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Process development of methylenedioxyphenyl-acetone chiral bioreduction

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Abstract

Talampanel is a non-competitive antagonist of AMPA receptor, and it is a drug studied for the treatment of epilepsy and cerebrovascular ischemia. The first step of an efficient synthesis of talampanel is the reduction of 3,4-methylenedioxyphenyl-acetone (MDA) to (*S*)-α-methyl-1,3-benzodioxole-5-ethanol (MBE) accomplished with the use of *Zygosaccharomyces rouxii* in the presence of XAD-7 resin. *Z. rouxii* was chosen for its resistance to higher substrate and product concentrations (<6 g/l) and its higher reductase activity in comparison to other yeasts. Application of the moderately polar adsorbent resulted in low and non-toxic concentration of both the substrate and product in water phase.

A low cost fermentation medium without any component of animal origin was elaborated to produce *Z. rouxii* biomass. The control of pH and dissolved oxygen concentration, temperature, antifoam system, the time of harvest, the rate of inoculations and the number of inoculation steps were also studied. The fermentation process in 1000 l fermentor provided cell paste, possessing satisfactory ketoreductase activity (95–99%) with excellent enantioselectivity.

A method was developed to measure enzyme activity and to determine the key parameters of bioreduction. Excellent enantioselectivity was found under all conditions studied. The yield was very sensitive to the quality of cell paste. In a rather wide range temperature and aeration did not affect the results of bioreduction.

Keywords: Corn steep liquor supernatant (CLS); *Zygosaccharomyces rouxii*; Stereoselective bioreduction; In vivo co-enzyme regeneration; Multi-functional sampler

1. Introduction

A novel class of orally active 2,3-benzodiazepines was recently discovered and patented by Hungarian researchers [1]. Biological activity studies led to the discovery of (8*R*)-7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo[4,5-h]-[2,3]benzodiazepine (talampanel), an investigational new drug with antiepileptic, neuroprotectant and skeletal muscle relaxant effects, as it was described in a review by Andrási [2].

The first stage of talampanel synthesis is a microbial stereospecific reduction of 3,4-methylenedioxyphenyl-acetone (MDA) to (*S*)-α-methyl-1,3-benzodioxole-5-ethanol (MBE, Fig. 1). In addition to excellent enantioselectivity, another advantage is the in vivo regeneration of the oxidized $NAD(P)^+$ co-enzyme, which is essential for the action of carbonyl reductase enzyme [3,4].

The scale-up of bioreduction in a Rosenmund-agitated filter dryer was published by Vicenzi et al. [5]. Our paper deals with an economical method of cell paste production on pilot plant scale and with further laboratory scale experiments on bioreduction.

The key parameters, from the stock culture to the application of cell pastes, were optimized experimentally. It has been found that the reductase activity of *Zygosaccharomyces rouxii* was very sensitive to the following factors: nitrogen source of fermentation medium, cultivation conditions in the bioreactor, method of cell separation, storage conditions of cell pastes and the parameters of bioreduction.

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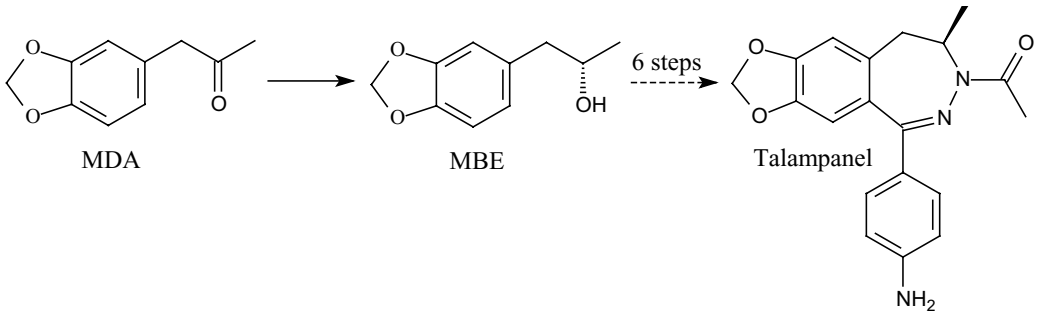

Fig. 1. Abbreviated synthetic scheme.

2. Experimental

2.1. Microorganism and growth conditions

Z. rouxii (ATCC 14462) is a generally recognized as safe (GRAS) type strain and was maintained on YM agar in a 1.2 l culture flask bottle. The cultures were cultivated for 3 days at 28 °C. In these experiments cultures not older than 1 month were used.

Seed cultures were grown in a bioreactor of 10 l working volume. The medium (4 × YM) consisted of 1.2% malt extract (Fluka), 1.2% yeast extract (Fluka), 2.0% vegetable peptone (Oxoid no. 1.) and 4.0% glucose. The following conditions were chosen: 28 °C temperature, 0.5 vvm bottom air flow, 450 rpm agitation and 0.2 bar overpressure. The seed culture was suitable for the inoculation of 1000 l fermentation medium after 22 h cultivation, when cell concentration reached 7×10^8 cells/ml and pH value was between 5.8 and 6.5.

In order to achieve optimal conditions, we conducted fermentations in a bioreactor of 10 l working volume. The following media seemed to be suitable for cultivating yeast cells with satisfactory reductase activity: the RR-1 medium consisted of 6.0% corn steep liquor with 50% dry matter content (Hungrana), 0.5% Gistex, 0.5% $(NH_4)_2SO_4$, 0.5% $CaCO_3$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% PPG-2000 (Neuber) and 6.0% glucose. The RR-2 medium contained 1.0% corn steep liquor powder (Roquette, Solulys HPP) instead of 6.0% corn steep liquor. In the RR-3 medium the corn steep liquor was exchanged for 18% corn steep liquor supernatant (CLS) with 15% dry matter content. The CLS was prepared as follows: raw corn steep liquor was warmed up to 60 °C, the pH was adjusted to 8.5, then the liquor was heated up to 95 °C and was maintained at this temperature for 10 min. Sedimentation was carried out at 6000 rpm in a Flottweg Zl type fast decanter. The supernatant was used as a component of the RR-3 medium.

In the 1000 l fermentor dissolved oxygen level was kept above 30% by means of bottom air inlet (0.2–1.0 vvm), agitation (200–300 rpm) and overpressure (0.2–0.4 bar). Optimal temperature was 26 °C. The pH value was not controlled. The reactor was inoculated with 10 l of 22 h old seed culture. The exponential phase continued up to the 16th hour of cultivation. The broth was harvested in the 24th hour of fermentation when the concentration of cells was between 2×10^9 and 3×10^9 cells/ml. The yeast biomass was separated from the fermentation broth in the Flottweg Z1 type fast decanter. From 1000 l broth, 65–70 kg biomass with 25–28% dry matter content could be obtained.

2.2. Resin-based bioreduction on laboratory scale and in a 250-l filter dryer

The conditions of laboratory scale bioreduction for testing the activity of cell pastes: 7.5 g resin (Amberlite, XAD-7), 1.0 ml $Na_2HPO_4 \cdot 2H_2O$ solution (10%) and 1.00 g MDA were added into a 100 ml Erlenmeyer flask. This mixture was agitated for 30 min in an orbital shaker. An aliquot from the 24 h old fermentation broth was centrifuged at 4000 rpm for 20 min, then 4.00–5.00 g cell paste was diluted with equal quantity of distilled water. After the addition of 7.5 g diluted cell paste and 2.0 g glucose, the mixture was shaken at 100 rpm. Samples were taken in the 2nd, 4th and in the 18th hour.

Reduction in the Rosenmund filter dryer was already reported [4,8], a mixture of 100 l XAD-7 resin, 8 kg MDA, 30 kg *Z. rouxii* paste and 14 kg glucose was raised to 200 l by the addition of tap water and maintained at 30 °C. The pH was kept above 7.0 with $Na_2HPO_4 \cdot 2H_2O$ solution. Bioreduction was completed in the filter dryer at low agitation speed (30–50 rpm) during a period of 16–42 h.

2.3. Sampling from resin-based bioreduction

A sampling device was developed (Fig. 2), suitable (i) for sampling from a reaction mixture with resin content, (ii) for separation of the resin and (iii) elution of the target compounds from the resin [6].

2.4. Determination of glucose concentration

Glucose concentration is of considerable importance upon the completion of fermentation. We also determined glucose concentration during the bioreductions by applying the widely used Somogyi–Nelson method [7].

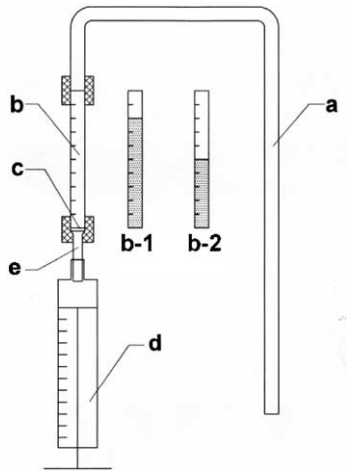

Fig. 2. Parts of sampler: (a) glass suction pipe, (b) graduated pipe, (c) stainless steel mash, (d) PP syringe, and (e) small PP pipe, connection between the mash and the syringe. Operation of this device: (i) sucking from a resin-added mixture, (ii) wet resin estimation by graduated pipe (b-1), (iii) elution the linked components by an organic solvent, (iv) air suction for drying up the resin to estimate the dry volume of it (b-2).

2.5. Thin layer chromatography (TLC) analysis of MDA and MBE

A quantitative TLC method was developed and recently published [6] in order to monitor the biotransformation during the bioreduction process. TLC was performed on 10 cm × 20 cm precoated plates (Merck Kieselgel 60 F_{254}, Art No. 5729). For mobile phase, a solvent mixture of hexane-ethyl acetate (7:3 v/v) was employed. Quantitation was performed by densitometry by scanning at 278 nm, using a computer controlled CAMAG TLC Scanner II.

Stereoselectivity was checked only in the first few experiments since *R* isomer was not produced in detectable amount. This result is in agreement with data published elsewhere [3].

3. Results and discussion

3.1. Optimisation of cell paste production

The cultures grown in a Roux-type culture flask were stored at 4 °C for a month without any loss of reductase activity. No degradation in enzyme activity was observed after 30 transfers of the original stock culture [8].

The 22 h old seed culture proved to be best for inoculation of the fermentation medium. Inoculation was also attempted with 16 and 36 h old seed cultures. The fermentation broth, inoculated with seed cultures cultivated for long time, reached the expected cell concentrations, the reductase activity of cell pastes was, however, not satisfactory (Table 1).

The effect of two-stage seed culture production was studied using the same seed medium under identical fermentation conditions. Inoculation rate of the second stage seed culture medium was 1%, and 22 h propagation time proved to be most effective. The cell paste obtained from a fermentation broth inoculated with a seed culture of the second stage showed sufficient reductase activity.

Table 1
The effect of different age seed cultures on reductase activity

	Propagation time of seed culture (h)		
	16	22	36
Cell concentration in the seed culture (cells/ml)	(3–4) × 10^8	(7–11) × 10^8	(10–15) × 10^8
Cell concentration in the 24th hour of fermentation (cells/ml)	(6–9) × 10^8	(18–25) × 10^8	(15–25) × 10^8
Conversion rate in the 18th hour of bioreduction (%)	60–76	95–99	84–89%

The seed culture medium published earlier contained peptone of animal origin [5]. It was found that the same amount of vegetable peptone no. 1 (purchased from Oxoid) resulted in a seed culture of same quality. Efforts were made to reduce the amount of expensive components, but no suitable seed culture could be obtained. Under the conditions described in the Section 2, dissolved oxygen level was kept over 40% in the 10 l bioreactor.

The cost of fermentation medium without component of animal origin could be reduced to 1/10th of the amount given in an earlier publication [5]. Initially, raw corn steep liquor was applied (RR-1), which led to contamination of the resin by corn steep liquor sediments. Later corn steep liquor powder was examined, which did not contaminate the XAD-7 resin, but the yield of reduction was not satisfactory. Finally, all problems have been solved by the use of CLS.

During the exponential phase ammonia and sodium hydroxide solution was used to maintain the pH value above 6.0. No significant difference was observed related to cell growth in a fermentation medium without pH control. Cell concentrations were similar in all cases, but lower enzyme activities were measured with yeast cells cultivated in a medium containing ammonia solution (Table 2). The pH value decreased to 4.2–4.8 in the exponential phase without control, which did not cause any loss in the reductase activity of yeast cells.

The control of dissolved oxygen level in a 1000 l bioreactor was optimized. Up to the 6th hour airflow was kept low (0.2 vvm) because of intensive foaming. In the course of exponential growth, aeration rate could be raised up to 1.0 vvm without intensive foaming.

Cell concentration did not increase significantly after the 20th hour of cultivation, but higher reductase activity was measured using 24 h old cells for the bioreduction. Similar enzyme activity was measured in the process of cultivation up to the 48th hour (Table 3).

Table 2
The effect of pH control on enzyme activity

	pH control (≥6.0)		
	With ammonia	With NaOH	Without control
Cell concentration in the 24th hour of fermentation	1.5–2.2 × 10^9 cells/ml	1.5–2.5 × 10^9 cells/ml	1.5–2.5 × 10^9 cells/ml
Conversion rate in the 18th hour of bioreduction	75–85%	95–99%	95–99%

Table 3
The effect of cultivation time on enzyme activity.

Propagation time	20 h	24 h	48 h
Cell concentration in fermentation broth (cells/ml)	1.3–2.5 × 10^9	1.8–2.5 × 10^9	1.8–3.0 × 10^9
Conversion rate in the 18th hour of bioreduction (%)	80–90	95–99	80–99

The cell concentrations are more similar to each other, contrary to reductase activities.

3.2. Optimisation of the bioreduction process

Complete reduction was achieved between 25 and 36 °C independently of aeration conditions. The accumulation of carbon dioxide could lead to a lower rate of bioreduction, owing to a decrease in pH values. In addition to the quality of cell paste, the amount of resin applied also had a definite effect on the yield attained. Different amounts of resin were added to the reaction mixture. Optimal resin amount was determined experimentally [5]. By addition of 20% more or less resin than the optimal amount, lower conversion rates were measured. The decrease in conversion was due to a change in substrate and/or product concentration in the supernatant.

The resin was also affected by the method of stirring, the shape of impellers as well as to the rate of agitation. A magnetic stirrer at 100 rpm led to disintegration of the resin, which in turn caused the conversion to cease at approximately 55% conversion. On the other hand, agitation at the same rpm by use of a rolling tube or orbit shaker led to complete conversion within 18 h. The living cell number in the reaction mixture never decreased during the bioreduction.

Since the proportion of the results obtained by the different pathways of glucose catabolism is not known in this case, the amount of reduced co-factors formed during the glucose metabolism cannot be exactly calculated. For this reason, the amount of glucose required had to be determined experimentally. Glucose in 2, 4, 6, 8, 10 and 12% was added to the reaction mixture. Independently of the concentration of glucose, yeast cells consumed all amount of glucose in each case. If the reaction mixture contained 2 or 4% of glucose, the reduction was incomplete. By the addition of 10–12% glucose, ethanol production might be responsible for the interruption. An amount of 6–8% glucose was sufficient to attain complete reduction (Fig. 3).

In the initial experiments, contamination by different bacterium species caused repeated interruption in bioreduction in consequence of multiplication of aerobic bacteria in the reaction mixture. It could be avoided by applying lower temperatures and by the use of continuous nitrogen inlet.

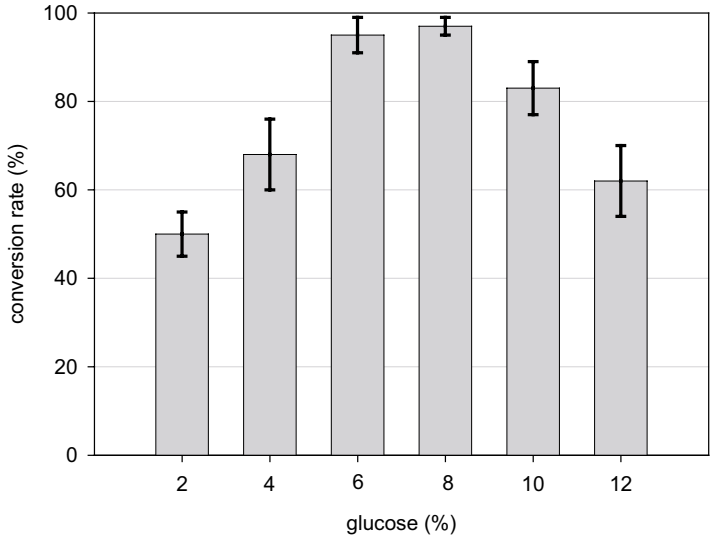

Fig. 3. The effect of different glucose concentrations on the rate of bioconversion.

4. Conclusions

An economical procedure was applied to produce yeast cell paste of excellent reductase activity in a 1000 l fermentor. Further scale-up of the fermentation process may not cause difficulties. Further steps of seed cultures did not lead to lower enzyme activity.

Investigation of this resin-based bioreduction gave further interesting results. Relative independence of the temperature and aeration facilitates further scale-up of the procedure. For closer information on the sensitivity of resin to the method of stirring, however further investigations are required. Resin can be floated by filtered air or nitrogen inlet on a larger scale however periodic agitation may offer an efficient method for preserving the adsorbing capacity of the resin.

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