Development of a Fermentation Process for the Manufacture of Riboflavin

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Introduction

Vitamin B₂ (riboflavin) is produced for large-scale commercial applications by chemical synthesis or by a combined fermentative/chemical process. In the last years, novel microbiological production processes for synthesis of riboflavin were developed, some of which represent attractive alternatives to the current production processes. The novel Roche process uses a recombinant Bacillus subtilis strain. Here, we describe the development of this strain, its use in fermentation, product purification, and analytics. In addition, we discuss other aspects relevant for the introduction of a novel biotechnology-derived product for application in animal feed and human nutrition, such as safety evaluation and environmental aspects. The Roche process offers the possibility to obtain a product of superior quality by using more environmentally friendly production technology. Riboflavin, thus, represents a good example to illustrate the contribution of modern biotechnology to replace traditional mineral-oil-based chemical processes by more natural ‘green’ production processes based on renewable resources like starch or vegetable oil.

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Commercial Application

Riboflavin is mainly used in animal feed and human food. In feed, riboflavin is important as a vitamin for live stock. In food, riboflavin like other vitamins is added mainly to compensate for the loss of vitamins resulting from industrial processing. In addition, riboflavin is used as a colorant for, e.g., soft drinks. Only a small amount of the riboflavin sold is used in human pharmaceutical applications to compensate for vitamin deficiencies. Each year, several thousand tons of riboflavin are produced worldwide, mainly by F. Hoffmann-La Roche Ltd. (Switzerland) and BASF (Germany).

Current Production Process

Currently, riboflavin is produced mainly by chemical synthesis starting from ribose, which is obtained fermentatively using Bacillus strains. This process has been developed and continuously improved in the last decades [1]. Development of biotechnological processes for the commercial production of riboflavin has been a focus for research for many years [2-4], however, until recently, these processes were not economically competitive. Riboflavin production strains were developed on the basis of eukaryotic fungi or prokaryotic bacteria. The fungus Ashbya goshypii is a naturally occurring over-

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Scheme 2. Comparison of Enzymatic and Conventional Chemical Resolution Processes for DL-PL

Chemical resolution

DL-PL → Concentration → Resolving agent → Resolution → DL-PL complex

- Extraction of resolving agent → L-PL complex
- Lactonization → Crystallization → L-PA

Enzymatic resolution

DL-PL → Enzymatic hydrolysis → Extraction → D-PA

- Lactonization → Crystallization → D-PL crystals
- Racemization → Lactonization → L-PL

Practical hydrolysis of the D-PL in a racemic mixture is carried out using immobilized cells of F. oxysporum as the catalyst. Stable catalyst with high hydrolytic activity can be prepared by entrapping the fungal cells into calcium alginate gels. When the immobilized cells were incubated in a reaction mixture containing 350 g/l DL-PL for 21 h at 30°C under the conditions of automatic pH control (pH 6.8-7.2), 90-97% of d-PL was hydrolyzed (optical purity, 90-97% ee). After repeated reactions for 180 times (i.e., 180 d), the immobilized mycelia retained more than 90% of their initial activity.

The overall process for the present enzymatic resolution is compared with the conventional chemical process in Scheme 2. The enzymatic process can skip several tedious steps which are necessary in chemical resolution and is highly advantageous for the practical purpose.

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## Commercial Application

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producer of riboflavin, which already accumulated high amounts of riboflavin in its culture medium prior to strain improvement [5]. The bacterial strains, which are under development now (mostly Bacillus subtilis), are natural riboflavin over-producers. For example, the wild type B. subtilis strain Marburg 168 secretes negligible amounts of riboflavin into the culture medium. The use of fermentation processes for riboflavin production offers significant advantages compared to chemical production, not only from an economic point of view, but also with regard to product quality and environmental aspects of production (see below). It is, thus, to be expected that chemical synthesis will be replaced by fermentation in the next few years.

**Biosynthetic Pathways Leading to Riboflavin**

Riboflavin biosynthesis starts from guanosine triphosphate (GTP) and ribose-5-phosphate and requires at least six enzymatic activities [6][7]. In B. subtilis, the genes for these six enzymes are clustered in the 4.3 kb riboflavin (rib) operon [8]. The rate of riboflavin production in B. subtilis is affected by the ribC gene located outside the rib cluster [9]. The ribC gene, which has been cloned recently, encodes a flavin kinase converting riboflavin into the biologically active cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [10]. The exact relationship between the kinase activity of RibC and repression of riboflavin biosynthesis is subject of ongoing investigations.

**Bacillus subtilis as Riboflavin Producer**

Bacillus subtilis can be converted into a commercially attractive producer of riboflavin by a combination of ‘classical’ mutation-selection and ‘modern’ molecular biology approaches. The B. subtilis riboflavin overproducing strain RB50:(pRF69)ₙ:(pRF93)ₚ developed in a joint effort by OmniGene Bioproducts Inc., Boston, and F. Hoffmann-La Roche Ltd., Basel, contains various genetically introduced mutations affecting regulation of the purine pathway [2]. Another mutation resulting in resistance against the riboflavin analog roseoflavin relieves the strain from the repressive effects of RibC. These classical mutations lead to B. subtilis strains which already secrete significant amounts of riboflavin into the culture broth. The riboflavin productivity of the strains was further improved by applying molecular biology methods. First, expression of the rib genes was brought under the control of strong promoters resulting in the modified rib opera pRF69 and pRF93. Subsequently, after integration into the Bacillus subtilis genome the copy numbers of pRF69 and pRF93 were increased to copy numbers n and m. Based on B. subtilis strain RB50:(pRF69)ₙ:(pRF93)ₚ, a glucose limited fed-batch fermentation was developed to a commercially attractive process for large-scale riboflavin production. Because of the high riboflavin titers and the very short cycle time, this Bacillus process surpasses all other microbial riboflavin production processes available to date.

**Riboflavin Isolation and Product Quality**

Riboflavin accumulates in the culture medium, whereas only minor amounts of the product remain inside the biomass. Due to the low solubility of riboflavin in neutral aqueous solvents, the fermentation product accumulates as needle-like crystals, which can be easily separated from the biomass by differential centrifugation. Subsequent washing of the crystals with hot diluted acids results in a product with a minimum riboflavin content of 96% which is suited for feed applications. Further recrystallization from hot, concentrated acid solutions provides a more than 98% pure product suitable for all human applications. The recrystallization used to obtain the latter quality is identical to the process used to purify the chemically produced riboflavin.

**Safety Evaluation: The Concept of ‘Substantial Equivalence’**

In many countries, food and food ingredients obtained from genetically modified organisms require a formal premarket approval. This is primarily to ensure the safety of the thus produced products and to assess any possible public health effects. Concepts and guidelines for the safety evaluation of novel food products have been elaborated by various national and international organisations. The common idea of all these assessment concepts focuses on the principle of ‘substantial equivalence’, a term originally elaborated by the OECD [11]. For a complex food or food ingredient, substantial equivalence means identity or sufficient similarity with a traditional food or ingredient as regards composition, nutritional value, metabolism, intended use, and the level of undesirable substances contained therein. Therefore, the safety evaluation and hence the premarket approval of a novel product will depend and rely on a sufficient demonstration of the substantial equivalence.

**Comparison of ‘Chemical’ with ‘Fermentative’ Riboflavin**

The elaboration of the substantial equivalence for riboflavin obtained from fermentation, a key factor for obtaining premarket approval, has been addressed twofold. First of all, the recrystallization step leading to the final food quality material (minimum riboflavin content 98%) was deliberately chosen to remain identical to the process currently used to purify the chemically produced material. This was meant to yield a sufficiently similar product quality compared to the synthetic material. In a second step, the actual product quality was examined by means of HPLC analysis of several pilot production batches and compared with the existing synthetic material. All peaks detected in the fermentatively obtained material by HPLC could be identified and shown to be also present in the synthetic material. The fermentative material had slightly lower levels of impurities and higher levels of riboflavin. Although a riboflavin content of 96% and 98% is guaranteed, the analytical results showed that the actual measured values are close to 100%, e.g. the average measured riboflavin content for the declared 98% material is 99.9%. Furthermore, no DNA was detected in the fermentation samples applying highly sensitive PCR techniques. Riboflavin from fermentation is substantially equivalent or even slightly superior to riboflavin from chemical synthesis: it meets the same product specifications and corresponds to the same quality criteria that are valid for the synthetic product.

**Environmental Aspects**

The environmental impact of the novel fermentation process for riboflavin compared to the existing chemical synthesis was also assessed by comparing the raw materials and energy streams used for both processes. It was shown, that the fermentation process offers significant environmental and economic advantages, primarily, because it uses predominantly
natural renewable raw materials. 87% of the raw materials used in the fermentation is glucose as opposed to 23% glucose used in the chemical synthesis. This reduces the use of organic solvents and other chemical substances and yields 36% less air emissions and wastes. Furthermore, energy consumption can be reduced by 25%.

Conclusion

Development of a successful commercial process using 'modern' biotechnological approaches requires not only the construction of a high-performing strain and optimization of a fermentation process, but also major activities related to product quality, safety, and acceptance. The use of recombinant technology for strain improvement makes premarket approval mandatory in many countries, which is not necessarily the case for 'classical' mutation-selection approaches. The extensive reviews done by the various national authorities during the evaluation process ensure optimal product safety. Thus, despite its image with parts of the general public, use of recombinant DNA technology adds to the safety of the products produced.

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