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Commercial riboflavin production by recombinant *Bacillus subtilis*: down-stream processing and comparison of the composition of riboflavin produced by fermentation or chemical synthesis

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A novel process for riboflavin production using a recombinant *Bacillus subtilis* strain has been developed. Here we describe a down-stream processing procedure to obtain riboflavin qualities having a minimal content of 96% ('feed-grade') and 98% ('food/pharma-grade') riboflavin, respectively. Compared to riboflavin produced by chemical synthesis, products with improved chemical purity were obtained. All compounds representing more than 0.1% of the final products were identified. Feed-grade riboflavin material ex fermentation contained small amounts of amino acids and amino sugars and the biosynthetic riboflavin precursor dimethyl-ribityl-lumazine. All other side products found were derived from riboflavin, resulted from the purification procedure and were also found in riboflavin obtained by chemical synthesis. The *Bacillus*-produced riboflavin does not contain DNA. The data presented here were used to obtain product approval for the commercial application in the USA, Japan and the UK.

Keywords: recombinant *Bacillus subtilis*; riboflavin produced by fermentation; down-stream processing; analytics; registration

Introduction

Riboflavin (vitamin B_2) is currently produced for large-scale commercial applications by chemical synthesis or by a one-step fermentation process. In the chemical synthesis, the ribose used as a building block is mostly obtained by fermentation of a *Bacillus pumilis* strain lacking a functional transketolase. This chemical production process has been continuously improved in the last decades (reviewed in [8]). Two product qualities are currently produced by chemical synthesis: a riboflavin product with at least 96% chemical purity for the animal feed market ('feed-grade riboflavin') and a product with at least 98% chemical purity for the human food and pharmaceutical markets ('food/pharmagrade riboflavin'). Development of biotechnological processes for the commercial production of riboflavin has been a focus for research for years. Several organisms can be used to produce high levels of riboflavin: the ascomycetes *Eremothecium ashbyii* and *Ashbya gossypii*, the yeasts *Saccharomyces cerevisiae* and *Candida flaveri* and recombinant *B. subtilis* and *Corynebacterium ammoniagenes* strains [1,2,6,8,12,13,16,17]. The fungus *A. goshypii* is a naturally occurring overproducer of riboflavin, which already produced high amounts of riboflavin prior to strain improvement [9]. Riboflavin production by the ascomycetes and yeasts has been improved over many years using a mutation-selection and fermentation development approach. *A. gossypii* (BASF, Ludwigshafen, Germany) and *C. flaveri* (Archer Daniels Midland, Decatur, IL, USA) are currently used for large-scale commercial production of riboflavin. The products produced with these organisms include an 80% riboflavin product containing 20% biomass-derived compounds [8]. This product is then used directly in the animal feed market.

The novel fermentation process described here [12,13,18] uses the recombinant *B. subtilis* strain RB50::$(pRF69)_n$::$(pRF93)_m$, which has been developed in a collaboration between OmniGene Bioproducts Inc, Boston, USA and F Hoffmann-La Roche Ltd, Basel, Switzerland. This strain contains various classically introduced mutations affecting regulation of the purine pathway and one resulting in resistance against the riboflavin analog roseoflavin. The latter mutation resides in the *RIBC* gene. RibC is the kinase involved in conversion of riboflavin to the active co-factors FMN and FAD. The mutation results in reduced activity of RibC and, thus, reduces the repressive effects of FMN on riboflavin production [3,10]. In addition to these mutations, the expression of the *rib* genes of *B. subtilis* was drastically increased by replacing the *rib* promoters by strong bacteriophage promoters and thereby creating the modified *rib* opera in plasmids pRF69 and pRF93. Subsequently, the copy numbers (n and m, respectively) of pRF69 and pRF93 were increased after integration of the optimized operons into the *B. subtilis* genome.

Since a novel production procedure and particularly a recombinant production organism is used, approval of regu-

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latory authorities is required prior to commercial use of the riboflavin products produced. The key issues are: (a) substantial equivalence of the novel products compared with the already available riboflavin qualities; and (b) the absence of production strain DNA in the riboflavin ex fermentation. Riboflavin is mainly used in animal feeding, but also in human nutrition as vitamin or as colorant and in pharma applications. Each of these applications, even within the same country, may require a different approval approach.

In this paper, we show a down-stream processing procedure and chemical analysis of the riboflavin products obtained using recombinant *B. subtilis*.

Materials and methods

Production of riboflavin by fermentation

B. subtilis strain RB50::$(pRF69)_n$::$(pRF93)_m$ (n and m represent the copynumbers of each plasmid integrated into the *B. subtilis* RB50 genome) [12,13] was grown in a glucose-limited fed-batch fermentation at a 35-m^3 scale. After pasteurisation of the fermentation broth, riboflavin crystals, which accumulate in the fermentation medium, were separated from the *B. subtilis* cells by differential centrifugation. The next steps were washing of the crystals with 0.2 M hot mineral acid, eg hydrochloric or sulphuric acid [7], followed by recrystallisation of riboflavin from hot concentrated acid, eg hydrochloric acid >25%. All steps were performed under conditions representative for those to be used at the final production scale. The material obtained from three representative fermentations was analysed.

Analysis of riboflavin(-related) compounds

HPLC analysis was done using a Hewlett-Packard model 1090 equipped with diode array detector. The separation was achieved using a 250×4.6 mm column with Supercosil™ LC8DB, 5-μm reverse-phase material (pore size 100 Å). For elution, a gradient elution was formed using, as mobile phase A, 0.05 M potassium dihydrogenphosphate pH 6, as mobile phase B, acetonitrile and as mobile phase C, methanol: water in a 1:1 ratio. The starting composition A:B:C was 92:0:8. Between 0 and 12 min after injection, the eluent composition changed to A:B:C = 71:21:8. At 20 min the ratio was A:B:C = 51:41:8 and at 30 min A:B:C was 11:81:8. The flow rate was 1.5 ml min^{-1} and column temperature 38°C. UV detection was done at 240 nm enabling the detection of molecules with low-absorbing chromophores. This procedure, in which the eluents were polar at the beginning of the separation and apolar at the end, allows simultaneous separation of polar, water-soluble impurities and also of lipophilic compounds. Known amounts of dry riboflavin samples were obtained by weight determination. All standards and samples were prepared under subdued light to avoid degradation and were dissolved in dimethyl sulfoxide (DMSO) by heating at 80°C for 10 min. When appropriate, 2 mg 1,3-dinitrobenzene ml^{-1} was used as internal standard. The by-products present in chemically-synthesized riboflavin have all been chemically characterised (J-L Meinrad and Ch Gerber, F Hoffmann-La Roche AG, Basel, Switzerland, unpublished). Riboflavin, 8-hydroxymethylriboflavin and lumichrome present in riboflavin ex fermentation were identified by comparing their retention times and UV spectra with those of the pure (co-injected) reference compound, obtained by chemical synthesis. Because of their instability, formyl-methyl-flavin acetal and ribityl-oxo-chinoxalic acid were identified by comparison with the HPLC profiles and UV spectra of a synthetic riboflavin sample containing these compounds. For quantitation a highly purified riboflavin standard, obtained by chemical synthesis, was used, having an $E_{1\%,\,1\,cm}$ of 328 at 444 nm. The limit of detection was 0.05% of the total chromatographic peak area. The relative standard deviations were better than 1.5% ($n = 11$, σ_s equal to or smaller than 1.3%) and 10% ($n = 40$, σ_s equal to or smaller than 5%) for riboflavin and the detected impurities, respectively.

Amino acid analysis

Amino acid analysis was done according to Spackman *et al* [15], using a Liquimat III Amino Acid Analyser (Biotech Kontron AG, Basel, Switzerland), with a 270×4 mm column packed with 7 mm CK10F resin cation-exchanged (Mitsubishi Ltd, Tokyo, Japan) and a step gradient of 0.16–0.35 M sodium citrate, pH 3.21–4.24. The riboflavin sample was incubated with 6 M hydrochloric acid at 110°C for 24 h and dried in a dessicator for 5 h over potassium hydroxide. The residue was dissolved in sodium citrate buffer pH 2.20 and centrifuged. Quantitation was done using norleucin as internal standard.

DNA stability during product purification

Stability of *B. subtilis* RB50::$(pRF69)_{25}$::$(pRF93)_m$ DNA under conditions representative for purification of feed-grade riboflavin were determined. Purified production strain DNA or intact cells were incubated at the indicated conditions. Incubation was stopped by neutralisation and rapid cooling of the samples. Prior to PCR analysis, DNA was passed through Chroma Spin-10 TE columns (Clontech Laboratories Inc, Palo Alto, CA, USA). PCR was performed at 48°C with 10 pmol of each 20 mer oligonucleotide using standard protocols [14]. The primers used were 5′-ATTGGAAGAGAAAAGAGATA-3′ and 3′-TAAGACAAACACTACCAATA-5′. The 5′-positions of the primers correspond to positions 1288 and 1845 respectively of the *CAT* gene of pC194 [5]. These are specific for the chloramphenicol resistance gene present on plasmid pRF69, resulting in amplification of a 557-bp DNA fragment. For functional analysis of DNA, *B. subtilis* strain 1012 was transformed as described [5] and transformants resistant to 5 μg chloramphenicol per ml were selected.

Analysis of DNA in riboflavin samples

Riboflavin samples were dissolved in 1% deoxycholate in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (1 ml per 50 mg of riboflavin). Riboflavin was then extracted by addition of five volumes of buffer-saturated phenol. After centrifugation, the aqueous phase was collected and passed through Chroma Spin-10 TE columns prior to PCR analysis (see above). In control experiments, known amounts of production strain DNA were added to riboflavin samples and dried *in vacuo* prior to addition of deoxychlorate-containing buffer.

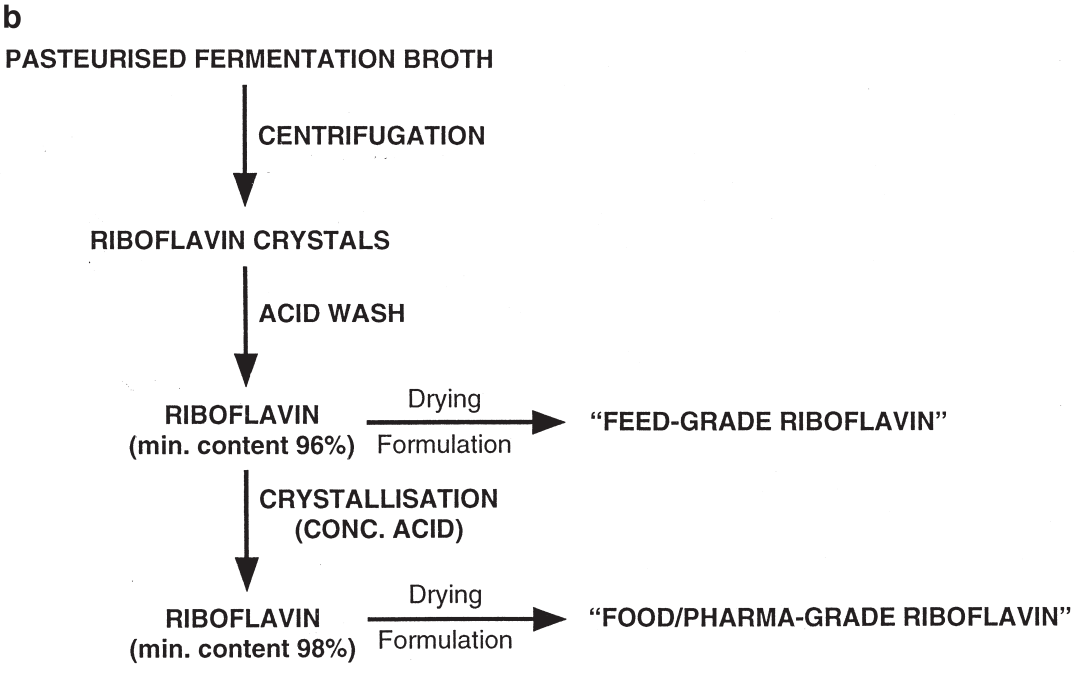

Figure 1 Riboflavin purification from fermentation broth. (a) Needle-like riboflavin crystals (brownish-yellow) accumulate in the fermentation medium. The *B. subtilis* cells are seen as small rods in the background. Bar marker 20 μm. (b) The procedure used for purification of riboflavin [7] is summarised (for details, see Materials and Methods). The % values given refer to the minimal guaranteed riboflavin content of the product.

Results

Riboflavin crystals accumulate in the culture supernatant

A glucose-limited fed-batch fermentation was used for large-scale riboflavin production. Riboflavin was excreted into the culture medium, no accumulation was observed within the *B. subtilis* cells. Due to the low solubility of riboflavin in the fermentation medium, the product accumulated as needle-like crystals (Figure 1), which could easily be separated from the biomass by differential centrifugation. After centrifugation, the riboflavin content of the effluent was about 90%. By washing with hot dilute acid a product was obtained with a riboflavin content of about 98% (Table 1). Subsequent recrystallisation from hot, concentrated acid then provided a product with more than 99%

Table 1 Analysis of riboflavin produced by fermentation of *B. subtilis* or by chemical synthesis. The contents (expressed in % weighed samples) of the riboflavin(-related) compounds was determined after HPLC separation (see Figures 2 and 3). Amino acids were quantified by direct analysis, while analysis of DNA was done using PCR (see Figure 4)

Substance	Acid-washed riboflavin ex fermentation, determined contents (%)[a]	Recrystallised riboflavin ex fermentation, determined contents (%)[a]	'Food/pharma-grade riboflavin' ex chemical synthesis, determined contents (%)[a]
Riboflavin	98.4	99.7	98.8
Lumichrome	0.12	0.15	0.2
HR	traces[b]	0.2	0.2
Lumiflavin	not detected	not detected	0.5
DMRL	0.4	not detected	not detected
FMFA	traces[b]	traces[b]	traces[b]
ROC	not detected	not detected	traces[b]
Amino acids	0.9	0.06	–
DNA	not detected	not detected	–
Sum of measured products	99.82	100.11	99.7

HR, 8-hydroxymethyl-riboflavin; DMRL, dimethyl-ribityl-lumazine; FMFA, formylmethyl-flavin acetal; ROC, ribityl-oxo-chinoxalic acid.
[a]Average of the values obtained for three representative fermentations.
[b]Detected, but below limit for quantitative determination of 0.1%.

riboflavin content (Table 1). Importantly, the recrystallisation step used here is identical to the last down-stream processing step currently used to purify the chemically-produced 'food/pharma-grade riboflavin' (Figure 1).

Comparison of riboflavin produced by chemical synthesis and fermentation

The chemical purity of the riboflavin samples obtained by fermentation was determined using three 35-m^3 pilot scale production batches. Both the acid-washed and recrystallised riboflavin samples obtained by fermentation contained fewer riboflavin-related contaminants than the 98% 'food/pharma-grade riboflavin' obtained by chemical synthesis (Figure 2). The acid-washed riboflavin contained 0.9% amino acids including small amounts of the amino sugars glucosamine and galactosamine (Table 1). These compounds were virtually absent in the recrystallised riboflavin and are not present in the chemically-produced riboflavin.

All impurities seen in the HPLC profiles could be unambiguously identified by comparing the retention times and UV spectra to those of co-injected reference compounds (Figure 3). All impurities except dimethyl-ribityl-lumazine and the amino acids were also detected in the chemically-produced riboflavin (Figure 2 and Table 1). Dimethyl-ribityl-lumazine is a naturally-occurring precursor in riboflavin biosynthesis, which was present in the acid-washed riboflavin ex fermentation, but was almost completely removed upon recrystallisation. In contrast, 8-hydroxymethyl-riboflavin and lumichrome were present in increased concentrations in the recrystallised riboflavin compared to acid-washed material. The presence of 8-hydroxymethyl-riboflavin and formylmethyl-flavin acetal in both the recrystallised riboflavin materials ex fermentation and ex chemical synthesis was not surprising since these products exclusively result from acid degradation of riboflavin (J-L Meinrad and Ch Gerber, F Hoffman-La Roche AG, Basel, Switzerland, unpublished). Lumiflavin was not detected in riboflavin ex fermentation as expected, since this product was generated from chemically-produced riboflavin upon incubation at basic pH. In contrast to the chemical synthesis, such incubation is not part of the downstream processing of riboflavin ex fermentation.

Quantitative analysis of the individual compounds identified here (Table 1) suggests that all material in both riboflavin qualities could be accounted for by riboflavin and the identified compounds (Table 1).

Production strain DNA was not detected in acid-washed or recrystallised riboflavin ex fermentation

The presence or absence of DNA in the final products is an issue in product registration procedures in various countries (see Discussion). Direct analysis by PCR, for the presence of DNA of the production strain *B. subtilis* RB50::$(pRF69)_{25}$::$(pRF93)_m$ in the acid-washed or recrystallised riboflavin samples from pilot-scale production, was performed. DNA was not detected in any of the riboflavin production samples (Figure 4b, lanes 1 and 3). In model experiments known amounts of production strain DNA were subjected to the acid treatment used in riboflavin purification. Survival of DNA was tested by PCR and by transformation of wildtype *B. subtilis* cells. No DNA was detected after acid treatment for less than 5 min at 90°C or for 1 h at 25°C, showing an at least 10^7-fold reduction in the amount of DNA amplifiable with PCR (data not shown). In line with the PCR results, a short treatment with hot acid also rendered DNA incompetent for *B. subtilis* transformation (data not shown). Similar results were obtained when intact *B. subtilis* cells were used: DNA was also rapidly degraded and did not survive the acid treatment (data not shown). Thus, no DNA is present in any of the fermentative riboflavin samples.

Discussion

Chemical analysis of riboflavin products

The novel fermentation process for riboflavin production uses a recombinant *B. subtilis* strain. Chemical analyses of

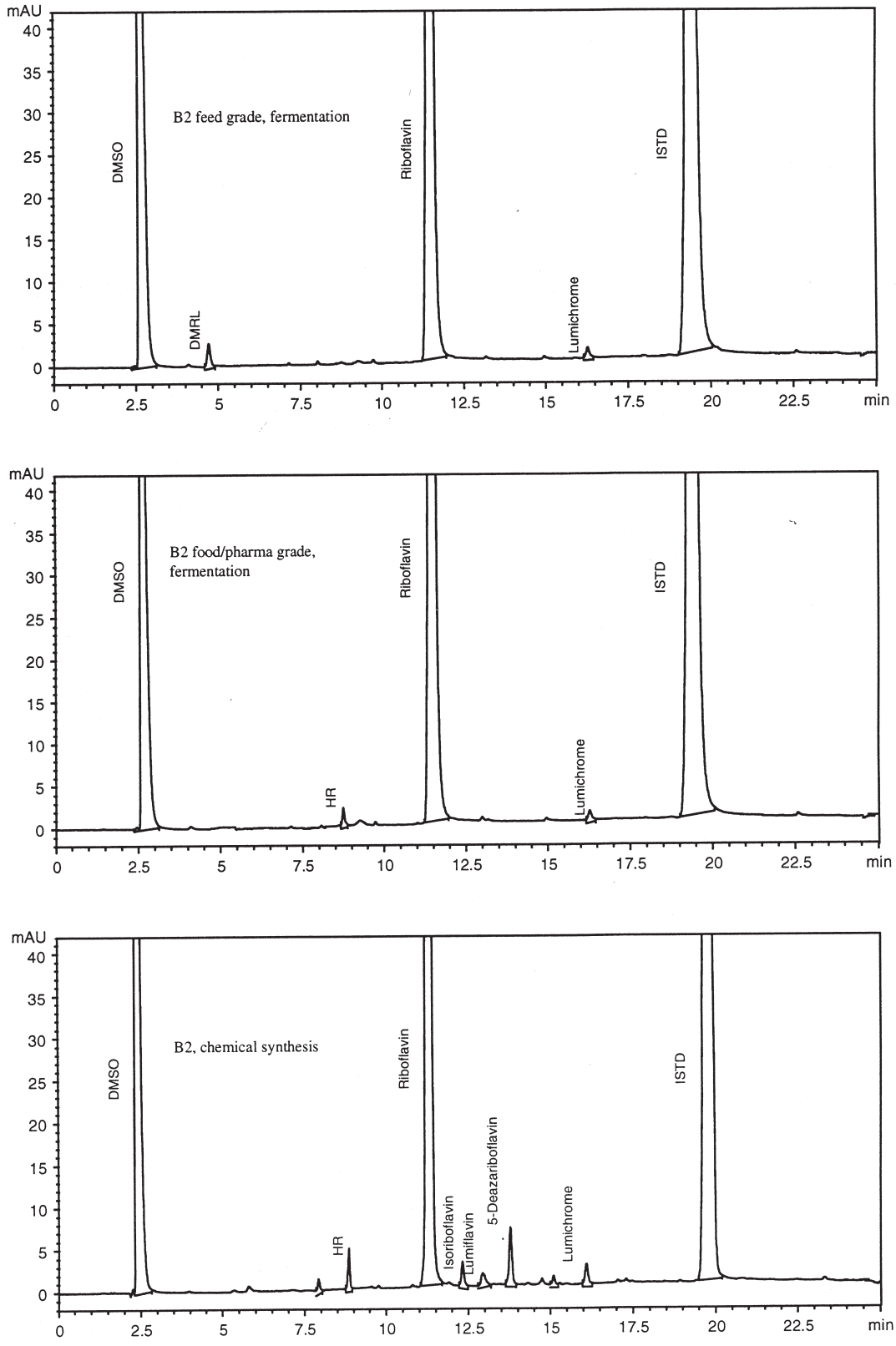

Figure 2 Analysis by HPLC of riboflavin products obtained by fermentation or chemical synthesis. Top panel, 'feed-grade riboflavin' ex fermentation; middle panel, 'food/pharma-grade riboflavin' ex fermentation; bottom panel, 'food/pharma-grade riboflavin' ex chemical synthesis. B2, riboflavin; DMSO, dimethyl sulfoxide; HR, 8-hydroxymethyl-riboflavin; DMRL, dimethyl-ribityl-lumazine; ISTD, internal standard.

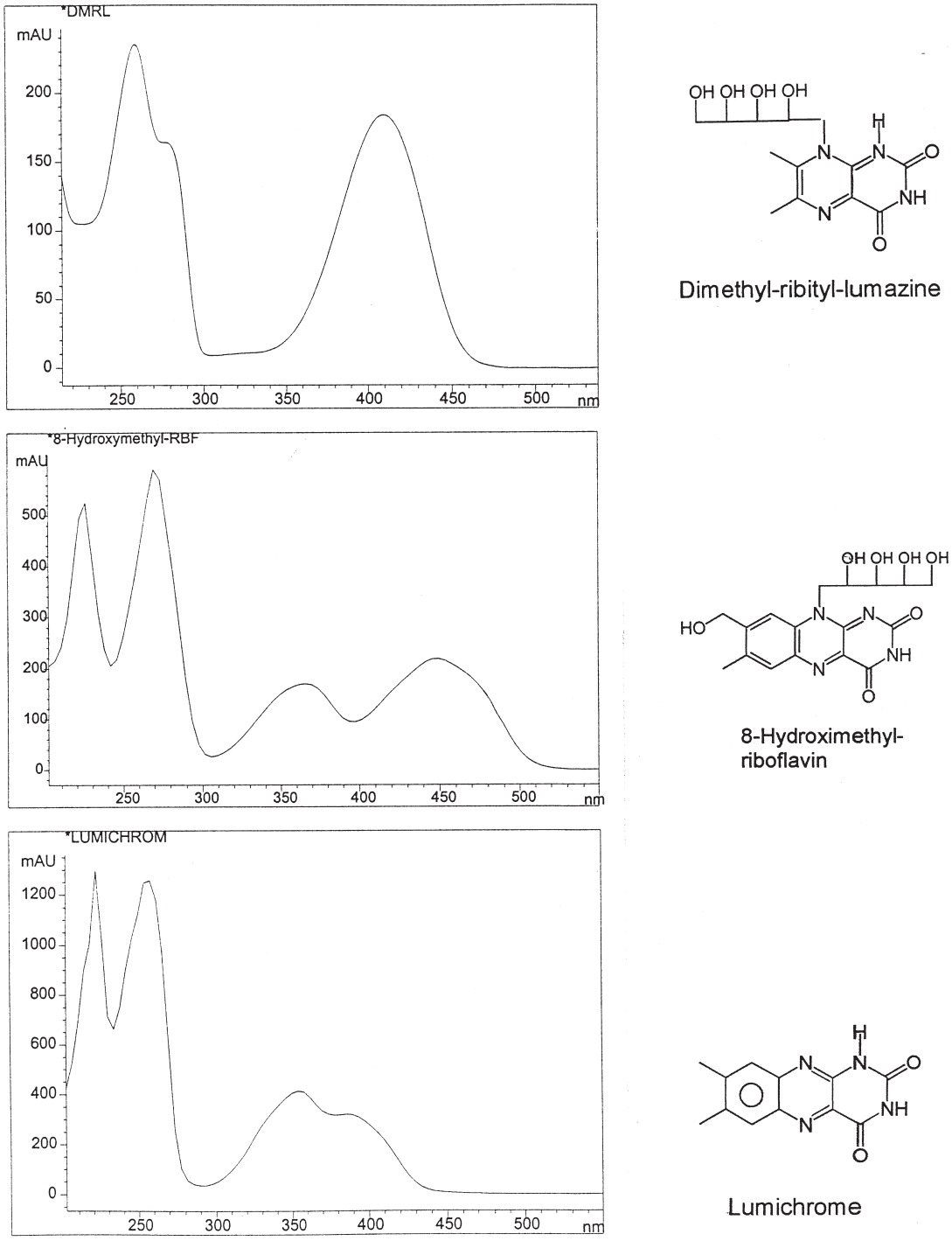

Figure 3 The UV spectra and chemical formula of side-products in riboflavin ex fermentation. DMRL, dimethyl-ribityl-lumazine, top panel; 8-hydroxymethyl-riboflavin, middle panel; lumichrome, bottom panel. The spectra shown are those obtained for the compounds present in the riboflavin samples ex fermentation. They are identical to those of the reference compounds (not shown).

the riboflavin obtained were done using material from 35-m^3 pilot scale fermentations. The riboflavin contents of the riboflavin products ex fermentation exceed the 98% chemical purity of the highest grade riboflavin available from chemical synthesis. The analytical results show that the actual measured values in the riboflavin products obtained by *B. subtilis* fermentation for the acid-washed material is 98.4% and for the recrystallised material 99.7%. In addition, fewer contaminating compounds are present. Chemical characterisation of all compounds present in more than 0.1% of the final product showed that they are either biomass derived (the amino acids and amino sugars), a biosynthetic precursor of riboflavin (DMRL) or degradation products of riboflavin which are also present in the

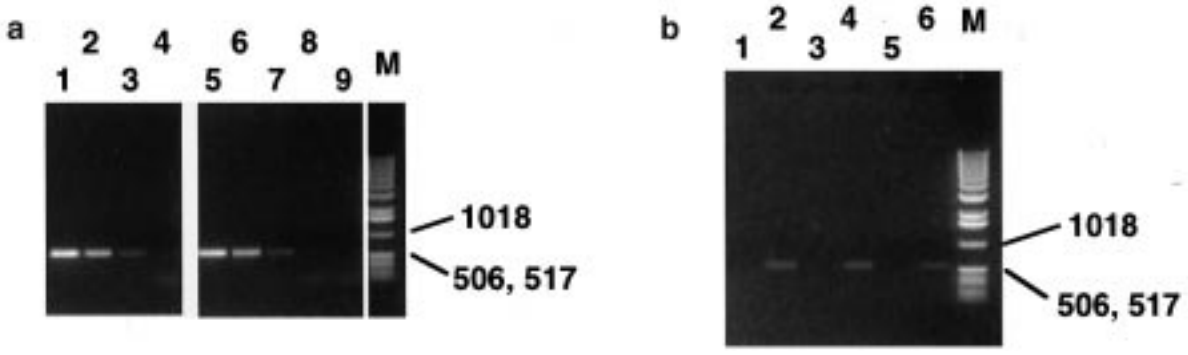

Figure 4 DNA was not detected in riboflavin ex fermentation. Panel (a): Recovery of DNA added to riboflavin samples and sensitivity of the PCR assay used. Control DNA of the riboflavin-producing *B. subtilis* strain RB50::$(pRF69)_{25}$::$(pRF93)_m$ (50 pg mg^{-1} riboflavin) was added to 'feed-grade riboflavin' (lanes 1–4) or 'food/pharma-grade riboflavin' samples (lanes 5–8). After re-extraction of the riboflavin, PCR was performed using either undiluted or diluted samples to detect a 557-bp fragment of the chloramphenicol resistance gene residing on plasmid pRF69. The detection limit of 500 fg of DNA corresponds to about 100 molecules of the *B. subtilis* RB50::$(pRF69)_{25}$::$(pRF93)_m$ genome, each containing 25 copies of pRF69. Lanes 1 and 5: 50 pg DNA; lanes 2 and 6: 5 pg DNA; lanes 3 and 7: 500 fg DNA; lanes 4 and 8: 50 fg DNA; lane 9: blank, no DNA. Panel (b): Assay for absence or presence of DNA in riboflavin samples ex fermentation. Riboflavin samples obtained from the pilot scale production ('feed-grade riboflavin', lanes 1 and 2; 'food/pharma-grade riboflavin', lanes 3 and 4) were directly tested by PCR. Lanes 1 and 3, no control DNA added; lanes 2 and 4, 500 fg control DNA added per mg riboflavin; lane 5, blank, no DNA used; lane 6, direct PCR assay using 500 fg DNA.

chemically-produced riboflavin and result from the acid treatments in the purification procedure. The procedures presented here are directly relevant for the commercial fermentation production process used for riboflavin production.

Importantly, the recrystallisation step leading to highest quality material ex fermentation was kept identical to the last process step currently used to purify the 'food/pharma-grade riboflavin' quality from chemically-produced 'feed-grade riboflavin'. Using the same last step in product purification allows optimal identity between the 'food/pharma-grade riboflavin' qualities obtained by chemical synthesis and fermentation. This process step identity allows optimizing product safety, since it drastically reduces the chances to introduce novel unknown and potentially toxic compounds in the final 'food/pharma-grade riboflavin' product. Also direct analysis of both riboflavin qualities obtained by fermentation in rats in standard sub-chronical (12 weeks) toxicology studies gave no indication of any toxicological effects (not shown).

Product and production process approval by regulatory authorities

National regulations in many countries, eg all European countries, require extensive review of production processes which use recombinant organisms: the product approval procedures focus on the production process. Interestingly, no similarly strict approval process is required nor used for novel production processes using non-recombinant organisms. In the USA, recombinant and non-recombinant organisms are treated similarly, since regulations focus on product quality rather than on the production process as is the case in Europe. According to FDA guidelines for human use [4], riboflavin 'may be prepared by chemical synthesis, biosynthetically by the organism *Eremothecium ashbyii*, or isolated from natural sources'. Since our novel process does not meet this requirement, evaluation of *B. subtilis* as production organism and of the riboflavin produced was required. The products produced should meet 'the specifications of the Food Chemicals Codex, 3rd edn, (1981) p 262' [4]. For feed application in the USA no production procedure has been specified in regulations. Thus, for feed use only the product quality of the new product was considered in the evaluation.

Safety evaluation: the concept of 'substantial equivalence'

Food and food ingredients require a formal premarket approval. This is primarily to ensure the safety of the products produced 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 to or sufficient similarity with a traditional food or ingredient with regards to 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. For riboflavin, 'substantial equivalence' relates to comparison with the currently available commercial products.

The regulatory situation for animal feed differs somewhat from that for the food in the way that at national levels there are no clearly defined regulations in place. However, the safety principle also applies for products to be used in animal feeding. Therefore, also for feed applications the safety assessment focussed on the establishment of the substantial equivalence principle. In this paper, we showed that riboflavin ex fermentation fullfilled the criteria for 'substantial equivalence'.

Absence of DNA and its relevance to safety and labelling

In those countries in which the recombinant nature of the production organism is a key issue, eg in European countries, impurities derived from the production organism itself

also have to be considered. An appropriate and accurate risk evaluation of these products therefore also requires information regarding impurities related to the genetic modification of the production strain. Furthermore, the use of genetic engineering in food production may generate moral and ethical concerns for some consumers. To address such concerns, guidelines for product registration in some countries include product labelling requirements. Product labelling would be required eg in case the food produced still contains DNA of the recombinant production organism. We showed here that no production strain DNA was present in any of the riboflavin produced by fermentation.

Conclusions

In summary, riboflavin obtained by fermentation of the recombinant *B. subtilis* strain RB50::$(pRF69)_n$::$(pRF93)_m$ fullfils the criteria of 'substantial equivalence' to the currently available riboflavin produced by chemical synthesis. In fact, it meets at least the same product specifications and exceeds the quality criteria that are valid for the chemically-synthesized products. The recombinant *B. subtilis* strain described is currently being used for commercial production of riboflavin.

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