Full Length Research Paper

Microorganisms' mediated reduction of β-ketoesters

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Several microorganism strains of genera *Saccharomyces*, *Candida*, *Hansenula*, *Aspergillus* and *Lactobacillus* were screened for their ability to perform the reduction of γ-chloro-β-ketobutyric acid ethyl ester to γ-chloro-β-hydroxybutyric acid ethyl ester. The optimal conditions for both stages of the bioprocess were established. Both stereoisomers of γ-chloro-β-hydroxybutyric acid ethyl ester were obtained in different biotransformation conditions. While in aqueous medium the S isomer was obtained, in organic media the product had predominantly the R configuration.

Key words: microbial reduction, β-keto-esters, *Hansenula polymorpha*, ethyl alcohol.

INTRODUCTION

The enantiomeric pure products occupy an increasingly important position in the development of the modern biochemical technologies, especially in pharmaceutical industry. Worldwide sales of chiral drugs in single-enantiomer dosage forms continued growing at more than 13% annual rate to $133 billion in 2000, according to the consulting firm Technology Catalysts International. At a future growth rate estimated by TCI, the figure could hit $200 billion in 2008. In a second growth trend, 40% of all dosage-form drugs sales in 2000 were of single enantiomers. In 1999, the share was one third (Stinson, 2001).

The use of whole cell biocatalysts is increasingly applied for the synthesis of small molecules, especially for the preparation of optically pure compounds (D'Arrigo et al., 1997). Purified hydrolytic enzymes are among the most popular biocatalysts for this purpose along with the reducing enzymes found in microorganisms. However, purified oxidoreductases require expensive cofactors for performing the catalytic step (Kula and Peters, 1996; Ishigara et al., 1994).

Therefore, cells are preferentially used, thereby circumventing the difficulty of effective recycling of the cofactor (Baraldi et al., 1992).

Whole cells usually express a multitude of enzymatic activities; therefore an important limitation of this me-thodology is selectivity (Bommarius and Riebel, 2004; Bertau, 2002). In order to achieve a more selective system in microorganisms mediated reductions, it is of great importance to be able to control the conditions of reaction so that the desired enzyme activity is favored and/or the undesired ones are suppressed (Chin-Joe et al., 2002; Medson et al., 1997; Jayasinghe et al., 1993; Shieh and Sih, 1993; Nakamura et al., 1991).

Optically active 4-halo-3-hydroxybutiric acid esters are important intermediates for the synthesis of various medicinal compounds, optically and biologically active substances and their derivatives (Fuganti and Grasselli, 1985).

We like to report our results in the production of the key chiral compound for L-carnitine synthesis by the reduction of γ-chloro-β-ketobutyric acid ethyl esters using whole microbial cells.
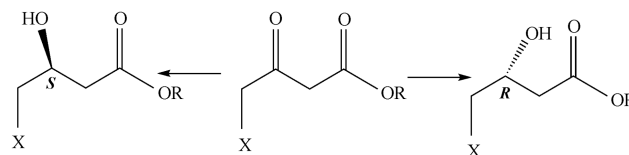
MATERIALS AND METHODS

Strains

All the screened strains are part of the Collection of Industrial Microorganisms from the National Institute for Chemical-Pharmaceutical R & D Bucharest registered WFCC 232.

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Materials

Ethanol, methanol, glucose, kalium dihydrogen phosphate, magnesium sulphate and diammonium sulphate were all purchased from CHIMO-PAR Bucharest. Yeast extract, peptone, glucose and malt extract were obtained from SIGMA. γ-Chloro-β-ketobutiric acid ethyl ester (CAAE) and γ-chloro-β-hydroxybutiric acid ethyl ester (HE) were prepared by chemical synthesis, in our laboratory.

Media

The yeast strains were maintained on YPG solid medium at 4^oC and transferred every three weeks. The yeast strains growing on methanol or ethanol as carbon and energy source were maintained on modified YPG solid medium, containing 2% (w/v) alcohol.

The fungal strains were kept on malt agar medium at 4^oC and subcultured every month. The lactic acid bacterium strains were maintained on MRS medium at 4^oC and transferred every 2 weeks.

Each microorganism was cultivated for the biomass development on specific medium; the optimum composition being previously determined (Vamanu et al., 2001; Popa, 2000; Ionita et al., 1997).

Analytical methods

Evaluation of cell mass development was determined by means of dry cell weight. When the carbon source was glucose, its concentration was determined spectrophotometrically by o-toluidine reaction, on a Perkin-Elmer λ12 Spectrophotometer, at λ = 630 nm.

Methanol's and ethanol's concentrations were determined by gas-chromatography, on a Carlo-Erba 4200 gas-chromatograph with FID and a glass column (L x ϕ = 1.5 m x 2.4 mm), filled with PORAPAC Q (80 – 100 mesh).

During the biotransformation stage, the consumption of substrate and the product's formation were qualitatively appreciated by TLC on Kieselgel F_{254} (Merck) using dichloroethan as eluant and a solution 1:1 (v/v) $KMnO_4$ 0.1 M: NaOH 10% for spot visualization. The R_f values determined in these conditions were:

• 0.55 for CAAE
• 0.30 for HE.

The quantitative determination of CAAE and HE was made by gas-chromatography, using a Carlo-Erba 4200 gas-chromatograph with FID, equipped with a glass column (L x ϕ = 2 m x 4 mm), filled with Chromosorb W-A WD MCS (80 – 100 mesh), 10% (w/w) impregnated with silicon SE 52, which allowed a good separation of the two components.

Measured volumes of fermentation broth were centrifuged at 6000 rpm for 20 – 30 min, and the supernatant was extracted three times with an equal volume of ethylene chloride. The joined organic extracts were dried over anhydrous sodium sulphate and then the solvent was removed by evaporation under reduced pressure. The samples were analyzed by GC for CAAE and HE determination.

The optical rotation of partially purified final product samples was recorded using a Jasco-360 polarimeter in chloroform.

RESULTS AND DISCUSSION

Chemical synthesis of the substrate and the product

The substrate γ-chloro-β-ketobutiric acid ethyl ester (CAAE) and the product, γ-chloro-β-hydroxybutiric acid ethyl ester (HE), were prepared by chemical synthesis, in

our laboratory, via chloroacetic ethyl ester (CAE).

CAE was obtained by direct esterification of monochloracetic acid (MCA) with 96% ethanol in the presence of 96% sulphuric acid in a molar ratio MCA:EtOH:H_2SO_4 = 1:2:0.11.

CAE was recovered by vacuum distillation (50 - 51^oC, 20 mm Hg) with 89% yield against MCA. The purity of compound was established by GC as 98%.

CAAE synthesis was performed by condensation of CAE with MCA in the presence of Mg, by addition of iodine and mercury chloride in anhydrous ether in the molar ratio CAE:Mg:I_2:$HgCl_2$ = 1:0.5:0.001:0.001. After the hydrolysis of the organomagnesian compound, CAAE was recovered by vacuum fractionation (79 - 80^oC, 2 mm Hg).

In order to obtain a standard for GC and TLC, CAAE was reduced with $NaBH_4$ (CAAE: $NaBH_4$ = 1:4) with 80% yield against CAAE. The purity of HE was 98%.

The biotransformation process

The whole bioprocess is formed of two stages:

• Obtaining of the active biomass, further used as biocatalyst;
• Bioreduction of the substrate, CAAE, into optically active product HE with the help of the active cells.

The aim of the first stage of the bioprocess was to obtain a highly active biomass suitable to perform the bioreduction in the second stage. Microorganisms were cultivated in 500 ml Erlenmayer flasks, closed with cotton stoppers, containing 100 ml medium. Flasks of the first generation were inoculated from the agar slants (except for the *Lactobacillus* strains, which were kept on MRS liqiud medium) and cultivated for 24 h on an orbital shaker (240 rpm). The cultures were transferred (10%, v/v) to flasks of the second generation, where the cultivation was continued for the next 24 to 48 h.

Several microorganism's strains have been cultivated in order to obtain the active biomass; the optimal conditions of the growth phase are presented in Table 1.

The biomass was separated by centrifugation or filtration, washed twice with distilled water and separated again.

All the strains were screened for their ability to perform the biotransformation of γ-chloro-β-ketobutiric acid ethyl ester into the γ-chloro-β-hidroxi-butiric acid ethyl ester with **R** configuration, the intermediate for the synthesis of L-carnitine.

The standard conditions for the bioreduction stage were as follows:

• Wet cell weight = 200 g/l;
• Glucose concentration = 150 g/l;
• Time of biotransformation = 48 h;
• Temperature = the optimal for the strain development;

Table 1. The parameters for the optimal biosynthesis of cellular biomass.

Microorganism	Substrate concentration (g/l])	Time of cultivation (h)	pH	Temp (^{o}C)	DCW (g/l)
S. cerevisiae 3.37	Glucose 70	24	3.8 - 4.2	28	5.7
S. cerevisiae 3.20	Ethanol 2 - 3	24	3.8 - 4.2	28	8.8
C. boidinii 3.33	Methanol 1 - 2	48	4.0 - 4.5	28	6.1
C. tropicalis 3.5	Glucose 70	40	4.0 - 4.5	31	6.3
C. utilis 3.7	Glucose 70	30	4.0 - 4.5	28	7.05
C. lypolytica 3.16	Glucose 70	48	4.5 - 5.0	28	8.1
H. anomala 23	Glucose 70	40	4.5 - 5.0	31	7.8
H. polymorpha 28	Methanol 1 - 2	48	4.0 - 4.5	31	7.1
H. polymorpha 28	Ethanol 2 - 3	24	4.0 - 4.5	34	8.9
A. niger 19	malt extract 40	72	6.0 - 6.5	31	23.9
Lactobacillus sp. LB1	MRS	24	6.0 - 6.5	28	4.3

*The pH was kept at the mentioned values with aq. NH_3 12.5 (%) every 12 h. Glucose was added in the mentioned concentration at the beginning of the fermentation; the concentration of methanol/ethanol was kept at indicated values by periodical supplements so as to prevent the inhibitory effects of the alcohol on the microorganism.

Table 2. Screening the strains for their ability to perform the bioreduction of the β-keto group of the ester.

No.	Microorganism	Bioreduction conditions	Residual CAAE concentration (g/l)	HE concentration (g/l)
1	*S.cerevisiae* 3.37	Aerobic wet biomass	0.84	1.1
2	*S. cerevisiae* 3.20	Aerobic wet biomass	0.8	1.35
3	*S.cerevisiae* 3.20	Anaerobic wet biomass	1.5	0.49
4	*S. cerevisiae* 3.20	Aerobic lyophilized biomass	0.9	1.15
5	*C.boidinii* 3.33	Aerobic wet biomass	1.05	0.85
6	*C. tropicalis* 3.5	Aerobic wet biomass	0.75	1.24
7	*C. utilis* 3.7	Aerobic wet biomass	1.74	1.01
8	*C. lypolytica* 3.16	Aerobic wet biomass	1.65	0.70
9	*H. anomala* 23	Aerobic wet biomass	0.75	1.20
10	*H. polymorpha* 28	Aerobic wet biomass	0.7	1.42
11	*H. polymorpha* 28	Anaerobic wet biomass	2.1	0.65
12	*Aspergillus niger* 19	Aerobic wet biomass	1.75	0
13	*Lactobacillus* sp. LB1	Aerobic wet biomass	2.06	0.47
14	*Lactobacillus* sp. LB1	Anaerobic wet biomass	1.75	0.81

• pH = the optimal for the strain development;
• CAAE concentration = 4 g/l; the substrate, dissolved in ethanol (1:2, v/v) was added after one hour of adaptation of the microorganism and at 24 h of cultivation, in equal aliquots.

The biotransformation was performed in Erlenmayer flasks (V_u/V_g = 50/200, v/v), closed with cotton stoppers, on a New Brunswick shaker, at 250 rpm.

The results of the screened strains were presented in Table 2. Some of the tested microorganisms did not produce any HE, most of them showing low or medium ability for the required bioreduction.

Previous research showed that the bioreduction could be conducted in the presence (Matsuyama et al., 1996; Norimasa et al., 1995) or in the absence of the air

(Masayasu et al., 1990); therefore we also tested this ability for some of the screened strains (experiments number 2 - 3, 10 - 11, 13 - 14 in Table 2). For two of the tested strains, *S. cerevisiae* 3.20 and *H. polymorpha* 28, the ratio HE $_{produced}$ / CAAE$_{residual}$ was favorable for aerobic system and only for the strain *Lactobacillus* sp. LB1, the anaerobic conditions for the bioreduction lead to better results, comparatively.

The use of fresh wet cell biomass or lyophilized cells did not significantly influence the ratio of biotransformation (experiments number 2 and 4 in Table 2). The analysis of the results proved the best performances were obtained with two yeast strains *S. cerevisiae* 3.20 and *H. polymorpha* 28, both capable of using other substrates than glucose, for growth. Consequently, we tested other energy sources different from glucose, like ethanol

Table 3. The influence of carbon and energy source on the $HE_{produced}$ / $CAAE_{residual}$ ratio.

Strain	Substrate	$HE_{produced}$/ $CAAE_{residual}$
S. cerevisiae 3.20	Glucose (150 g/l)	1.68
	Ethanol* (70 g/l)	2.27
H. polymorpha 28	Glucose (150 g/l)	2.02
	Ethanol* (70 g/l)	2.74
	Methanol* (70 g/l)	1.95

*The alcohol was added in fed-batch system, so that its concentration in the medium is less than 2 - 3 g/l.
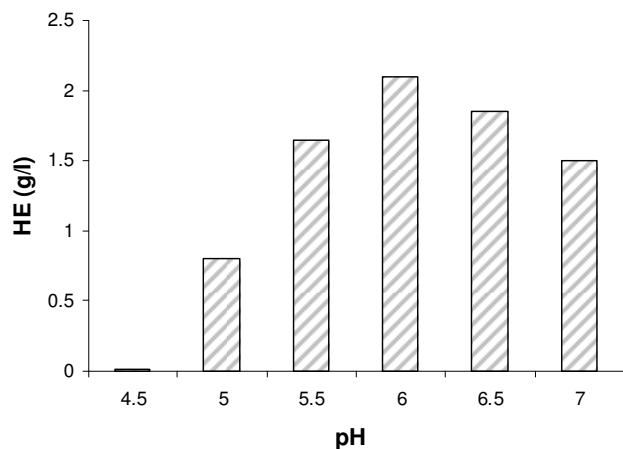
Figure 1. The influence of pH value on the concentration of HE obtained.
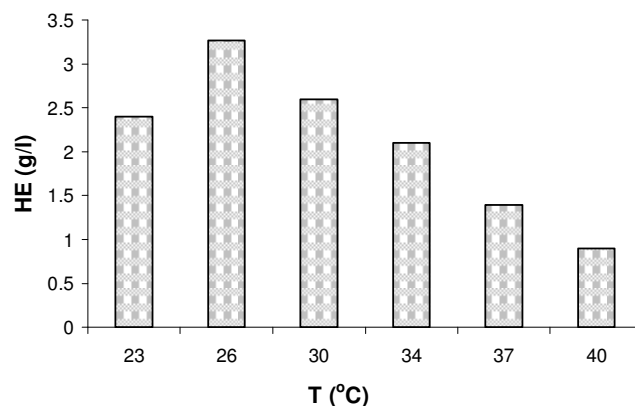

Figure 2. The influence of the temperature on the concentration of the HE obtained.

and/or methanol for the bioreduction phase (Table 3).There are only a few references in the literature mentioning the use of other carbon and energy source than glucose for yeast mediated biotransformation of carbonyl compounds (Matsuno et al., 1993; Baraldi et al., 1992).

The use of ethanol as energy source lead to a greater ratio of biotransformation better than in the case of glucose as substrate, for the same strain. One explanation could be the possible inactivation of yeast cells by the initial high concentration of glucose. Therefore it may be necessary to test the fed-batch system for glucose addition in future experiments. Another possible explanation could be the better solubility of the substrate to be reduced, CAAE, in ethanol.

The yeast strain selected for performing the next experiments was *Hansenula polymorha* 28, using ethanol as carbon and/or energy source in both stages of the bioprocess.

Our previous experience has shown that the optimal temperature and pH values obtained for the yeast development phase were not the same with those for the biotransformation phase. Therefore, we also determined the optimal values for the bioreduction stage. Data presented were the average of three experiments.

The experiments for determining the influence of pH on the $HE_{produced}$ were performed in phosphate-citrate buffer

medium for pH values between 4.5 and 7.0. From the data presented in Figure 1, it was obvious that the optimum pH value was 6.0, for which the greatest concentration of HE was obtained.

The further experiments were performed in the same conditions as above, except the pH was kept at 6.0. The optimal temperature for the bioreduction phase was found to be much lower than the optimum for growth, that is, 26°C (Figure 2).

The optimal bioreduction time was determined to be 48 h (data not shown), the prolongation of the biotransformation over this value did not lead to a greater $HE_{produced}$/ $CAAE_{residual}$ ratio.

The substrate to be reduced by the yeast cells, 4-chloro-3-keto-butyric acid ethyl ester, is quite toxic to the microorganism: the cells' viability drastically decreased from 100%, just after the addition of CAAE to about 18% after 20 h of biotransformation. The best way to prevent the lethal effects of the substrate on the yeast cells was to keep its concentration between 0.5 and 1.5 g/l.

The parameters of the bioreduction phase, as established in our experiments, are as follows:

- WCW = 200 g/l;
- Medium = phosphate-citrate buffer pH: 6.0;
- Temperature = 26°C;
- Energy source/ total concentration = ethanol at 70 g/l,

Table 4. The results of the bioreduction stage (in aqueous medium).

Parameter	Value
Concentration of $CAAE_{residual}$ (g/l)	0.7
Concentration of HE_{final} (g/l)	3.3
$[\alpha_D]^{20}$ (3% $CHCl_3$)	-4.25
Configuration	**S**

Table 5. The influence of the bioreduction conditions and of some specific treatments on the stereoselectivity of the bioprocess.

Variant	Specific conditions	HE_{final} (g/l)	$[\alpha_D]^{20}$ (3% $CHCl_3$)	Configuration
1	T = 26°C, pH 6.0 medium: phosphate-citrate buffer	3.3	- 4.25	"S"
2	T = 26°C, medium: n-hexane : buffer pH 6.0 = 2.5:1 (v/v)	2.85	+ 7.50	"R"
3	T = 26°C, medium: n-octanol : buffer pH 6.0 = 2.5:1 (v/v)	3.1	+8.95	"R"
4	1 h incubation of cells at 50°C before the bioreduction stage	2.7	+4.65	"R"
5	1 h incubation of cells (26°C) with chloroacetic acid ethyl ester, before the bioreduction stage	2.6	+3.9	"R"
6	1 h incubation of cells (26°C) with allylic alcohol before the bioreduction stage	2.2	≈ 0	-

added in fed-batch system such that the ethanol's concentration in the medium to be kept between 2 - 3 g/l;
• Substrate (CAAE) feeding system = 4.0 g/l added as follows; 1.5 g/l at 1 h, 1.5 g/l at 16 h, and 1.0 g/l at 32 h.
• Bioreduction total time = 48 h.

In the conditions described above, 4-chloro-3(**S**)-hydroxybutyric acid ethyl ester was obtained. During the bioreduction, the product formation and the substrate consumption was followed by TLC. The final concentrations were established by GC and the optical configuration by polarimetry, as shown in Table 4.

The configuration was established by the comparison of the obtained value with those presented in the literature (Sih and Madison, 1987; Fuganti and Grasselli, 1985).

The separation of the residual CAAE was realized by the precipitation of Cu^{2+} complex of the ketoester.

As L-carnitine, the physiologically active stereoisomer of carnitine, is obtained starting from the R-hydroxy ester, we tried several methods to change the optical configuration of the obtained product. The results are presented in Table 5. The conditions for performing the bioreduction stage were the same as above, except for the specified modifications.

The best results were obtained in the variants 2 and 3,

which used as medium for the bioreduction stage a two phases system, comprising an organic solvent and a phosphate buffer.

We tested several variants for the two tested organic solvents by modifying the volumetric ratio between 1:1 and 7.5:1 (organic solvent : phosphate buffer pH 6.0, v/v). The data presented in Figure 3 showed the best results were obtained with n-octanol as organic solvent in the volumetric ratio of 5:1 with phosphate buffer pH 6.0. The influence of the volumetric ratio for the other solvent, n-hexane, was not different between 2.5:1 and 7.5:1 volumetric ratio. Using as bioreduction medium a mixture of n-octanol : phosphate buffer 6.0 = 5:1 (v/v), we succeeded in obtaining 3.45 g/l 4-chloro-3(**R**)-hydroxybutyric acid ethyl ester, $[\alpha_D]^{20}$ = 13.50.

Conclusions

Several microorganism strains of different genera were screened for their ability to perform the reduction of γ-chloro-β-keto-butyric acid ethyl ester to γ-chloro-β-hydroxy-butyric acid ethyl ester. The best results were obtained with the yeast strain *Hansenula polymorpha* 28, using ethanol as carbon and/or energy source. This has not been previously reported. The bioprocess consists of two stages: obtaining the active biomass of *H. polymor-*
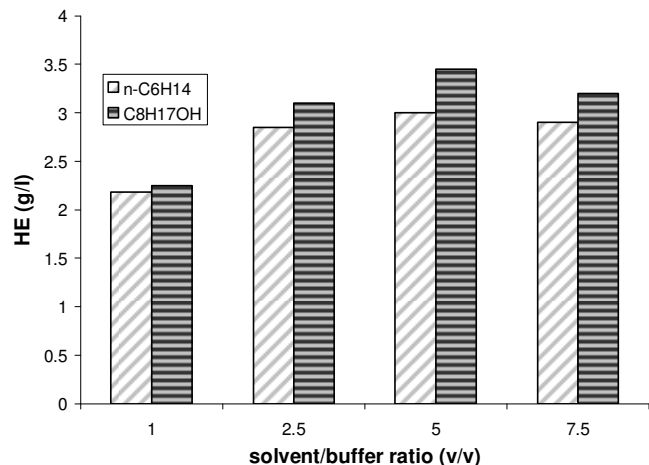
Figure 3. The influence of the solvent/buffer pH 6.0 ratio on the concentration of the HE obtained

pha 28, and using it as active biocatalist in the second stage of bioreduction of CAAE. We succeeded in obtaining both stereoisomers, using different conditions for the bioreduction stage: (**S**)-HE with a final concentration in aqueous medium of 3.3 g/l and (**R**)-HE, at 3.45 g/l in a two-phase biotransformation medium.

ACKNOWLEDGMENTS

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