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Biotechnological Production of 20-alpha-Dihydrodydrogesterone at Pilot Scale

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Abstract The human sex hormone progesterone plays an essential and complex role in a number of physiological processes. Progesterone deficiency is associated with menstrual disorders and infertility as well as premature birth and abortion. For progesterone replacement therapy, the synthetic progestogen dydrogesterone is commonly used. In the body, this drug is metabolized to 20α-dihydrodydrogesterone (20α-DHD), which also shows extensive pharmacological effects and hence could act as a therapeutic agent itself. In this study, we describe an efficient biotechnological production procedure for 20α-DHD that employs the stereo- and regioselective reduction of dydrogesterone in a whole-cell biotransformation process based on recombinant fission yeast cells expressing the human enzyme AKR1C1 (20α-hydroxysteroid dehydrogenase, 20α-HSD). In a fed-batch fermentation at pilot scale (70 L) with a genetically improved production strain and under optimized reaction conditions, an average 20α-DHD production rate of 190 μM day^{-1} was determined for a total biotransformation time of 136 h. Combined with an effective and reliable downstream processing, a continuous production rate of 12.3±1.4 g 20α-DHD per week and fermenter was achieved. We thus established an AKR-dependent whole-cell biotransformation process that can also be used for the production of other AKR1C1 substrates (as exemplarily shown by the production of 20α-dihydroprogesterone in gram scale) and is in principle suited for the production of further human AKR metabolites at industrial scale.

Keywords 20α-hydroxysteroid dehydrogenase · AKR1C1 · Aldo-keto reductase · Dydrogesterone · *Schizosaccharomyces pombe* · Whole-cell biotransformation

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Introduction

Steroids and steroid hormones currently represent one of the largest sectors in the pharmaceutical industry. Due to the overwhelming demand for these substances, new synthesis routes have to be developed. In this context, biotransformations of steroids catalyzed by recombinant microorganisms are of steadily increasing interest. One major family among the different types of enzymes that are known to allow specific modifications of the steroid backbone are the aldo-keto reductases (AKRs). They represent a diverse collection of cytosolic, soluble NAD(P)H-dependent oxidoreductases that reduce aldehydes and ketones to yield primary and secondary alcohols, respectively [1]. Animal AKRs are involved in a range of cellular processes that include both the biosynthesis of steroid hormones in classical steroidogenic tissues as well as the inactivation of steroids in target tissues [2, 3]. In addition to their value as a metabolite producing enzymes, AKRs are also a suitable biological tool for specific changes on the steroid backbone that are difficult to realize by chemical means. However, these enzymes have not been used at industrial scale so far. With respect to the production of larger quantities of metabolites, whole-cell biotransformations are often superior to the use of purified enzymes due to easier scale-up and cofactor regeneration by the endogenous metabolism of the expression host. Recombinant fission yeast offers a great potential for the production of various enzymes of industrial interest, and its suitability for whole-cell biotransformations make this organism very promising for many biotechnological applications [4, 5]. In a recent study, we demonstrated the first functional expression of a heterologous AKR in *Schizosaccharomyces pombe* using human AKR1C1 (20α-hydroxysteroid dehydrogenase) as the model enzyme [6]. We reported the general feasibility of a biotechnological use of this system for steroid modification and showed that the natural substrate progesterone as well as the synthetic progestogen dydrogesterone are regio- and stereospecifically reduced to 20α-dihydroprogesterone (20α-DHP) and 20α-dihydrodydrogesterone (20α-DHD), respectively. A whole-cell biotransformation process for 20α-DHP production using an AKR1C1 expressing fission yeast strain in a laboratory scale bioreactor was established, but substrate solubility and substrate transport into the cell seemed to limit the rate of steroid reduction. From a commercial point of view, the synthetic progesterone derivative dydrogesterone (brand name Duphaston®) is arguably the most interesting AKR1C1 substrate. It is a potent, orally active progestogen that is widely used in a range of gynecological conditions, which include menstrual disorders, infertility, threatened and habitual abortion, endometriosis, and hormone replacement therapy, respectively. Dydrogesterone is indicated in all cases where patients suffer from a relative or absolute endogenous progesterone deficiency. Although similar in molecule structure and pharmacological effects to endogenous progesterone, dydrogesterone has the advantage of being orally active at far lower dosages. Dydrogesterone also avoids many disadvantages (lacking potency, stability and bioavailability) or undesired adverse effects of the natural hormone or other synthetic progestogens. Moreover, its major human metabolite 20α-DHD has extensive pharmacological effects and hence could act as therapeutic agent itself [7]. Thus, the availability of a production process for this compound is a desirable objective. However, chemical reduction of the C20 keton with tetrabutylammonium borohydride (nBu_4NBH_4), which was described for progesterone by D'Incan [8], in case of dydrogesterone exclusively leads to 20β-DHD (Messinger et al., unpublished results). Two other approaches for synthesis of 20α-DHD via biotransformation have been described [9, 10], but the development of an efficient procedure for production and purification at gram scale was so far never aspired. Thus, it was the aim of this study to

improve our previously published system in order to establish a process that allows the production of 20α-DHD at technical scale. To this end, genetic improvement of the production strain, an increase of substrate solubility by addition of β-cyclodextrin, and the development of a sophisticated high-cell density fermentation at pilot scale were employed. In this way, we developed the first process that permits the biotechnological production of several grams of 20α-DHD per week and fermenter. Furthermore, by usage of the exemplary substrate progesterone, we show that this innovative fission yeast-based whole-cell biotransformation process is transferable to the conversion of other AKR1C1 substrates without special adaptation.

Materials and Methods

Substrates, Chemicals, and Reagents

Steroids were from Sigma-Aldrich (Deisenhofen, Germany) except for dydrogesterone which was from Abbott Products GmbH (Hannover, Germany). All other chemicals and bio-chemicals used were obtained from Roth (Karlsruhe, Germany) and were of analytical grade.

Construction of Fission Yeast Strains CAD300 and CAD302

AKR1C1 cDNA and Vectors

General DNA manipulation methods were done using standard techniques [11]. The AKR1C1 expression plasmid pREP1-AKR1C1 has already been described [6]. For the generation of strain CAD300 and CAD302, two further expression plasmids bearing the cDNA of human AKR1C1 were constructed. For construction of the new expression vector pMWIK2, the *Bam*HI site located in fission yeast *his3* gene was mutated by site-directed mutagenesis in the promoter-less screening vector pBG1 (a kind gift of K. Gould) [12] to yield the new plasmid pMW2. An expression construct consisting of the *nmt1* promotor in front of the AKR1C1 cDNA was cut out from pREP1-AKR1C1 using *Pst*I/*Bam*HI and cloned into the *Pst*I/*Bam*HI digested pMW2, yielding the new expression plasmid pMWIK2-AKR1C1. Next, the expression cassette of pREP1 [13] was excised using *Pst*I/*Sac*I and ligated to the *Pst*I/*Sac*I digested vector pREP42GFP-C [14] to yield pREP1ura. The AKR1C1 cDNA was then excised from pREP1-AKR1C1 with *Nde*I/*Bam*HI and ligated into pREP1ura to yield expression plasmid pREP1ura-AKR1C1. The correctness of all constructs was confirmed by automated sequencing.

Strains and Media

The preparation of media and basic manipulation methods of *S. pombe* were carried out as described [15]. The AKR1C1 expression strain JMN8 has been described [6]. Fission yeast strain ATCC96115 with the genotype *h⁻ ade6-M210 leu1-32 ura4-D18 his3-Δ1* [12] was transformed using pREP1-AKR1C1 and pREP1ura-AKR1C1 to yield CAD300. Strain CAD300 was subsequently transformed using pMWIK2-AKR1C1 yielding CAD302. Fission yeast transformations were done according to the lithium acetate method [16]. Transformed cells were plated on Edinburgh minimal media (EMM) with 5 μM thiamine and incubated at 30°C. Cells were grown in EMM with supplements as necessary. All

liquid cultures did not contain thiamine to keep the *nmt1* promoter active. Biomass production was scaled up by factor 10 to yield 100 mL or 1 L cell suspensions in batch cultures. Usually, the final cell density was around 5×10^7 cells mL^{-1} with cells being in the stationary growth phase. Cells were generally centrifuged at 5,000×*g* at room temperature for 5 min. The medium used for fermentation was DM1 [6] containing 0.4% (*w*/*v*) glucose and 0.01% (*w*/*v*) adenin. The feeding medium was composed of 230 g L^{-1} glucose·H_2O, 40 g L^{-1} $(NH_4)_2SO_4$, 10 g L^{-1} KH_2PO_4, 8 g L^{-1} $MgSO_4$, 2 g L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 40 mL L^{-1} of salt stock solution, 2 mL L^{-1} of vitamin stock solution, and 200 μL L^{-1} of mineral stock solution supplemented by 2 g L^{-1} adenin.

AKR1C1 Activity Assay for Strain Selection

For strain selection, 7 mL of EMM with supplements as necessary were inoculated with a clone of the fission yeast strains to be tested. Cultures were incubated at 30°C and 70 rpm for 48 h. Three 2 mL samples of each culture were centrifuged (3,000×*g*, 5 min, RT) and the supernatants discarded. One cell pellet was used for dry weight determination while the other two were used for the activity assays. Each of these two pellets was resuspended in 1 mL fresh EMM with supplements as necessary. Cell suspensions were incubated with 500 μM progesterone in sealed deep well microtiter plates at 750 rpm and 30°C for 24 h. After whole-cell biotransformation, the content of each well was completely extracted and product formation determined by HPLC analysis as described below.

Growth Assays

Biomass dry weight in cultures was used as measurement parameter for cell growth. For growth assays, 100 mL DM1 with supplements as necessary and glucose concentrations as indicated were inoculated with CAD302 biomass to a concentration of 1.5 g L^{-1}. Cultures were incubated for 4 h at 30°C at 150 rpm in Erlenmeyer shaking flasks and hourly sampled for the determination of biomass dry weight. The specific growth rate μ was calculated using software supported by Gnuplot.

Whole-Cell Biotransformation in Shaking Flasks

For biotransformation experiments, 10^8 cells L^{-1} were incubated in 50 mL of EMM in 250 mL Erlenmeyer flasks with 1 mM dydrogesterone and varying concentrations of β-cyclodextrin as indicated. Cultures were incubated for 24 h at 150 rpm and 30°C. After biotransformation, cultures were centrifuged (5 min, 5,000×*g*, 4°C), culture supernatants and cells (resuspended in 5 mL of medium) were separately extracted, and 20α-DHD concentrations determined by HPLC analysis as described below. All experiments were done twice in duplicates.

Whole-Cell Biotransformation in a Pilot Bioreactor

Fed-batch cultivation was done in a pilot bioreactor (LP 351, Bioengineering AG; Wald, Switzerland) at 30°C and a stirrer speed of 300 rpm. The pH was continuously measured by a pH electrode (Ingold single-rod measuring cell, Bioengineering) and kept constant at pH 5.3 via automatic dosing of 25% NaOH. The dissolved oxygen concentration was monitored continuously with an oxygen measuring system (IL/Ingold amperometric oxygen electrode and IL-pO_2 measurement amplifier, Bioengineering) and was kept continuously

above 20% by flushing the bioreactor with air at flow rates rising in the course of fermentation from 9 up to 35 L min^{-1}. The bioreactor was pre-filled with an initial volume of 22 L. After pH electrode calibration, the bioreactor was autoclaved *in situ* at 115°C for 10 min. Following a polarizing phase, the oxygen electrode was two-point calibrated using N_2(g) for the zero signal and atmospheric air pumped at a rate of 9 L min^{-1} for the 100% signal. Cells of strain CAD302 were raised for 72 h in eight 1-L cultures to a biomass density of approximately 4 g L^{-1}. After harvest and resuspension of the biomass in 200 mL of medium, the bioreactor was aseptically inoculated.

Feeding was carried out in an open-loop fashion for approximately 42 h according to the feed rate formula (equation 1) given by Jansen et al. [17] where the maximum growth rate constant was set to 0.06 h^{-1}, the biomass yield coefficient on glucose set to 0.33, and an assumed maintenance of 0.02 g glucose L^{-1} h^{-1}. The feeding pump rate was pulse-width controlled at a period of 5 min with a variable duty cycle set by the feeding rate formula. Subsequent culture feeding was done linearly with flow rates manually adjusted as required. After consumption of 20 L of feeding medium, it was replaced by a concentrated glucose solution (600 g L^{-1}). For optimum cell growth during the initial biomass growth phase, the glucose concentration was kept in the range of 0.1–0.5 g L^{-1}. After a biomass growth phase of about 24 h, 72.7 g β-cyclodextrin (64 mmol) dissolved in 400 mL dH_2O with 5 mL 25% NaOH and subsequently 20 g substrate (64 mmol) dissolved in 350 mL ethanol were added. Hence, the initial concentration of both substrate and β-cyclodextrin was around 2.9 mM. During the biotransformation process, glucose concentration was kept in the range of 0.5–5.5 g L^{-1}. Fermentation was continued for 136 h to yield a total runtime of 160 h. Samples were frequently taken and analyzed for glucose concentration, biomass dry weight, and product formation. The product concentration was determined by HPLC analysis as described below.

Downstream Processing

S. pombe cells were removed from the fermentation broth by centrifugation (4,000×*g* for 7 min at 4°C) and both the fermentation broth and the cell pellet suspension were subjected liquid–liquid extraction with ethyl acetate at a ratio of 3:1. The combined organic phases were evaporated using a rotary evaporator (Büchi; Flawil, Switzerland) and after the volume of the extract was reduced to approximately 500 mL, a first crude pre-purification was performed using silica-gel columns (Macherey-Nagel, 240 silica-gel Flash columns; Düren, Germany). In this step, major impurities from the fermentation broth that exhibit a high affinity to the silica-gel matrix and would disturb the final product purification were removed. The whole extract was continuously loaded onto the column with a flow of 30 mL min^{-1}. Afterwards, both product and substrate were eluted from the column using ethyl acetate and a flow rate of 70 mL min^{-1}. The elution was monitored by UV/Vis detection (BT 8200 UV/Vis HPLC-detector; Jasco Corporation, Tokyo, Japan). Fractions that showed an absorbance at 240 nm (progesterone and 20α-DHP) or 290 nm (dydrogesterone and 20α-DHD) were combined and concentrated to a final volume of approximately 350 mL.

The final part of the purification was another chromatography step using silica-gel columns from Macherey-Nagel (240 silica-gel Flash columns). The solvent used for this procedure was ethyl acetate/*n*-hexane at a ratio of 3:1 for dydrogesterone or at a ratio of 9:11 for progesterone, respectively. The flow was 70 mL min^{-1}. The concentrated ethyl acetate extract was applied in 10 mL fractions onto the column using a manual injector (Knauer; Berlin, Germany). The substrates dydrogesterone and progesterone eluted from

the column after approximately 7–8 and 9 min, respectively, whereas the products 20α-DHD and 20α-DHP were removed from the column after 9–13 min. A single purification run was typically completed after 16 min. Fractions containing the product were pooled and evaporated to dryness using a rotary evaporator (Büchi). The progress of the purification and the purity of the products were monitored by reversed phase HPLC throughout the production process.

Analytical Methods

High Performance Liquid Chromatography Analysis

For high-performance liquid chromatography (HPLC) sample preparation, 1 mL of a cell suspension was extracted with 500 μL of ethyl acetate, and the organic phase was evaporated to dryness. The dry residue was reconstituted in 1 mL acetonitrile and analyzed using a Waters HPLC system consisting of an Alliance 2690 separation module and the photodiode array detector PDA966. If not indicated otherwise, the stationary phase was a Macherey-Nagel EC 125/4 Nucleosil 100-5C18 column (4×125 mm, 5 μm) with a Macherey-Nagel CC 8/4 Nucleosil 100-5 C18 guard column (4×8 mm, 5 μm). The mobile phase consisted of a mixture of acetonitrile and water 50:50 (*v*/*v*). The injected sample volume was 20 μL, the column temperature was 40°C, and the flow rate was 1.0 mL min^{-1}, yielding a total run time of 11 min. Absorption was recorded at 240 nm (progesterone and 20α-DHP) or 290 nm (dydrogesterone and 20α-DHD).

NMR Analysis

For structural confirmation of the purified 20α-DHD by NMR, solutions were prepared in $CDCl_3$ (concentration approx. 10 mg mL^{-1}). ^{1}H and ^{13}C spectra of 20α-DHD were recorded on a Bruker DRX400 spectrometer at 400 and at 100 MHz, respectively. The chemical shift was referred to TMS for ^{1}H NMR and the $CDCL_3$ signal at 77.05 ppm for ^{13}C NMR. The chemical shifts are given in δ values (ppm). Basis of the determination of the stereochemistry of the hydroxyl group at C20 is the publication of Robinson and Hofer [18], where a 0.07–0.11 ppm downfield shift of the C21 methyl group was established when comparing α and β derivatives; also the C19 methyl group was identified a further characteristic signal.

Results and Discussion

Due to their essential biological functions, steroid hormones and their metabolites have great importance as pharmaceuticals, and consequently, there is a large interest in processes that permit regio- and stereo-specific modifications of the steroid backbone. For many of these reactions, chemical synthesis is tedious, and natural microorganisms that permit the respective biotransformations are not always known or applicable. Hence, the use of recombinant microorganisms is an economically reasonable alternative [19] that has so far mainly focused on cytochrome P450 (CYP) enzymes [20–24]. Although some enzymes of the AKR superfamily are also promising for specific steroid modifications, they have not yet been applied in industrial scale. In large-scale biotransformations, whole-cell systems are often superior to the use of (partly) purified enzymes because enzyme expression as well as cofactor regeneration are done by the microbes themselves and scale-up of

production is in principle only limited by the size of the available fermentation vessels. With respect to the choice of the host, recombinant fission yeasts of the species *S. pombe* have repeatedly been shown to be well suited for whole-cell biotransformation of steroids [6, 22, 25–29] and for biotechnological production of both steroidal and non-steroidal drug metabolites [5, 30–34]. In a recent study, we described the first biotechnological use of human AKR1C1 (20α-HSD) heterologously expressed in *S. pombe* and demonstrated the reduction of progesterone as well as the synthetic progestogen dydrogesterone at the keto group at C20 position. A successful fed-batch fermentation process for production of 20α-DHP at lab scale was reported, but no further development towards technical scale production had been done. Therefore, it was the aim of the present study to enhance productivity of the existing production process and to optimize it for efficient production of 20α-DHD.

Construction and Characterization of *S. pombe* Strains CAD300 and CAD302

The first step was the genetic improvement of the production strain. Fission yeast strain ATCC96115 was transformed using pREP1-AKR1C1 and pREP1ura-AKR1C1 to yield the new strain CAD300 (*h⁻ ade6.M210 his3.Δ1/pREP1-AKR1C1 pREP1ura-AKR1C1*) that contains two AKR1C1 expression units, and this strain was in turn transformed with pMWIK2-AKR1C1 to yield strain CAD302 (*h⁻ ade6.M210/pREP1-AKR1C1 pREP1ura-AKR1C1 pMWIK2-AKR1C1*) which harbors three AKR1C1 expression units. In order to compare the biotransformation activity of the newly created strains CAD300 and CAD302 to that of the older strain JMN8, all strains were grown in the absence of thiamine to induce the strong *nmt1* promoter and tested in whole-cell biotransformation assays with the natural AKR1C1 substrate progesterone in microtiter plate (MTP) format (Fig. 1). Under these conditions, the single AKR1C1 expressor strain JMN8 showed an average specific 20α-DHP production rate of 42±13 μmol g^{-1} day^{-1}. In comparison, the double expressor CAD300 showed a considerably higher rate (116±23 μmol g^{-1} day^{-1}), and the triple expressor CAD302 displayed almost a fourfold increase in activity (157±27 μmol g^{-1} day^{-1}), thus suggesting a gene dose effect. In all strains tested, the formation

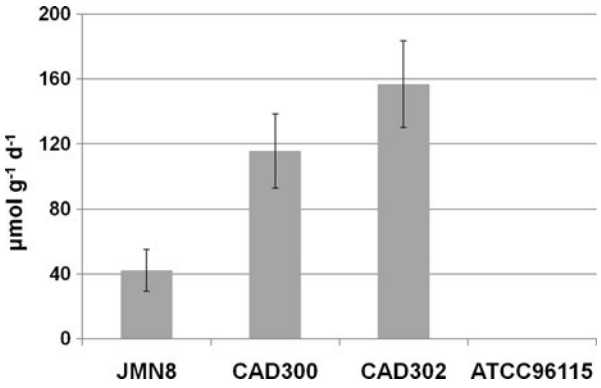

Fig. 1 Comparative analysis of biotransformation activity of *S. pombe* strains JMN8, CAD300, CAD302, and ATCC96115 in MTP format. Specific 20α-DHP formation activity was determined after 24 h incubation with 500 μM progesterone at 30°C and 750 rpm, resp. After whole-cell biotransformation, the product formation was determined by HPLC analysis. All assays were done in triplicates, and the resulting data were normalized to biomass dry weight

of byproducts or a reverse reaction towards the substrate was not observed, and no activity was detected in the control experiment with the parental strain. On the basis of these data, the new strain CAD302 was chosen for all further experiments.

Process Improvement

In order to achieve high biomass concentrations and, thus, high biotransformation rates, the influence of several parameters on the growth of CAD302 was examined. The investigation of the dependence of the specific growth rate on the dydrogesterone concentration in the culture medium revealed a distinct inhibitory effect of substrate concentrations above 0.5 mM on cell growth (data not shown). Next, we examined the influence of glucose concentration in the culture medium on growth rate. For this purpose, 1.5 g L^{-1} CAD302 cells were incubated for 4 h at 30°C in DM1 containing glucose concentrations in the range of 0–20 g L^{-1}. Cultures were hourly sampled, and the specific growth rate was determined (Fig. 2). As expected, almost no cell growth was detectable in the absence of glucose (μ= 0.05±0.04 h^{-1}). With glucose concentrations between 2 and 4 g L^{-1}, a maximal specific growth rate of 0.16±0.05 h^{-1} was observed. A further increase in glucose concentration did not lead to a significant raise in growth rate, indicating that a glucose concentration of about 4 g L^{-1} is sufficient for maximum cell growth of strain CAD302. Hence, a relatively low glucose concentration (max. 5.5 g L^{-1}) was chosen for the production process. We speculated that low substrate availability due to low substrate solubility could be a limiting factor in this biotransformation process, and therefore, it was attempted to increase the substrate solubility by addition of β-cyclodextrin. To investigate the feasibility of this approach, the influence of β-cyclodextrin on the solubility of dydrogesterone in the process media was determined. DM1 (2 mL) were spiked with 2 mM dydrogesterone and varying concentrations of β-cyclodextrin in the range of 0–6 mM, respectively. Samples were well mixed and subsequently centrifuged (15 min, 13,000×g, room temperature), and the supernatants were analyzed by HPLC (Fig. 3). In this experiment, the substrate solubility in DM1 was highest (1.7±0.1 mM) at a β-cyclodextrin concentration of 3 mM, while both higher and lower concentrations of β-cyclodextrin resulted in reduced dydrogesterone solubility. A reasonable substrate solubility can hence be reached with dydrogesterone:β-cyclodextrin ratios in the range of 1:1 to 1:2. We next examined the influence of different molar ratios of dydrogesterone to β-cyclodextrin on the biotransformation rate in shaking

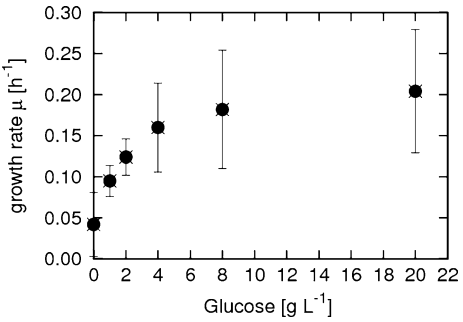

Fig. 2 Dependency of the specific growth rate μ of *S. pombe* strain CAD302 on glucose concentration in the culture medium. Cells (1.5 g L^{-1} biomass dry weight) were incubated for 4 h at 30°C in DM1 containing glucose concentrations in the range of 0–20 g L^{-1}. Cultures were hourly sampled, and the specific growth rate was determined. Growth assays were done in triplicates

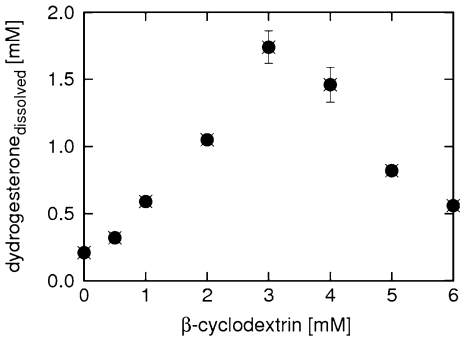

Fig. 3 Dependency of the solubility of dydrogesterone on β-cyclodextrin concentration in the culture medium. DM1 (2 mL) was spiked with 2 mM dydrogesterone and concentrations of β-cyclodextrin in the range of 0–6 mM, resp. Samples were well mixed and subsequently centrifuged (15 min, 13,000×*g*), and concentrations of dissolved dydrogesterone in the supernatants were analyzed by HPLC. The experiment was done in triplicate and obtained data were averaged

flasks. For this purpose, CAD302 was incubated for 24 h with 1 mM dydrogesterone and different β-cyclodextrin concentrations in the range of 0–10 mM. After biotransformation, product concentrations in the culture supernatants and in the cell pellets were determined by HPLC analysis (Fig. 4). These data show that the addition of β-cyclodextrin in single and double molar ratio to the substrate is indeed conducive to the biotransformation rate, with product concentrations in the culture supernatant as well as in the cell pellet of these mixtures being about 20% to 50% increased. In contrast, higher β-cyclodextrin concentrations had a negative effect on dydrogesterone transformation. Accordingly, β-cyclodextrin was added to the substrate in equimolar ratio for biotransformations in the bioreactor. The reason for the distinct inhibitory effect of higher β-cyclodextrin concentrations on dydrogesterone biotransformation remains obscure. It might be caused

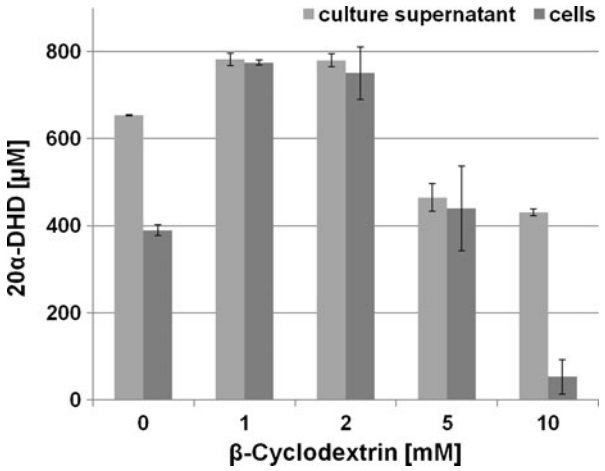

Fig. 4 Dependency of the biotransformation activity on β-cyclodextrin concentration in the culture medium. 10^8 cells mL^{-1} of strain CAD302 were incubated for 24 h at 30°C in 50 mL EMM containing 1 mM dydrogesterone and β-cyclodextrin concentrations in the range between 0 and 10 mM, respectively. After biotransformation, product concentrations in the culture supernatants and in the cell pellets were determined by HPLC analysis. Data shown are from two independent experiments done in duplicates

by aggregate formation of the cyclodextrins that lead to lower substrate availability; alternatively, higher concentrations of cyclodextrin could be detrimental to the fission yeast cells, as they are known to be for mammalian cells [35].

Production of 20α-DHD in Pilot Scale

Based on the existing data for 20α-DHP production with the single AKR1C1 expression strain JMN8, an efficient pilot scale production process for 20α-DHD with the improved strain CAD302 was developed. All details of the process successfully used for repeated 20α-DHD production in a pilot fermenter are described in the "Materials and Methods" section. Briefly, the biotransformation of dydrogesterone was performed as a fed-batch cultivation under strictly controlled conditions. Preliminary experiments indicated that a comparably high substrate amount of 20 g (2.9 mM) is advantageous to reach a balance between good conversion and sufficient product amounts (data not shown). At indicated time points, samples were taken and analyzed for glucose concentration and biomass dry weight. Also, cell suspensions were extracted and analyzed by HPLC to monitor product formation. Culture feeding was carried out for 42 h via an automated growth rate adjusted feed strategy and afterwards continuous with flow rates manually adjusted as and when required. After 89 h, the feeding medium was replaced by a concentrated glucose solution. Data from a representative fermentation are shown in Fig. 5. The biomass concentration increased during the whole fermentation up to 15 g L^{-1}. Product formation ran almost parallel to biomass growth: the first 24 h of the biotransformation were the phase of the highest conversion activity (about 700 μM day^{-1}). Overall, an average 20α-DHD production rate of approximately 190 μM day^{-1} was determined for the whole biotransformation period of 136 h and a culture volume of about 36 L. The system productivity was about 2.1 g day^{-1}, which corresponds to a total production of 12 g 20α-DHD per fermentation run. In comparison, the previously reported fed-batch experiment with strain JMN8 yielded a 20α-DHP productivity of 95 mg day^{-1} and a total product amount of 286 mg [6]. Thus, the daily productivity of the new optimized process with CAD302 is increased by a factor of 22, and due to the almost doubled biotransformation period, the total product amount produced per fermentation is even 42-fold higher. In

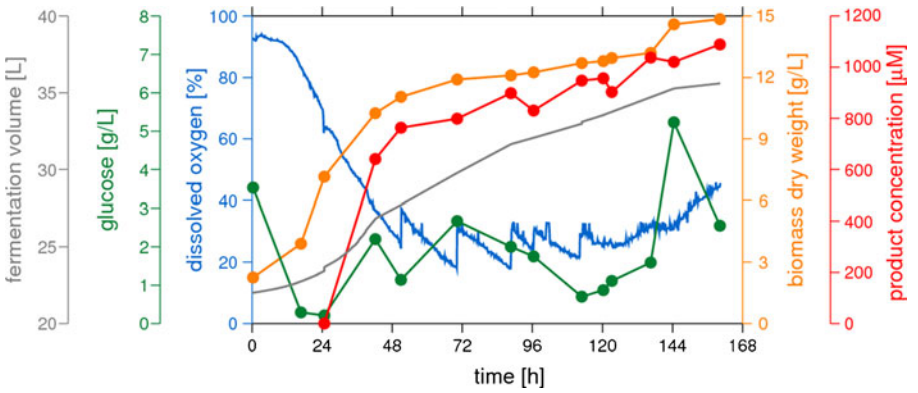

Fig. 5 Optimized 20α-DHD production by whole-cell biotransformation in a pilot bioreactor. Fed-batch cultivation of the production strain CAD302 in DM1 at 30°C. Dydrogesterone (20 g) as substrate was added after 24 h biomass growth; pH value and dissolved oxygen were measured continuously. Samples were taken periodically and analyzed for glucose concentration, biomass dry weight, and product formation

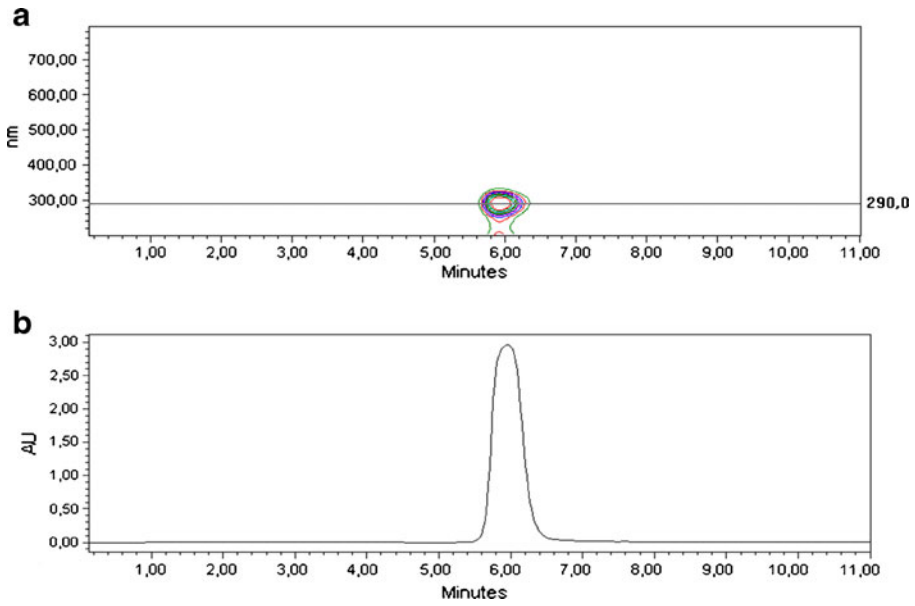

Fig. 6 HPLC quality analysis of purified 20α-DHD (1 mg mL^{-1}). **a** UV/Vis wavelength scan from 200 to 700 nm. **b** Extracted chromatogram at 290 nm. According to this diagram, the purity of the product is about 99%

addition to the scaling factors, the improvement mainly resulted from the considerably higher biotransformation activity of the production strain and the increase in substrate solubility. Repeated runs of this process documented a very high reproducibility concerning fermentation characteristics and product formation (data not shown). After biotransformation, the product was successfully extracted and purified from the fermentation cultures by a multistep process as outlined in the "Materials and Methods" section. The purification yield and purity of the produced 20α-DHD were monitored by reversed phase HPLC. By liquid–liquid extraction, approximately 90–95% of the product could be recovered from the fermentation broth. During two chromatographic purification steps, approximately 10% of the product was lost. Therefore, the overall purification yield from a single fermentation was always 80% or higher of the theoretical maximum yield of 20α-DHD. From three independent fermentation runs, an average of 12.3±1.4 g purified product (corresponding to 400 mg L^{-1} 20α-DHD and a substrate turnover of 63%) with a purity of 99% was obtained (Fig. 6). Subsequent NMR structure analysis unambiguously proved the identity

Table 1 ^{1}H NMR and ^{13}C NMR spectral data of 20α-DHD (= (9β, 10α, 20*S*)-20-hydroxy-pregna-4,6-dien-3-one) recorded in $CDCl_3$ on a Bruker DRX400 spectrometer

Atom	δ ppm ^{1}H	δ ppm ^{13}C
18	0.76 (s)	11.4
20	3.75 (m)	70.0
21	1.24 (*d*, 6.1 Hz)	23.6

The positions were numbered according to the IUPAC recommended atom numbering of steroids. Chemical shift referred to TMS for ^{1}H NMR (400 MHz) and the $CDCl_3$ signal at 77.05 ppm for ^{13}C NMR (100 MHz)

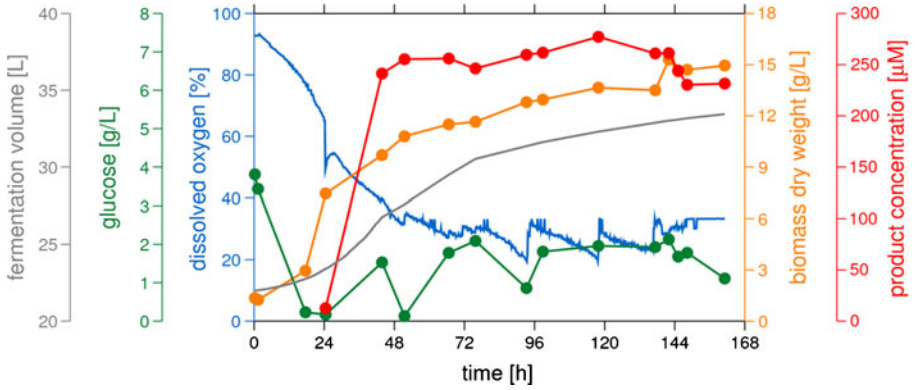

Fig. 7 20α-DHP production by whole-cell biotransformation in a pilot bioreactor. Fed-batch cultivation of the production strain CAD302 in DM1 at 30°C. All conditions were as for the 20α-DHD production. The substrate (20 g progesterone) was added after 24 h of biomass growth. pH value and dissolved oxygen were measured continuously. Samples were taken periodically and analyzed for glucose concentration, biomass dry weight, and product formation

of the produced 20α-DHD (Table 1). The results of ^{1}H NMR and ^{13}C NMR analyses of the isolated 20α-DHD were as follows: δ ppm 0.76 (*s*, H, H-18), 1.24 (*d*, *J*=6.1 Hz, 3H, H-21), 3.70–3.80 (*s*, 1H, H-20). The ^{1}H and ^{13}C chemical shifts observed for the 20α-DHD produced by biotransformation are identical to those published previously [18].

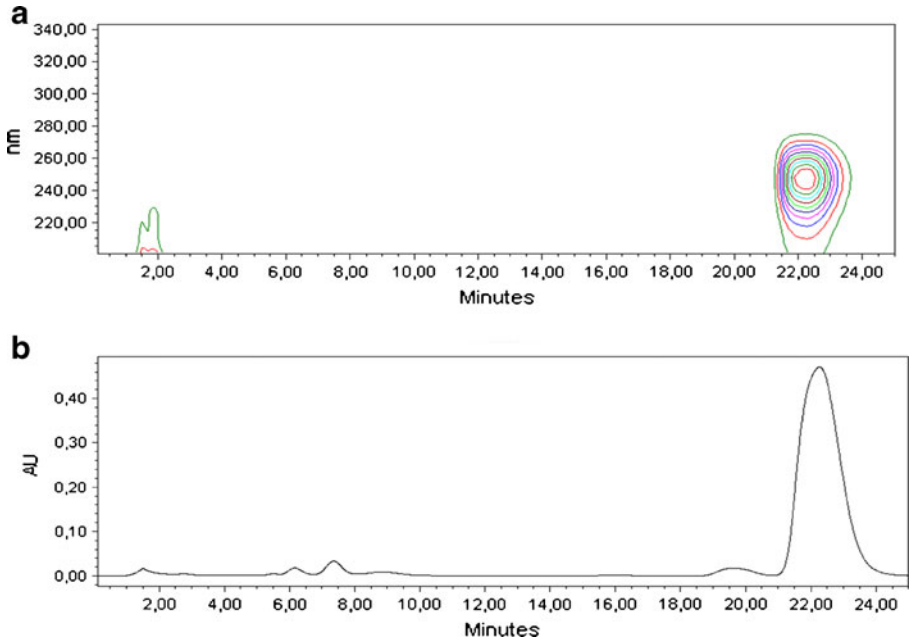

Fig. 8 HPLC quality analysis of purified 20α-DHP (1 mg mL^{-1}; mobile phase, 53% MeOH, 47% dH_2O (*v*/*v*); flow rate, 1 mL min^{-1}; solid phase, LiChroCART® 125-4, LiChrospher® 100 RP-8 (5 µm), Macherey-Nagel). **a** UV/Vis wavelength scan from 200 to 340 nm. **b** Extracted chromatogram at 240 nm. According to this diagram, the purity of the product is about 90%

Production of 20α-DHP in Pilot Scale

The main aim of this study was to establish an efficient process for the AKR1C1-dependent reduction of dydrogesterone in gram scale. However, in order to demonstrate the versatility of this new optimized production and purification procedure, we repeated the process without major modifications using progesterone as a substrate (Fig. 7). As above, culture feeding was carried out for 44 h via an automated growth rate adjusted feed strategy and afterwards, continuous feeding was employed with flow rates manually adjusted as required. After 93 h, the feeding medium was replaced by a concentrated glucose solution. With respect to biomass growth, culture feeding and glucose and oxygen requirements, the fermentation characteristics were most similar to the 20α-DHD production. However, due to the known lower activity of human AKR1C1 towards the substrate progesterone [6], the 20α-DHP productivity was considerably lower. Again, the highest conversion activity (about 230 μM day^{-1}) was observed during the first 24 h of the biotransformation. A final product concentration of approximately 235 μM (74 mg L^{-1} in a culture volume of about 34 L) was reached which corresponds to 12.4% substrate conversion. For the total biotransformation period of 137 h, the average 20α-DHP production rate was thus around 41 μM day^{-1}. According to these data, the theoretical product amount in the culture was about 2.5 g. The purification process developed for 20α-DHD was suited for the purification of 20α-DHP without major modifications as well. The purification yield and purity of the produced 20α-DHP were again monitored by reversed phase HPLC (Fig. 8) and the purification procedure was found to be nearly without product loss. Irrespective of the fact that progesterone is a poorer substrate for human AKR1C1 than dydrogesterone, these results demonstrate that the biotransformation process developed in this study is in principle also suited for the conversion of other AKR1C1 substrates. In summary, we report the first biotechnological pilot scale application based on fission yeast that recombinantly express human AKR1C1. The pilot scale process described in this study is an important intermediate stage in the transfer of the original lab scale biotransformation to possible industrial applications.

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