Two Biotechnological Approaches to the Preparative Synthesis of Natural Dihydrocoumarin

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Abstract: In this work, we describe two different biotechnological processes that provide the natural flavour dihydrocoumarin in preparative scale. Both the presented approaches are based on the enzyme-mediated reduction of natural coumarin. The first one is a whole-cell process exploiting the reductive activity of the yeast Kluyveromyces marxianus, a Generally Recognized As Safe (GRAS) microorganism that possesses high resistance to the substrate toxicity. Differently, the second is based on the reduction of natural coumarin by nicotinamide adenine dinucleotide phosphate (NADPH) and using the Old Yellow Enzyme reductase OYE2 as catalyst. NADPH is used in catalytic amount since the co-factor regeneration is warranted employing an enzymatic system based on glucose oxidation, in turn catalysed by a further enzyme, namely glucose dehydrogenase (GDH). Both processes compare favourably over the previously reported industrial method as they work with higher coumarin concentration (up to 3 g/L for the enzymatic process) yet allowing the complete conversion of the substrate. Furthermore, the two approaches have significant differences. The microbial reduction is experimentally simple but the isolated dihydrocoumarin yield does not exceed 60%. On the contrary, the enzymatic approach requires the use of two specially prepared recombinant enzymes, however, it is more efficient, affording the product in 90% of isolated yield.

Keywords: dihydrocoumarin; coumarin; natural flavours; biotransformation; Kluyveromyces marxianus; biocatalysis; ene-reductase; glucose dehydrogenase; whole-cell process; recombinant enzyme

1. Introduction

Coumarin (1) and dihydrocoumarin (2) (Figure 1) are compounds of great relevance in the flavour and fragrance industry [1,2]. These lactones, as well as melilotic acid (3) are natural occurring phenylpropanoid that contribute to the scent of different plant flowers and extracts [2–7]. Usually, they are present as a trace component with the relevant exception of coumarin, which is the main component of the Tonka beans (Dipteryx odorata Willd.) extract [3]. Dihydrocoumarin (also known as melilotol) and melilotic acid are strictly related as the hydrolysis of the lactone 2 gives the acid 3. This reaction is catalysed both by hydrolases and by organic acids; therefore, dihydrocoumarin and melilotic acid are usually present together in plant.

![chemical structures](image)

**Figure 1.** The chemical structures of coumarin (1), dihydrocoumarin (2) and melilotic acid (3).

Unfortunately, coumarin is toxic and the European authority has banned the use of this flavour as a food additive and has restricted the use of natural extracts in which it is
present, assessing the tolerable daily intake (TDI) to 0.1 mg/Kg [8]. Therefore, flavour and fragrance producers have been forced to select other natural compounds, considered as safe for human health, possessing a coumarin-like odour. In this context, the most used coumarin substitute is dihydrocoumarin, which possesses a pleasant sweet, herbaceous, coumarin, and coconut-like odour [1,2].

This compound has been recognized as safe for food flavouring [9], as certified by the Flavor and Extract Manufacturers Association (FEMA), which included dihydrocoumarin in the list of the substances Generally Recognized As Safe (GRAS) under the number 2381. Unfortunately, the amount of dihydrocoumarin extracted from natural sources is largely insufficient to satisfy the growing market request of this flavour. Therefore, the main part of the natural dihydrocoumarin is currently produced by biocatalysed reduction of natural coumarin. The processes based on the latter approach fulfill the requirements of the European [10] and USA [11] legislations concerning natural flavour production. Indeed, both regulations establish that the biotransformation of a natural precursor is a ‘natural method’ of synthesis [12] and the flavours obtained by these means possess the ‘natural’ status.

In light of the fact that extractive coumarin is an affordable natural product and that dihydrocoumarin obtained by biotechnological processes is hundreds of times as expensive as the flavour of synthetic origin, we have previously studied the stable isotope characterization of extractive coumarin and of melilotoic produced by baker’s yeast reduction [13]. Accordingly, the natural abundance $^2$H-NMR analysis proved to be a valuable method to differentiate the flavour of natural or synthetic origin.

A few years later, a research group from Degussa and the Technical University of Munich published both a research paper [14] and a patent [15] on the production of dihydrocoumarin by microbial reduction of natural coumarin using different Saccharomyces cerevisiae strains. Melilotic acid was the main product of the biotransformation that was converted into dihydrocoumarin by simple distillation in the presence of a catalytic amount of citric acid (Figure 2). The same study also underlined the main drawback of the claimed process, namely, the high toxicity of coumarin to yeast, which does not allow a proper transformation at a concentration superior to 0.5 g/L.

$$\text{1} \xrightarrow{\text{microbial reduction}} \text{2} \xrightarrow{\text{hydrolysis}} \text{3}$$

Figure 2. The whole-cell process for the preparation of natural dihydrocoumarin starting from natural coumarin.

To improve the affordability of the biotransformation, the industrial processes should be performed with higher substrate concentration. Therefore, we recently decided to select new microorganisms to be employed for dihydrocoumarin production. Accordingly, we first screened a variety of yeasts and filamentous fungi, isolated from different sources, in order to evaluate their ability to reduce selectively the conjugated double bond of coumarin [16]. Out of eighteen selected strains, the yeasts Torulaspora delbrueckii, Kluyveromyces marxianus and the fungus Penicillium camemberti displayed the higher activity and selectivity in the substrate transformation. Moreover, Kluyveromyces marxianus presented the best resistance to substrate toxicity, allowing the biotransformation process even with coumarin concentration up to 1.8 g/L. Overall, our previous results indicated that the problem of substrate concentration could be partially solved employing specific strains, specially selected for their resistance to coumarin toxicity. In addition, our previous studies
on the enzymatic mechanism of coumarin reduction [13,16] suggest that an enzymatic process could be a valid alternative to the microbial biotransformation.

In nature, yeasts perform the ‘biohydrogenation’ of the double bonds using NAD(P)H as reducing agent. The enzymes ene-reductases (ERs) [17] catalyse the asymmetric reduction of the activated C–C double bonds, such as those of the α,β-unsaturated lactones. The most predominant family of ERs present in yeasts are the FMN-containing Old Yellow Enzyme (OYE) family of oxidoreductases (EC 1.6.99.1) [18–20]. We have already studied the biocatalytic reduction of substituted olefins using both fermenting baker’s yeast [21–25] and isolated reductases [26]. We envisage that a merely enzymatic process, devoid of living microorganisms and exploiting OYE catalyst can overcome the problem of coumarin toxicity.

In the present work, we presented two preparative biotechnological processes for the synthesis of the flavour dihydrocoumarin in natural form. First, we developed a whole-cell process based on the transformation of natural coumarin by the yeast *Kluyveromyces marxianus*, a GRAS microorganism that possesses high resistance to the substrate toxicity. Hence, we devised a second preparative approach in which natural coumarin is reduced by NADPH thanks to the catalytic activity of the ene-reductase OYE2. The latter process makes use of an enzymatic system of co-factor regeneration based on the oxidation of glucose, catalysed by a further enzyme, namely a glucose dehydrogenase (GDH).

2. Results and Discussion

As mentioned before, the microbial transformation of coumarin is hampered by the toxicity of the substrate itself that inhibits cell growth along with the cell metabolic activity. Therefore, the substrate concentration strongly affects the biotransformation efficiency. This aspect rises a further important problem: coumarin and dihydrocoumarin are separable with difficult, either by distillation or by chromatography. To design an affordable preparative process for dihydrocoumarin synthesis, it is necessary to reduce the incidence of the separation costs as much as possible. Thus, we decided to find out an experimental procedure allowing the complete reduction of the substrate.

To this end, we selected the yeast *Kluyveromyces marxianus* (DSM 70073) as the microorganism of choice to perform coumarin reduction. At least two different reasons have addressed our selection. First, this yeast well suits the safety requirements for an industrial process in flavour production [27,28] since it has been recognized as safe for human health and has been included in the list of GRAS microorganisms. The second reason concerns its biocatalytic potential. As described in our previous research [16], *Kluyveromyces marxianus* possesses high resistance to coumarin toxicity.

In order to select the experimental procedure that could give the best results both in term of yield and substrate concentration, we first ran a number of experiments using different concentration of coumarin and different biotransformation conditions (Table 1). Accordingly, the yeast was grown in liquid media, using the universal Medium for Yeasts (YM). At the end of the exponential growth phase, the cultures were inoculated with the suitable amount of the coumarin, dissolved in ethanol. The experiments were performed in aerobic conditions, sealing the flasks with cellulose plugs. In these conditions, the Thin Layer Chromatography (TLC) analysis showed only the presence of the melilotic acid and/or of coumarin, indicating that dihydrocoumarin was hydrolysed as soon as it was formed. In addition, the reduction of the conjugated double bond did not proceed further after five days since the substrate addition. From that time onward, the melilotic acid/coumarin ratio kept a constant value. Therefore, the progresses of the biotransformation processes described in Table 1 were measured after five days of contact time. The Gas Chromatographic analyses were performed using a Mass detector (GC-MS) and the results were given as a dihydrocoumarin/coumarin ratio, since the melilotic acid is transformed quantitatively into dihydrocoumarin in the GC injector (see experimental).
Table 1. Reduction of coumarin using *Kluyveromyces marxianus* (DSM 70073) in different experimental conditions.

| Entry | Experimental Conditions | Coumarin Concentration | Dihydrocoumarin/Coumarin Ratio |
|-------|--------------------------|------------------------|---------------------------------|
| 1     | a                        | 1.0 g/L                | 76/24                           |
| 2     | a                        | 1.5 g/L                | 73/27                           |
| 3     | a                        | 2.0 g/L                | 65/35                           |
| 4     | a                        | 2.5 g/L                | 22/78                           |
| 5     | a                        | 3.0 g/L                | 2/98                            |
| 6     | b                        | 1.0 g/L                | 93/7                            |
| 7     | b                        | 2.0 g/L                | 55/45                           |
| 8     | c                        | 2.0 g/L                | 99/1                            |

1 Experimental conditions: *Kluyveromyces marxianus* (DSM 70073) was grown in Erlenmeyer flasks at 28 °C using the universal Medium for Yeasts (YM) and shaking at 130 rpm. Coumarin was added according to conditions a, b, or c. (a) Coumarin was added to the yeast culture as soon as it reached the stationary phase of growth; (b) Coumarin was added to the yeast culture during the exponential phase of growth (16 h after the inoculum); (c) Coumarin (1 g/L) was added after 16 and 24 h after the yeast inoculum, glucose (7.5 g/L) was added after 16, 24 and 40 h after the yeast inoculum. 2 Ratio between total coumarin added and volume of the biotransformation medium. 3 Ratio are determined by GC-MS analysis after five days of contact time.

According to our experiments (Table 1, entries 1–5), we can observe that using a starting coumarin concentration between 1.0 to 2.0 g/L the dihydrocoumarin/coumarin ratio remains rather constant (from 76/24 to 65/35) whereas decreases significantly with concentrations superior to 2 g/L. Finally, using a concentration of coumarin of 3 g/L, the reduction reaction was almost completely inhibited. Overall, we could conclude that the selected *Kluyveromyces marxianus* strain was active even with coumarin concentration up to 3 g/L, but the investigated conditions of biotransformation did not allow the complete transformation of the substrate. Hence, we decided to change another experimental condition, namely the time of coumarin addition. More specifically, in a new set of trials, the substrate was added at once when the yeast was in its exponential phase of growth (Table 1, entry 6 and 7). This approach gave promising results, as it allowed very good conversion results using a substrate concentration of 1 g/L. Unfortunately, increasing the substrate concentration to 2 g/L, the dihydrocoumarin/coumarin ratio decreased from 93/7 to 55/45.

These experimental results can be explained taking into account that during the exponential phase of growth, the yeast metabolises glucose generating a large amount of NAD(P)H through Krebs cycle. In these conditions, the ene-reductases present in *Kluyveromyces marxianus* catalyse the addition of NAD(P)H to the conjugated double bond of the substrate. When coumarin is added in higher concentrations, the toxic effect of substrate inhibits the metabolism, thus, decreasing NAD(P)H formation and in turn coumarin reduction.

Taking advantage of the above-described results, we further modified the biotransformation procedure. More specifically, we decided to add the substrate in two equal portions, after 16 and 24 h after the microbial inoculum whereas further glucose was added after 16, 24, and 40 h (Table 1, entry 8). According to the latter protocol, we observed complete conversion of the substrate with a dihydrocoumarin/coumarin ratio of 99/1. As a final point, we evaluated the actual yield of our procedure. Indeed, the latter ratio value indicates the progress of the ‘biohydrogenation’ reaction, which is only partially correlated with the yield of product obtainable after work-up and purification steps.

Therefore, we performed the biotransformation on a preparative scale (500 mL, 2 g/L), isolating the melilotic acid/coumarin mixture by extraction with ethyl acetate. The following distillation under reduced pressure of the crude extract, in presence of a catalytic amount of citric acid, afforded pure almost pure dihydrocoumarin (2% of coumarin) in 58% overall yield.

This experiment confirmed the complete reduction of the substrate as well as the scalability of the procedure. The yield (58%) indicated that coumarin and/or melilotic acid
were partially degraded during the fermentation process. This result is not surprising as phenylpropanoids containing a phenol functional group (as melilotic acid) are prone to both microbial and chemical oxidation in aerobic conditions [16]. Overall, we can conclude that our whole-cell process compares favourably over the previously reported industrial method, based on the use of baker’s yeast, in which the working coumarin concentration does not exceed 0.5 g/L. Its main drawback concerns the requirement of specific experimental conditions that are mandatory to achieve complete coumarin reduction.

In order to overcome this issue, we studied a new approach based on the use of isolated enzymes. We envisage that an enzymatic process can be standardized easily, overcoming both the problems related to the microbial metabolism and those related to coumarin toxicity.

According to the generally recognized mechanism of biocatalysed reduction of \( \alpha,\beta \)-unsaturated lactones, ERs catalyse the conjugated addition of an hydride from NAD(P)H to the \( \beta \) position of the double bond followed by the addition of a proton (abstracted from water) to the carbon in \( \alpha \) position. Overall, the ‘biohydrogenation’ reaction consists in an enantioselective anti addition of hydrogen to the C-C double bond [23, 26].

The ERs present in yeasts are FMN-containing Old Yellow Enzyme (OYE) oxidoreductases (EC 1.6.99.1). Since OYE2 is the most representative ER present in baker’s yeast [29], we decided to employ OYE2 from Saccharomyces cerevisiae as the enzyme of choice to perform coumarin reduction. Of course, due to high cost of the cofactor, preparative processes cannot make use of NAD(P)H in stoichiometric amount. Therefore, the use of a catalytic amount of the cofactor combined with a suitable regeneration system becomes mandatory. In this context, the oxidation of glucose to \( \delta \)-gluconolactone catalysed by glucose dehydrogenase (GDH) with simultaneous reduction of NADP to NADPH has proved to be a very efficient system [30]. In addition, glucose is a cheap sacrificial substrate, and the formed lactone is irreversibly hydrolysed in an aqueous environment (Figure 3).

![Figure 3. The enzyme-catalysed reduction of coumarin to dihydrocoumarin/melilotic acid.](image)

The reduction of \( \alpha,\beta \)-unsaturated aldehydes catalysed by OYE2 and using NADPH and glucose/GDH as regeneration system has been already described by Parmeggiani [29] that successfully exploited this method for the synthesis of the pharmaceutical intermediate (S)-2-ethoxy-3-(\( \rho \)-methoxyphenyl)propanoate. In the latter study, His-tagged OYE2 from
Saccharomyces cerevisiae and His-tagged GDH from Bacillus megaterium were cloned, overexpressed in Escherichia coli and purified. Thanks to the availability of these two modified E. coli strains, decided to design a biocatalytic process for coumarin reduction employing the latter two enzyme. Accordingly, we performed a number of experiments to first verify the applicability of the latter approach and then to optimize the experimental parameters for bioconversion (Table 2).

Table 2. Reduction of coumarin using NADP+/glucose/GDH/OYE2 in different experimental conditions.

| Entry | Experimental Conditions ¹ | Reaction Time (h) | Coumarin Concentration ² | Dihydrocoumarin/ Coumarin Ratio ³ |
|-------|---------------------------|------------------|--------------------------|----------------------------------|
| 1     | a                         | 42               | 1.0 g/L                  | 0/100                            |
| 2     | b                         | 18               | 1.0 g/L                  | 52/48                            |
| 3     | b                         | 42               | 1.0 g/L                  | 54/46                            |
| 4     | c                         | 42               | 1.0 g/L                  | 55/45                            |
| 5     | d                         | 17               | 0.5 g/L                  | 100/0                            |
| 6     | d                         | 17               | 1.0 g/L                  | 100/0                            |
| 7     | d                         | 17               | 2.0 g/L                  | 100/0                            |
| 8     | d                         | 22               | 3.0 g/L                  | 67/23                            |
| 9     | d                         | 22               | 5.0 g/L                  | 35/65                            |
| 10    | d                         | 22               | 8.0 g/L                  | 18/82                            |
| 11    | d                         | 72               | 3.0 g/L                  | 100/0                            |

¹ Experimental conditions: Coumarin (10% w/v solution in ethanol) was added to a reaction mixture containing glycerol (10% v/v), ethanol (2% v/v), glucose (eight equivalents vs. coumarin), NADP⁺ (0.15 mM), and potassium phosphate buffer (25 mMol, pH 7.4). For coumarin concentrations superior to 2 g/L, the ethanol content was increased up to 18% v/v, in order to warrant the homogeneity of the reaction mixtures. The reactions were performed in sealed tubes, previously flushed with nitrogen, at 23 °C under shaking (100 rpm). (a) GDH (0.3 mg/mL) from recombinant E. coli lysate; (b) GDH (0.3 mg/mL) from recombinant E. coli lysate and OYE2 (0.25 mg/mL) from recombinant E. coli lysate; (c) Phenylmethanesulfonyl fluoride (0.5 mM), GDH (0.3 mg/mL) from recombinant E. coli lysate and OYE2 (0.25 mg/mL) from recombinant E. coli lysate; (d) purified GDH (0.3 mg/mL) and purified OYE2 (0.2 mg/mL).

² Ratio between total coumarin added and volume of the biotransformation medium.

³ Ratio is determined by GC-MS analysis.

Since we needed to develop a preparative process, we first investigated the direct use of E. coli cells overexpressing GDH and OYE2 as a source of the latter enzymes. Accordingly, we first employed the clear E. coli lysates, containing the recombinant proteins, as biocatalysts for coumarin reduction. The experiments (Table 2, entry 1–4) indicate the efficacy of the biocatalytic approach although with a defined limit in term of conversion. Since the first biotransformation trial (entry 1) did not contain OYE2, no dihydrocoumarin was formed, confirming that the presence of the latter enzyme is essential to catalyse the reduction reaction. Using the same experimental conditions, the introduction of both enzymes rapidly brought to formation of dihydrocoumarin (entry 2, 52% conversion, 18 h) but the reaction did not proceed further even considerably prolonging the reaction time (entry 3, 54% conversion, 42 h). We supposed that the presence of hydrolytic enzymes (such as the proteases) in the lysates could slowly reduce the enzyme activities, thus stopping the catalytic cycles. Unfortunately, our assumption was wrong as indicated by one further trial (entry 4). Indeed, the addition of a protease inhibitor (phenylmethanesulfonyl fluoride) showed no improvements.

Hence, we decided to test the biotransformation process using purified enzymes, at different substrate concentrations (entries 5–11). The experiments ran with a coumarin concentration up to 2 g/L (entries 5–7), gave excellent results showing complete conversion within 17 h of reaction. Higher substrate concentrations reduced noticeably the yields as indicated by trials described in entries 8–10, where conversions decreased from 67% to 18% increasing substrate concentrations from 3 g/L to 8 g/L. These results can be explained taking in account that the catalytic activity of the enzymes could be partially inhibited by high concentrations of the substrate/product. In addition, for coumarin concentrations superior to 2 g/L, the experimental conditions were slightly modified, as ethanol content...
had to be increased up to 18% v/v in order to warrant the homogeneity of the reaction mixtures. Overall, we established that a working concentration of substrate of 3 g/L represented a good compromise between coumarin concentration, reaction time and process efficiency. The last trial (entry 11) indicated that using the above-described experimental conditions and prolonging the reaction time (72 h), it is possible to achieve the complete reduction of the substrate.

Another relevant aspect related to the combined use of OYE2 and NADPH/GDH concerns the relative efficiency of the two enzyme-catalysed reactions. GDH from Bacillus megaterius has high catalytic activity [29] and the reduction rate of NADP⁺ is greatly enhanced using a large excess of glucose. Therefore, the conjugated addition of the NADPH hydride to the substrate is the bottleneck of the entire enzymatic process. From a biotechnological standpoint, it would be important to determine the OYE2 concentration that can ensure complete conversion of the substrate within a proper reaction time. To this end, we devised a new set of experiments in which each trial contained the same amount of coumarin, NADP⁺, glucose, and GDH but with different concentrations of OYE2 (Table 3). In these experimental conditions, OYE2 concentrations superior to 0.25 mg/L allowed complete conversions in less than 24 h (entries 1 and 2). Furthermore, the reduction of coumarin using a OYE2 concentration of 0.2 and 0.15 mg/L took 48 h to achieve complete conversion (entries 3–5). Finally, we observed that the trials containing OYE2 concentrations inferior to 0.1 mg/L required very long time to reach good substrate conversions (entries 6–10).

Table 3. Reduction of coumarin using NADP⁺/glucose/GDH/OYE2 using different concentration of OYE2.

| Entry | OYE2       | Reaction Time (h) | Dihydrocoumarin/Coumarin Ratio ² |
|-------|------------|-------------------|----------------------------------|
| 1     | 0.50 mg/L  | 24                | 100/0                            |
| 2     | 0.25 mg/L  | 24                | 100/0                            |
| 3     | 0.20 mg/L  | 24                | 90/10                            |
| 4     | 0.20 mg/L  | 24                | 100/0                            |
| 5     | 0.15 mg/L  | 24                | 100/0                            |
| 6     | 0.10 mg/L  | 24                | 78/22                            |
| 7     | 0.10 mg/L  | 24                | 100/0                            |
| 8     | 0.05 mg/L  | 24                | 96/4                             |
| 9     | 0.025 mg/L | 24                | 50/50                            |
| 10    | 0.0125 mg/L| 24                | 35/65                            |

¹ Experimental conditions: The reaction mixture was made up of coumarin (2 g/L), glycerol (10% v/v), ethanol (2% v/v), glucose (10 equivalents vs. coumarin), NADP⁺ (0.16 mM), purified GDH (0.35 mg/mL), purified OYE2 (concentrations in Table 3), and potassium phosphate buffer (25 mMol, pH 7.4). The reactions were performed in sealed tubes, previously flushed with nitrogen, at 23 °C under shaking (100 rpm). ² Ratio is determined by GC-MS analysis.

Overall, these experiments allow us to conclude that the proposed biotransformation process, with a coumarin concentration superior to 2 g/L, need a OYE2 concentrations of at least 0.2 mg/L to achieve complete reduction of the substrate within three days of reaction time.

Taking advantage of the previous described results, we evaluated the scalability of the proposed enzymatic process as well as its efficiency in terms of dihydrocoumarin yield. Accordingly, we performed the biotransformation on a preparative scale (500 mL, 3 g/L of coumarin, 23 °C) using 0.3 mg/mL of GDH, 0.2 mg/mL OYE2, NADP⁺ (0.15 mM), and glucose (10 equivalents vs. coumarin). After three days of reaction, the coumarin was completely reduced. The work up procedure afforded a mixture of melilotic acid and dihydrocoumarin whose distillation under reduced pressure, in presence of a catalytic amount of citric acid, afforded pure dihydrocoumarin in 90% overall yield.

In conclusion, we developed two different preparative processes that provide the natural flavour dihydrocoumarin starting from natural coumarin. The first approach is a whole-cell biotransformation that makes use of the yeast Kluyveromyces marxianus, a GRAS microorganism possessing high resistance to the substrate toxicity. The latter process
works with coumarin concentration up to 2 g/L and allows the complete conversion of the substrate in five days, to afford dihydrocoumarin in 58% isolated yield. The second preparative approach exploits the catalytic activity of the ene-reductase OYE2 from Saccharomyces cerevisiae. NADPH is used as reducing agent and the co-factor regeneration is warranted employing an enzymatic system based on glucose oxidation, in turn catalysed by the glucose dehydrogenase from Bacillus megaterius (GDH). According to the latter process, coumarin (3 g/L) is completely reduced within 72 h and dihydrocoumarin is obtained in 90% of isolated yield. Both processes were studied using synthetic coumarin. For sake of completeness, the two preparative procedures were repeated using natural coumarin (from fava Tonka beans) affording almost identical results.

Overall, we can conclude that both processes compare favourably over the previously reported industrial method [14,15], based on the use of baker’s yeast, in which the working coumarin concentration does not exceed 0.5 g/L. Furthermore, our two approaches have significant differences. The microbial reduction is experimentally simple but the isolated dihydrocoumarin yield does not exceed 60%. On the contrary, our process employing OYE2 and GDH needs the specific preparation of the latter recombinant enzymes but overcomes the problem of the interference of the microbial metabolism, allowing a working coumarin concentration of 3 g/L with very high product yield (90%).

3. Materials and Methods

3.1. Materials and General Methods

All air and moisture sensitive reactions were carried out using dry solvents and under a static atmosphere of nitrogen. All solvents and reagents, including kanamycin sulphate, ampicillin, phenylmethanesulfonyl fluoride, and coumarin, were of commercial quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA); NADP\(^+\) (sodium salt hydrate, 97% purity, lot. A0404841) was purchased from Acros Organics; riboflavin was purchased from Health Leads UK Ltd., (Horeb, UK); natural coumarin from fava Tonka beans (composition 94% coumarin and 6% dihydrocoumarin) was purchased from Mane SA (Le Bar-sur-Loup, France).

Reference standard samples of dihydrocoumarin and melilotic acid were prepared starting from coumarin, as described previously [16].

3.2. Microorganism and Growth Media

The growth and expression media used in this work are YUM (Yeasts Universal Medium), LB and ZYM-5052 implemented with 20 mg/L of riboflavin.

YUM medium composition: yeast extract (3 g/L), malt extract (3 g/L), peptone from soybeans (5 g/L), glucose (10 g/L).

LB medium composition: NaCl (5 g/L), tryptone (10 g/L), yeast extract (5 g/L). pH was adjusted to 7.0.

ZYM-5052 medium composition: ZYM-0, 50X 5052 solution (20 mL/L) and trace elements solution (1 mL).

The bulk composition of ZYM medium (ZYM-0) is casein peptone (10 g/L), yeast extract (5 g/L), Na\(_2\)HPO\(_4\) (3.6 g/L), KH\(_2\)PO\(_4\) (3.5 g/L), NH\(_4\)Cl (2.7 g/L), Na\(_2\)SO\(_4\) (0.7 g/L) and MgSO\(_4\) (0.25 g/L). pH was adjusted to 7.0. ZYM-0 is then autoclaved for 15 min at 121 °C.

Trace elements solution: FeCl\(_3\) (50 mM), CaCl\(_2\) (20 mM), MnCl\(_2\) (10 mM), ZnSO\(_4\) (10 mM), CoCl\(_2\) (2 mM), CuCl\(_2\) (2 mM), NiCl\(_2\) (2 mM), Na\(_2\)MoO\(_4\) (2 mM), Na\(_2\)SeO\(_3\) (2 mM), H\(_3\)BO\(_3\) (2 mM).

50X 5052 solution composition: glycerol (250 g/L), glucose (25 g/L), α-lactose (100 g/L). Once prepared, the solution was filtered on Sartorius\textsuperscript{TM} Minisart\textsuperscript{TM} Plus Syringe Filters 0.2 µm pore size and stored at 4 °C.

\textit{Kluyveromyces marxianus} DSM 70073 was grown using YUM medium at 25 °C.
3.3. Protein Expression and Purification of OYE2 (OYE2)

Recombinant *E. coli* BL21(DE3) cells, containing the pET-30a-OYE2 plasmid harbouring the gene of *Saccharomyces cerevisiae* (ATCC 204508) coding for OYE2 (UniProtKB accession number Q03558) [29], were inoculated in a 1 L Erlenmeyer flask containing LB medium (100 mL). The flask was supplemented with kanamycin (100 µg/mL) and was incubated at 37 °C overnight with shaking (220 rpm). Hence, the culture was centrifuged at 3220 g for 5 min (4 °C), the supernatant was removed, and the cells were resuspended in 10 mL of fresh LB medium. The obtained suspension was added to a fermenter vessel of the 5 L bioreactor containing ZYM-0 medium (2 L) supplemented with kanamycin (50 µg/mL), 50X 5052 solution (40 mL) and trace elements solution (2 mL). The temperature, the stirring speed and the pH were set to 22 °C, 250 rpm and 7.0, respectively. The pH was controlled by dropwise addition of sterilized aqueous solutions (10% w/w in water) of either sulfuric acid or ammonia. The fermentation was stopped 72 h since the inoculum.

The centrifugation (8000 rpm, 15 min, 4 °C) of the whole fermentation broth allowed obtaining 18.9 g of wet cells. The pellet was resuspended in 25 mM potassium phosphate buffer, pH 7.0, containing 500 mM NaCl and 20 mM imidazole. At this point, cells were disrupted by ultrasonication (15 cycles, 20”ON/120” OFF pulses in ice).

Clear lysate (160 mL) was obtained by centrifugation (11,000 rpm, 30 min) and the protein content titer was assessed by Bradford assay (22 mg/mL). A sample of the aforementioned clear lysate (40 mL) was purified by incubation with Ni Sepharose 6 Fast Flow agarose resin (Ni-NTA) (GE Healthcare, Milan, Italy) under mild shaking for 90 min at 4 °C. The resin was then loaded onto a glass column (10 × 110 mm) and washed with 20 mL of wash buffer (20 mM imidazole, 500 mM NaCl, 20 mM potassium phosphate buffer). Elution of His-tagged proteins was achieved by using a 3-step gradient (15 mL washing buffer containing 100, 200, and 300 mM imidazole, respectively). To purified protein solution was added glycerol (10% v/v). The obtained clear solution (88 mL) contained 2.5 mg/mL of OYE2, which appeared as a single band by SDS-PAGE analysis. The aforementioned solution was stored at −80 °C.

Protein concentration was determined using the Bio-Rad Protein Assay according to the method of Bradford, and SDS-PAGE analyses (12% T, 2.6% C) were performed to assess protein purity. Molecular weight protein standards were from Bio-Rad (Karlsruhe, Germany), and gels were stained with Coomassie Brilliant Blue for protein detection.

3.4. Protein Expression and Purification of Glucose Dehydrogenase (GDH)

Recombinant *E. coli* BL21(DE3) cells, containing the pKTS plasmid harboring the gene of *Bacillus megaterium* DSM 509 coding for glucose dehydrogenase (UniProtKB accession number P40288) [29], were inoculated in a 1 L Erlenmeyer flask containing LB medium (100 mL). The flask was supplemented with ampicillin (100 µg/mL) and was incubated at 37 °C overnight with shaking (220 rpm). Hence, the culture was centrifuged at 3220 g for 5 min (4 °C), the supernatant was removed, and the cells were resuspended in 10 mL of fresh LB medium. The obtained suspension was added to a fermenter vessel of the 5 L bioreactor containing ZYM-0 medium (2 L) supplemented with ampicillin (100 µg/mL), 50X 5052 solution (40 mL) and trace elements solution (2 mL). The temperature, the stirring speed, and the pH were set to 22 °C, 250 rpm and 7.0, respectively. The pH was controlled by dropwise addition of sterilized aqueous solutions (10% w/w in water) of either sulphuric acid or ammonia. The fermentation was stopped 72 h since the inoculum.

The centrifugation (8000 rpm, 15 min, 4 °C) of the whole fermentation broth allowed obtaining 25.1 g of wet cells. The pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.0, containing 500 mM NaCl and 20 mM imidazole. At this point, cells were disrupted by ultrasonication (10 cycles, 20”ON/120” OFF pulses in ice).

Clear lysate (228 mL) was obtained by centrifugation (11,000 rpm, 30 min) and the protein content titer was assessed by Bradford assay (11.7 mg/mL). The presence of soluble glucose dehydrogenase was assessed by SDS-PAGE (12% T, 2.6% C). A sample of the aforementioned clear lysate (29 mL) was purified by incubation with Ni Sepharose 6 Fast Flow
agarose resin (Ni-NTA) (GE Healthcare, Milan, Italy) under mild shaking for 90 min at 4 °C. The resin was then loaded onto a glass column (10 × 110 mm) and washed with 20 mL of wash buffer (20 mM imidazole, 500 mM NaCl, 20 mM potassium phosphate buffer). Elution of His-tagged proteins was achieved by using a 3-step gradient (15 mL washing buffer containing 100, 200, and 300 mM imidazole, respectively). To the purified protein solution was added glycerol (10% v/v). The obtained clear solution (37 mL) contained 3.6 mg/mL of GDH, which appeared as a single band by SDS-PAGE analysis. The aforementioned solution was stored at −80 °C.

3.5. Analytical Methods and Characterization of the Products Deriving from the Biotransformation Experiments

3.5.1. Instruments and Analytic Condition

Nuclear Magnetic Resonance spectroscopy (NMR): 1H- and 13C-NMR Spectra and DEPT experiments: CDCl3 solutions at RT using a Bruker-AC-400 spectrometer (Billerica, MA, USA) at 400, 100, and 100 MHz, respectively; 13C spectra are proton decoupled; chemical shifts in ppm relative to internal SiMe4 (=0 ppm).

TLC: Merck silica gel 60 F254 plates (Merck Millipore, Milan, Italy); column chromatography: silica gel.

GC-MS analyses: HP-6890 gas chromatograph equipped with a 5973 mass detector, using a HP-5MS column (30 m '0.25 mm, 0.25 µm film thickness; Hewlett Packard, Palo Alto, CA, USA) with the following temp. program: 60° (1 min), then 6°/min to 150° (held 1 min), then 12°/min to 280° (held 5 min); carrier gas, He; constant flow 1 mL/min; split ratio, 1/30; tR given in min: tR(1) 17.82, tR(2) 16.49.

The biotransformations were monitored by means of GC-MS analysis. To this end, an aliquot of the biotransformation mixture was acidified at pH 4 and filtered on celite. The aqueous phase was then extracted three times with ethyl acetate and the combined organic layer was washed with brine and dried on Na2SO4. The obtained solution was analyzed by GC-MS in order to determine the dihydrocoumarin/coumarin ratio.

3.5.2. General Procedure for the Study of the Biotransformation of Coumarin Using Kluyveromyces marxianus

A small amount of active yeast (DSM 70073) was picked-up from a petri dish, was suspended in 1 mL of sterile water, and then was inoculated in a 100 mL conical pyrex flask containing 30 mL of the suitable medium. The flask was shaken for four days at 25 °C and 130 rpm. After this period, 2 mL of the yeast suspension were inoculated in 1 L conical biotransformation flasks, each containing 200 mL of fresh medium. In order to ensure aerobic conditions, the flasks were sealed with cellulose plugs. Coumarin (100 g/L solution in ethanol) was added to the latter trials according to the experimental conditions described in Table 1. After five days since substrate addition, the reactions were analysed as described above.

3.5.3. General Procedure for the Study of the Biotransformation of Coumarin Using OYE2/NADP+/Glucose/GDH and Variable Substrate Concentrations

A reaction mixture (25 mL) made up of PB buffer (pH 7.4, 25 mM), glucose (8 equivalents vs. coumarin), NADP+ (0.15 mM), glycerol (10% v/v), and ethanol (2% v/v) was placed in a hungate tube, which was nitrogen flushed and sealed. The suitable enzymes (GDH, OYE2), phenylmethanesulfonyl fluoride, and coumarin (100 g/L solution in ethanol) were injected into the sealed tube using a syringe (Table 2). For coumarin concentrations superior to 2 g/L, further ethanol (up to 18% v/v) was added to obtain homogeneous reaction mixture. Each trial was shaken at 23 °C (100 rpm). After the indicated time (Table 2), the reaction was acidified with concentrated HCl aq. (500 µL) then was extracted with ethyl acetate (4 mL). The organic phase was separated and was analysed as described above.
3.5.4. General Procedure for the Study of the Biotransformation of Coumarin Using OYE2/NADP\(^+\)/Glucose/GDH and Variable OYE2 Concentrations

A reaction mixture (6 mL) made up of PB buffer (pH 7.4, 25 mM), glucose (10 equivalents vs. coumarin), NADP\(^+\) (0.16 mM), purified GDH (0.35 mg/mL) glycerol (10% v/v), ethanol (2% v/v), and coumarin (2 g/L) was placed in a hungate tube, which was nitrogen flushed and sealed. Purified OYE2 (Table 3) was injected into the sealed tubes using a syringe. Each trial was shaken at 23 °C (100 rpm). After the indicated time (Table 3), the reaction was acidified with concentrated HCl aq. (500 µL) then was extracted with ethyl acetate (4 mL). The organic phase was separated and was analysed as described above.

3.6. Preparative Procedure for the Synthesis of Dihydrocoumarin Using Kluyveromyces marxianus

A small amount of active yeast (DSM 70073) was picked-up from a petri dish, was suspended in 1 mL of sterile water, and then was inoculated in a 100 mL conical pyrex flask containing 30 mL of the suitable medium. The flask was shaken for four days at 25 °C and 130 rpm. After this period, 5 mL of the yeast suspension were inoculated in a 3 L conical biotransformation flask, containing 500 mL of fresh medium. In order to ensure aerobic conditions, the flask was sealed with a cellulose plug. Two aliquots of coumarin (each one consisting of 5 mL of a 100 g/L solution in ethanol) were added after 16 and 24 h after the yeast inoculum, while three aliquots of glucose (each one consisting of 6 mL of a 250 g/L solution in water) were added after 16, 24, and 40 h after the yeast inoculum. The flask was shaken at 25 °C and 130 rpm for five days since the first coumarin addition. Then, the biotransformation was quenched by adding concentrated HCl aq. (5 mL) and was filtered on a celite pad. The filtered biomass was washed with ethyl acetate (100 mL) and the aqueous phase was extracted with further ethyl acetate (2 × 100 mL). The combined organic phases were dried (Na\(_2\)SO\(_4\)) and concentrated in vacuo. Citric acid (50 mg) was added to the residue, which was bulb-to-bulb distilled. Dihydrocoumarin was obtained as a colourless oil (585 mg, 58% yield, 98% purity) showing 99/1 dihydrocoumarin/coumarin ratio by GC-MS analysis (chromatogram in the Supplementary Materials section).

An identical preparation experiment was performed starting from natural coumarin from fava Tonka beans (see paragraph 3.1). Dihydrocoumarin (96% purity, 99/1 dihydrocoumarin/coumarin ratio) was obtained in 55% yield.

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta = 7.28 - 7.16\) (m, 2H), 7.13 - 7.01 (m, 2H), 3.00 (br t, \(J = 7.3\) Hz, 2H), 2.78 (br t, \(J = 7.3\) Hz, 2H).

\(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta = 168.4\) (C), 151.9 (C), 128.2 (CH), 127.9 (CH), 124.3 (CH), 122.6 (C), 116.8 (CH), 29.1 (CH\(_2\)), 23.6 (CH\(_2\)).

GC-MS (EI): \(m/z\) (%) = 148 [M\(^+\)] (100), 120 (84), 106 (10), 91 (75), 78 (47), 63 (12), 51 (15).

3.7. Preparative Procedure for the Synthesis of Dihydrocoumarin Using OYE2 and NADP\(^+\)/Glucose/GDH

A solution of coumarin (15 mL of a 100 g/L solution in ethanol) was added under nitrogen to a mechanically stirred mixture (500 mL) made up of PB buffer (pH 7.4, 25 mM), glucose (10 equivalents vs. coumarin), NADP\(^+\) (0.15 mM), purified GDH (0.30 mg/mL), OYE2 (0.2 mg/mL), glycerol (15% v/v), and ethanol (5% v/v). The temperature was kept at 23 °C by means of a thermostatic bath and stirring was prolonged for further 72 h. Hence, the reaction was quenched by addition of HCl aq. (10 mL of a 10% w/v solution) followed by extraction with ethyl acetate (3 × 100 mL). The combined organic phases were dried (Na\(_2\)SO\(_4\)) and concentrated in vacuo. Citric acid (50 mg) was added to the residue, which was bulb-to-bulb distilled. Dihydrocoumarin was obtained as a colourless oil (1.36 g, 90% yield, 98% purity). The GC-MS analysis showed a dihydrocoumarin/coumarin ratio >99/1.

An identical preparation experiment was performed starting from natural coumarin from fava Tonka beans (see Section 3.1). Dihydrocoumarin (95% purity, dihydrocoumarin/coumarin ratio >99/1) was obtained in 91% yield (chromatogram in the Supplementary Materials section).

\(^1\)H NMR, \(^13\)C NMR, GC-MS are described in Section 3.6.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/catal12010028/s1, Figure S1: GC-MS analysis of the dihydrocoumarin obtained using Kluyveromyces marxianus (preparative procedure, Section 3.6), Figure S2: GC-MS analysis of the dihydrocoumarin obtained using OYE2 and NADP+/glucose/GDH (preparative procedure, Section 3.7), Figure S3: GC-MS (EI) spectrum of dihydrocoumarin, Figure S4: GC-MS (EI) spectrum of coumarin.

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Data Availability Statement: Data is contained within the article or Supplementary Materials.

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