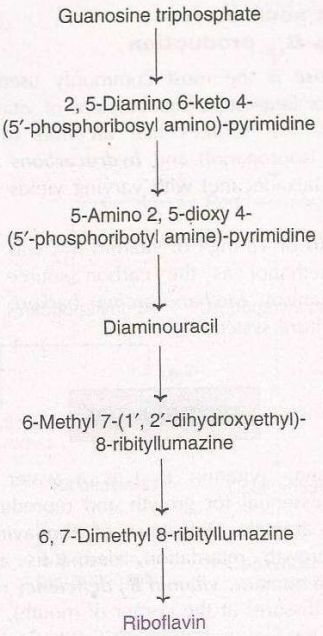

Fig. 27.2 : Biosynthesis of riboflavin.

COMMERCIAL PRODUCTION OF RIBOFLAVIN

There are three processes employed for the large scale production of riboflavin. The worldwide requirement of riboflavin is estimated to be around 2,500 tones per year.

1. **Biotransformation :** About 50% of the world's requirement of riboflavin is produced by biotransformation, followed by chemical synthesis. For this purpose, glucose is first converted to D-ribose by mutant strains of *Bacillus pumilus*. The D-ribose so produced is converted to riboflavin by chemical reactions.

2. **Chemical synthesis :** Approximately 20% of the world's riboflavin is produced by direct chemical synthesis.

3. **Fermentation :** At least one third of world's riboflavin requirements are met by direct fermentation processes.

Microorganisms and yields of riboflavin

Several microorganisms (bacteria, yeasts and fungi) can be employed for the production of riboflavin. In the acetone-butanol fermentation, employing the organisms *Clostridium acetobutylicum* and *Clostridium butylicum*, riboflavin is formed as a byproduct.

Commercial production of riboflavin is predominantly carried out by direct fermentation using the ascomycetes. The different organisms used and the corresponding yields of riboflavin are given in ***Table 27.2***. The two plant pathogens namely *Ashbya gossypii* and *Eremothecium ashbyii* are most commonly employed due to high yield. Among these two organisms, *A. gossypii* is preferred as it is more stable with a high producing capacity of riboflavin.

Genetically engineered strains for riboflavin production : High yielding strains of *Ashbya gossypii* have been developed by genetic manipulations. Such strains can yield as high as 15 g/1 riboflavin.

Production process of riboflavin

Industrial production of riboflavin is mostly carried out with the organism, ***Ashbya gossypii*** by using simple sugars such as glucose and corn steep liquor. Glucose can be replaced by sucrose or maltose for the supply of carbon source. In recent years, lipids such as corn oil, when added to the medium for energy purpose, have a profound influence on riboflavin production. Further, supplementation of the medium with yeast extract, peptones, glycine, inositol, purines (not pyrimidines) also increase the yield of riboflavin.

It is essential to carefully sterilize the medium for good yield of riboflavin. The initial pH of the culture medium is adjusted to around 6-7.5. The

TABLE 27.2 Microorganisms with corresponding yields of riboflavin

Microorganism	*Yield (mg/l)*
Clostridium acetobutylicum	0.097
Clostridium butylicum	0.120
Mycobacterium smegmatis	0.060
Mycocandida riboflavina	0.200
Candida flareri	0.575
Eremothecium ashbyii	2.500
Ashbya gossypii	7.500

fermentation is conducted at temperature 26–28°C with an aeration rate 0.3 vvm. The process is carried out for about 5-7 days by submerged aerated fermentation.

Riboflavin fermentation by *Eremothecium ashbyii* is comparable to that described above for *Ashbya gossypii*. *Candida* sp can also produce riboflavin, but this fermentation process is extremely sensitive to the presence of iron. Consequently, iron or steel equipment cannot be used. Such equipment have to be lined with plastic material.

Fermentation through phases

Some studies have been carried out to understand the process of fermentation of riboflavin particularly by ascomycetes. It is now accepted that the fermentation occurs through ***three phases.***

Phase I : This phase is characterized by rapid growth of the organism utilizing glucose. As pyruvic acid accumulates, pH becomes acidic. The growth of the organism stops as glucose gets exhausted. In phase I, there is no production of riboflavin.

Phase II : Sporulation occurs in this phase, and pyruvate concentration decreases. Simultaneously, there is an accumulation of ammonia (due to enhanced deaminase activity) which makes the medium alkaline. Phase II is characterized by a maximal production of riboflavin. But this is mostly in the form of FAD and a small portion of it as FMN.

Phase III : In this last phase, cells get disrupted by a process of autolysis. This allows release of FAD, FMN and free riboflavin into the medium.

Recovery : Riboflavin is found in fermentation broth and in a bound form to the cells. The latter can be released by heat treatment i.e. 120°C for about 1 hour. The cells can be discarded after filtration or centrifugation. The filtrate can be further purified and dried, as per the requirements.

Other carbon sources for riboflavin production

Besides ***sugars***, other carbon sources have also been used for riboflavin production. A pure grade of riboflavin can be prepared by using *Saccharomyces* sp, utilizing ***acetate*** as sole carbon source. Methanol-utilizing organism *Hansenula polymorpha* was found to produce riboflavin. The other carbon sources used with limited success for riboflavin production are aliphatic hydrocarbons (organism *Pichia guilliermoudii*) and n-hexadecane (organisms — *Pichia miso*).

β-CAROTENE

β-Carotene is the ***provitamin A***. When ingested, it gets converted to vitamin A in the intestine. Vitamin A is a fat soluble vitamin required for vision, proper growth and reproduction. The ***deficiency of vitamin A causes night blindness***, changes in the skin and mucosal membranes.

Occurrence and chemistry

β-Carotene is found in many animal and plant tissues. However, it originates exclusively from plants or microorganisms. Yellow and dark green vegetables and fruits are rich in β-carotene e.g. carrots, spinach, amaranthus, mango, papaya.

Carotenoids are isoprene derivatives. Chemically, they are tetraterpenoids with eight isoprene residues. There are around 400 naturally occurring carotenoids. The most important carotenoids are β-carotene, α-carotene, δ-carotene, lycopene and zeaxanthin.

Carotenoids are mainly used as colouring agents e.g., β-carotene, lycopene, xanthophylls. Several foods (cheese, meat, egg products) can be made attractive by coloration. It may be noted that the demand for β-carotene as the provitamin A is comparatively less.

Biosynthesis

The pathway for the biosynthesis of β-carotene and some other important carotenoids, elucidated in plants and fungi, is shown in ***Fig. 27.3***.

COMMERCIAL PRODUCTION OF β-CAROTENE

β-Carotene can be produced by microbial fermentation. However, for economic reasons, direct chemical synthesis of vitamin A is preferred rather than using its provitamin (β-carotene).

Microorganisms

The organisms *Blakeslea trispora*, *Phycomyces blakesleeanus* and *Choanephora cucurbitarum* are most frequently used for the production of

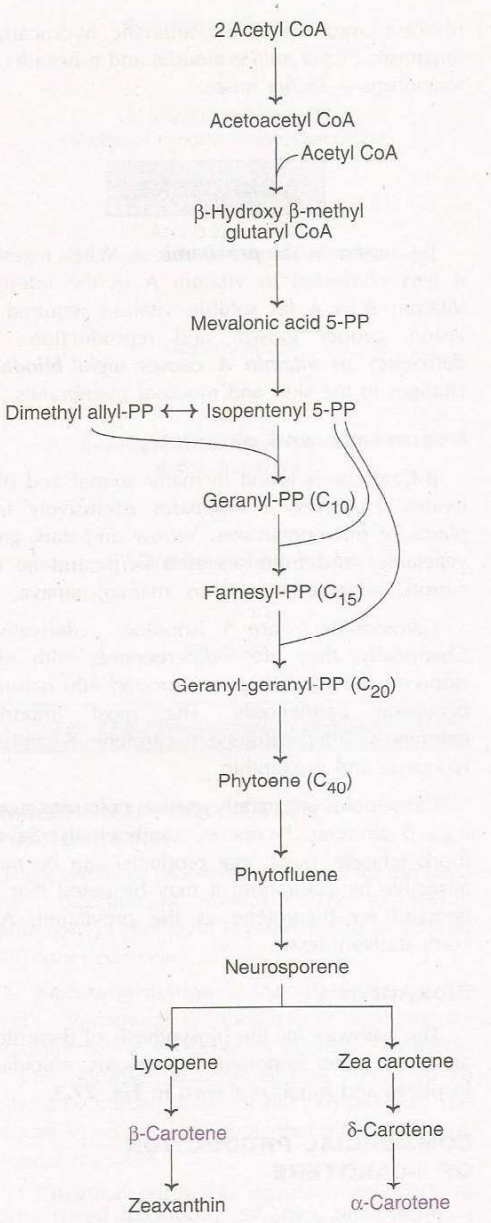

Fig. 27.3 : Biosynthesis of carotenes.

β-carotene. Among these, *Blakeslea trispora* is preferred due to high yield. In the ***Table 27.3***, some important carotenoids, the organisms and the production yields are given.

TABLE 27.3 Microbial production of important carotenoids

Carotenoid	*Organism*	*Production yield (g/l)*
β-Carotene	*Blakeslea trispora* (mixed cultures of + and – sexual forms)	3.0
Lycopene	*Blakeshlea trispora* (mixed culture)	0.4
	Streptomyces chrestomyceticus	0.5
Zeaxanthin	*Flavobacterium* sp	0.4

Production process of β-carotene

As already stated, the industrial production of β-carotene is mostly carried out by *Blakeslea trispora*. The fermentation medium contains corn starch, soybean meal, β-ionone, antioxidants etc. Addition of antioxidants improves the stability of β-carotene with in the cells. The ***fermentation is carried out by submerged process.***

The fermentation is usually started by mixing the cultures of both sexual forms, (+) and (–) strains of *B. trispora*. The yield of β-carotene is significantly higher with mixed cultures, compared to + or – strains (***Fig. 27.4***). This is due to the fact that

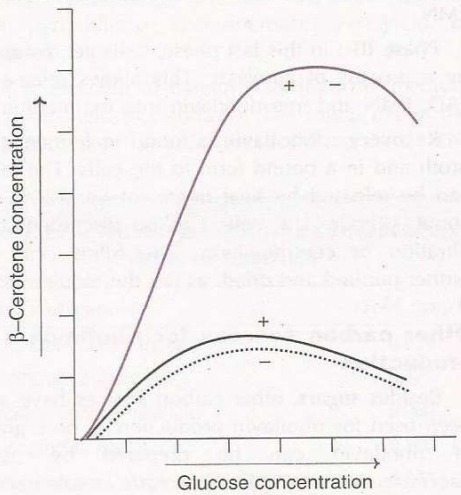

Fig. 27.4 : Yield of β–carotene by +, – and mixed (+ –) cultures of Blakesba trispora.

β-carotene production predominantly occurs during the process of zygospore formation. It may be stated here that the use of mixed strains does not improve the yield for other microorganisms (as observed in case of *Blakeslea trispora*).

Factors affecting production : Trisporic acid which can act as a microbial sexual hormone improves production yield of β-carotene. β-Ionones enhance β-carotene synthesis by increasing the activity of enzymes, and not by their direct incorporation into β-carotene. When the fermentation medium is supplemented with purified kerosene, β-carotene production is almost doubled. Kerosene increases the solubility of hydrophobic substrates.

Recovery : The mycelium rich in β-carotene can be directly used as a feed additive. For purification, mycelium is removed, subjected to dehydration (by methanol) and extracted in methylene chloride. This product is of 70–85% purity which can be further purified as per the requirements.

GIBBERELLINS — PLANT GROWTH STIMULANTS

Gibberellins are plant hormones that stimulate plant growth. They promote growth by cell enlargement and cell division. The observable effects of gibberellins include stimulus to seed germination, flowering and lengthening of stems.

MICROBIAL PRODUCTION OF GIBBERELLINS

So far only one microorganism, ***the fungus*** namely ***Gibberella fujikuroi has been found to produce gibberellins***. This is actually a pathogenic fungus of rice seedlings.

Gibberellin production can be carried out by using a glucose-salt medium at pH 7.5 and temperature 25°C for 2-3 days. The fermentation process is conducted in aerated submerged process. After the growth of the fungus is maximum, the production of gibberellins commences.