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# STEREOSELECTIVE BIOREDUCTION OF ARYL- AND ARALKYL-METHYL KETONES BY YEASTS

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**PhD School/Program**

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**The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.**

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## Introduction

The enantiomerically enriched products and intermediates have become more and more significant in the pharmaceutical processes. Since enzymes are available in an increasing number and their application circumstances are mild, the chemical catalysators are more and more adumbrated.

Talampanel is a drug candidate targeted for the treatment of epilepsy. Till now not any side effects of talampanel was reported, and its third clinical trial is forthcoming in the USA. Talampanel was discovered in our institute then all the licences were bought by Eli Lilly (USA). Our present owner (IVAX, Miami) decided to buy back the talampanel licences and after successfull clinical trials market it worldwide.

Talampanel has several well known beneficent effects. Brain tumors and other brain desaeses as well as Parkinson desease might treat effectively by talampanel in the future.

Our task was the development of talampanel synthesis on industrial scale. The first step, the key reaction of the seven-step long synthesis, is the stereoselective bioreduction of (3,4-methylenedioxyphenyl)acetone to (2*S*)-1-(3,4-methylenedioxyohenyl)propane-2-ol.

## Aims of R&D

*Optimalized production of the biocatalysator*

1. Examination of maintenance and enzyme stability of *Zygosaccharomyces rouxii*
2. Development of the seed and the fermentation media; preferably low cost media without any component of animal origin and worldwide available ingredients
3. Optimization of the parameters of the seed cultivation and the cell mass production

*Process development of the bioreduction*

1. Influence of temperature, pH and aeration on the yield of bioreduction
2. Examination of the agitation at resin-containing reaction mixtures
3. Influence of quality and quantity of the cosubstrate on the yield and ee

*Bioreduction of aryl- and aralkyl-methyl ketones*

1. Comparison of the ketoreductase activity of *Z. rouxii* and *Debaryomyces hansenii* by reducing 11 prochiral ketones
2. Examination of different freezing methods to obtain lyophilised yeast cells with good ketoreductase activity
3. Comparison of the reductase activity of resting and lyophilised cells

## Experimentals

*Biocatalysators*

*Zygosaccharomyces rouxii* (ATCC 14462) was used as biocatalysator for scale-up processes of (3,4-methylenedioxyphenyl)acetone chiral bioreduction. For reducing further prochiral ketones *Debaryomyces hansenii* (NCAIM Y00468) was also applied.

*Production of the biocatalysator*

The yeast strains were maintained on YM agar in Blake cultivation bottles. The seed cultures and biomasses were grown in agitated fermentors of different volume (10 up to 1000 litres). The cell pastes were separated from the fermentation broth in a laboratory centrifuge or in a fast decanter (pilot scale).

*Bioreduction*

Ketones, cosubstrate was added to the buffered cell suspension (glucose to *Z. rouxii* and 2-propanol to *D. hansenii*). Resin (XAD-7) was added to the reaction mixture to increase the ketone concentration to 40 g/l.

*Analytical methods*

The yield of bioreduction was monitored by densitometric thin-layer chromatography and the enantiomeric excess was measured by a HPLC method.

## Results

*Seed culture*

The exponential growth phase lasted for 22 hours, the increasing value of pH showed the end of this period. Best result was achieved when 22 hour-old culture was used as pre-culture.

Efficient cell concentration and reductase activity was achieved when 0.1% pre-culture was used. To avoid the growth of undesireable microorganisms 1.0% pre-culture was applied on pilot scale. Cell paste of low activity was obtained when 10.0% pre-culture was added to the fermentation medium, because of abundant number of the aged cells.

*Production of the cell paste*

Three requirements were precomposed for the development of the new medium:

- efficient reductase activity
- no animal origin component
- cost-effective composition.

The corn steep liquor was tried to be replaced by dried corn steep liquor (Roquette, France, Solulys HPP) a worldwide available component. The carbonyl reductase activity of the cells decreased significantly when Solulys was used instead of corn steep liquor. A simple method was developed to precipitate the contaminations of the raw corn steep liquor at high temperature and pH. The clear supernatant was suitable nitrogen source for the production of *Z. rouxii.*

*pH control*

The pH decreased to 4.0-4.5 in the exponential phase and then it increased up to 8.0 after the complete exhaustion of glucose. The effect of pH control on enzyme activity and on cell concentration was examined in 10 litres fermentors. Ammonia and sodium-hydroxid was added to keep the pH value above 6.0. Not any difference was measured related to cell concentration, but lower enzyme activity was measured when ammonia was fed to the culture. Efficient biocatalysators were also produced without pH control.

*DO control*

A novel dissolved oxygen control system was developed as a part of the new process control system. Two operation orders are optional: (i) aeration – agitation – pressure or (ii) agitation – aeration – pressure. In the case of yeast cultivation the first version was more efficient: the aeration could be increased from 0.2 vvm to 0.7 vvm, then agitation was raised from 250 rpm to 350 rpm, and last the pressure was increased from 0.2 to 0.4 bar. The acceptable lowest value was 30% which was achieved by the change of the parameters.

*Multi-functional sampler*

New sampler was developed adaptive to the resin-containing reaction mixture. It it suitable for estimation the volume of the wet and the dry resin and for the elution the substrate and product from the resin.

*Effect of temperature on the bioreduction*

Not any effect was noted between 25 and 36°C on the yield. The lowest optimal temperature is recommended for the scale-up to avoid the growth of the undesired aerob bacteria in the reaction mixture.

*Effect of aeration on the bioreduction*

The yield of bioreduction was independent of aeration. The only difficulty was the accumulation of carbon dioxide, because the enzyme activity diminished at low pH. It only happened when the reaction mixture was aseptically closed. No decrease in the viable cell concentration or in the yield of reduction was observed when nitrogen was led into the reaction mixture.

*Agitation of the resin-containing mixture*

Magnetic stirring and shaking on an orbital shaker was compared. The best result was obtained using an orbital shaker at 90 rpm. The resin was sensitive to the size ans the shape of the blades and to the rpm. When the resin was pulverized, it lost the adsorption ability, so the concentration of the reagents increased above 6.0 g/l in the water phase and the reaction was interrupted. The resin was not injured in the Rosenmund filter-dryer at 30 rpm after several use.

*Amount of the cosubstrate*

The most important advantage of the whole-cell system is the *in vivo* cofactor regeneration. The reduction of the cofactor (NADP) requires cosubstrate. NADPH is produced by degradation of cosubstrate. Several carbonhydrates and alcohols were examined as cosubstrate, but completed bioconversion was only obtained when glucose or sucrose were used. Between 2 and 12% amount of glucose was added to the reaction mixture. The optimal amount was 8%, less glucose was completely exhausted before completion of the reduction. More than 8% might lead for the accumulation of ethanol in the reaction mixture that also interrupted the reaction.

*Bioreduction of different methyl ketones with Z. rouxii cells*

Phenylacetone derivatives were reduced (i) with the aim of synthesising of novel benzodiazepines. (ii) Acetophenone and benzylacetone were reduced to examine the effect of the length of the side chain on enantiomer selectivity. The ketones investigated:

| | |
|---|---|
| **1a** | (3,4-methylenedioxyphenyl)acetone |
| **1b** | phenylacetone |
| **1c** | (2-methoxyphenyl)acetone |
| **1d** | (3-methoxyphenyl)acetone |
| **1e** | (4-methoxyphenyl)acetone |
| **1f** | (3,4-dimethoxyphenyl)acetone |
| **1g** | (2,4-dimethoxyphenyl)acetone |
| **1h** | (4-chlorophenyl)acetone |
| **2a** | benzylacetone |
| **2b** | anisylacetone |
| **3** | acetophenone |

Another biocatalysator is needed where less than 60% yield was obtained even at low substrate concentration (10 mmole, **1c, 1f, 1g, 2a, 2b, 3**). Complete reduction was obtained with **1b.** All the ketones substituted in the 4th carbon (**1e, 1h**) were reduced with efficient yield. The 2-substituted phenylacetones (**1c, 1g**) was reduced with low yield. The regio affected more on the yield than the quality of the substitution group.

Longer carbon chain containing ketone (**2a**) were reduced with poor ee, than the ketone containing shorter chain (**3**). The yields were the opposite: higher yield was performed on **2a**.

In comparison of **2a** *vs* **2b** and **1b** *vs* **1e** the similar 4-substitution effect could be observed: higher ee was obtained in the cases of 4-substituated alcohols in both substrate pair.

*Cosubstrate of Debaryomyes hansenii*

Primer alcohols and carbohydrates were not suitable cosubstrate of *D. hansenii.* High yield was resulted in reduction of **1a, 2a** added 2-propanole to the raction mixture. The quality of cosubtrate affected the yield of the whole-cell bioreduction but not the ee of the products.

*Bioreduction of different methyl ketones with D. hansenii cells*

The same ketones were reduced with *D. hansenii* than with *Z. rouxii*. Wider substrate tolerance was observed in the case of *D. hansenii.* All the ketones were transformed to alcohol between 57 and 99% yield, even the 2-substituted phenylacetones (**1c, 1g**) were reduced efficient. Lower yield was obtained in the case of **1e,** higher yield at **1f,** than *Z. rouxii* did. Weak ee was obtained when **2a** was transformed, but the 4-methoxy group (**2b**) also affected increased ee. The same ee increase was observed in the case of **1b**, and its 4-substituted

molecule (**1e**). The halogenized aromatic acetone (**1h**) was reduced with high yield and ee similar to *Z. rouxii.* The biggest difference between the carbonyl reductases of the yeasts was observed, when **3** was reduced. *D. hansenii* produced the alcohol with the same high ee and the yield was also good.

*Lyophilized biocatalysators*

*Effect of different freezing methods on the reductase activity*

Both the viable number and the reductase activity were monitored after lyophilization. Both the slow temperature decrease to -80°C and dip in fluid nitrogen caused significant viable cell number decrease and the relative enzyme activity also regressed. Freezing in carbon-dioxide ice resulted in cells possessing similar enzyme activity to the fresh cells. The weight of the lyophilised cell powder is 25-30% of the wet cell paste. When the wet cell paste was cleaned before freezing white powder was obtained.

*Comparison of the reductase activities of the fresh and the lyophilized cells*

*Z. rouxii*

Similar yields and ee values were obtained with lyophilized and fresh cells. In the case of **2b** higher ee was measured, and from **1g** optically clear alcohol was produced by lyophilized cells (with fresh cells ee was only 80%).

*D. hansenii*

Repeating the experiments with *D. hansenii* lyophilised cell similar yields were obtained as it was obtained by fresh cells (with the exception of **1a, 3**). Significant advance was observed considering the yield of bioreduction at **1b** and **2a**, in the case of **1e** and **2b** considerable higher ee was measured.

The lyophilisation improved the reductase character of *D. hansenii,* and similar activity was measured with fresh and lyophilised *Z. rouxii*.

**Conclusion and proposal**

The maintenance and the production of the biocatalysator required a prepared microbiological staff. We proved that the lyophilised cells are suitable inoculum for the production of biocatalysator. The developed process for the production of the biocatalysator and for the bioreduction is suitable for scale-up on industrial scale. Compared the reductase activity of *Z. rouxii* and *D. hansenii* we could state, that *D. hansenii* may an efficient biocatalysator in

reducing several aralkyl-methyl ketones. It possesses reductase activity of wider tolerance, this yeast produced all the alcohols with acceptable yield.

Efficient results were obtained when lyophilised yeasts were applied as biocatalyst after a short regeneration period. Nor the conversion rates not the ee values did not change compared to the enzyme activities of resting cells. The lyophilisation might be an efficient method to elongate the enzyme activity of yeasts, but significant improvement in the activity was not observed.

**New scientific results**

1. New media were developed for the industrial production of biocatalysator. The fermentation processes were optimized. The developed technology is suitable for the further scale-up.
2. Cosubstrates were found for optimal work of reductase activity of the yeasts. Glucose and saccharose are the best cosubstrates of *Z. rouxii*, since *D. hansenii* reduced the ketones efficient when 2-propanol was added to the reaction mixture.
3. New multi-functional sampler was invented suitable for sampling from resin-containing reaction mixtures.
4. The reductase activity of *Z. rouxii* was characterized throughout the bioreduction of 11 aryl- and aralkyl-methyl prochiral ketones.
5. The reductase activity of *D. hansenii* was characterized with bioreduction of the same ketones. Wider substrate tolerance was observed.
6. The lyophilization is an appropriate method to make suitable the yeast biocatalysators for the long time storage and transportation.
7. The shorter carbon chain containing acetophenone was reduced with higher ee, than the longer carbon chain containing benzylacetone by *Z. rouxii* and *D. hansenii*.

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