An efficient biotransformation of dialkyl esters of 2-oxoglutaric acid by *Rhodotorula minuta* whole cells

EDUARDO M. RUSTOY[1], PATRICIA CERRUTTI[2], MIGUEL A.GALVAGNO[2,3]

& ALICIA BALDESSARI[1,*]

[1]Departamento de Química Orgánica y UMYMFOR, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Piso 3, Ciudad Universitaria,C1428EGA, Buenos Aires, Argentina, [2]Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Buenos Aires, Pabellón de Industrias, Piso 1, Ciudad Universitaria, C1428EGA, Buenos Aires, Argentina, [3]IIB- Intech-CONICET- UNSAM, Colectora Avenida General Paz 5445 Edificio 24 y 19, C1650, San Martín, Buenos Aires, Argentina.

Shortened title:
Biotransformation of dialkyl 2-oxoglutarates

Eduardo M. Rustoy
Departamento de Química Orgánica
Facultad de Ciencias Exactas y Naturales
Universidad de Buenos Aires
Pabellón 2. Piso 3. Ciudad Universitaria
C1428EGA. Buenos Aires. Argentina
Phone/Fax: 54-11-4576-3385
E-mail: erustoy@qo.fcen.uba.ar

Patricia Cerrutti
Departamento de Ingeniería Química
Facultad de Ingeniería,
Universidad de Buenos Aires
Pabellón de Industrias. Piso 1.Ciudad Universitaria
C1428EGA, Buenos Aires.Argentina
Phone/Fax: 54-11-4576-3366
E-mail: cerrutti@di.fcen.uba.ar

Miguel A. Galvagno
Departamento de Ingeniería Química
Facultad de Ingeniería,
Universidad de Buenos Aires
Pabellón de Industrias. Piso 1.Ciudad Universitaria
C1428EGA, Buenos Aires.Argentina
Phone/Fax: 54-11-4576-3366
E-mail: mag@di.fcen.uba.ar

Alicia Baldessari
Departamento de Química Orgánica
Facultad de Ciencias Exactas y Naturales
Universidad de Buenos Aires
Pabellón 2. Piso 3. Ciudad Universitaria
C1428EGA. Buenos Aires. Argentina
Phone/Fax: 54-11-4576-3385
E-mail: alib@qo.fcen.uba.ar
alibhome@yahoo.com.ar

Abstract

Whole cells of the yeast *Rhodotorula minuta* were used in the biotransformation of dialkyl esters of 2-oxoglutaric acid. Almost 100% of conversion with 97-98% of enantiomeric excess of the (*S*) form of 2-hydroxydiesters was obtained through an enantioselective reduction of dimethyl and diethyl 2-oxoglutarate. When longer alkoxy chain 2-oxoglutarates were used as substrates, the corresponding 4-hydroxybutyric esters were obtained, suggesting a combination process including hydrolysis, decarboxylation and reduction. The cells showed a remarkable high productivity; very good results were obtained at 2 g wet weight/mmol substrate.

Keywords

Dialkyl 2-oxoglutaric acid esters, biotransformation, *Rhodotorula minuta*.

Correspondence

Alicia Baldessari, Prof., Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2. Piso 3. Ciudad Universitaria C1428EGA. Buenos Aires. Argentina. Fax: 54-11-4576-3385. E-mail: alib@qo.fcen.uba.a

1. INTRODUCTION

Optically pure 2-hydroxy carboxylic acids and their derivatives are important building blocks for the synthesis of pharmaceuticals and other fine chemicals (Coppola and Schuster 2000).

Many approaches have been developed to obtain enantiomerically enriched 2-hydroxy carboxylic acid esters. This includes dynamic kinetic resolution of racemic 2-hydroxy esters (Radosevich et al. 2005), hydrolysis of optically pure cyanohydrin (North 2003), etc. Another straightforward method to obtain enantiomerically pure 2-hydroxy carboxylic acid esters is the asymmetrical reduction of prochiral 2-keto esters (Ramachandran et al. 2002). Other viable option for this reduction is the heterogeneous enantioselective hydrogenation using cinchonidine as chiral modifier of supported and colloidal Pt (Baiker 2005). Generally, the catalytic asymmetric hydrogenation route employs harsh conditions such as high temperature and pressure, and often requires preparation of the asymmetric catalyst. Because of environmentally benign reaction conditions and unparellel selectivity, microbial reduction is attracting more attention from both academia and industry. In the last years, whole cells have been used in the transformation of steroids (Fernandes et al. 2003), alkaloids (Rathbone and Bruce 2002), terpenes (Martin et al. 2005) and other natural products. In particular, interesting work has been performed in the enantioselective reduction of ketones (Nakamura et al. 2003) and β-ketoesters (Mangone et al. 2002; Zhu et al. 2006), but studies on enzymatic reduction of α-keto esters have only scarcely reported (Rustoy et al. 2004; Kaluzna et al. 2004).

Several microorganisms have proven to be valuable biocatalysts for the asymmetric reduction of keto esters affording the (*R*) or the (*S*) hydroxyester, depending on the nature of the biocatalyst and reaction conditions (García-Urdiales et al. 2005). For

instance, while baker's yeast (Kayser et al. 1999) and *Aureobasidium pullulans* (Patel et al. 2002) afforded the chiral hydroxyester (*R*), *Streptomyces thermocyaneviolaceus* in the presence of glutamic acid gave the hydroxyesters of opposite configuration (*S*) (Ishihara et al. 2000). In a previous report we found that, working on ethyl 2-oxoglutarate as substrate, fresh whole cells of the fungus *Mucor rouxii* produced the corresponding (*S*)-hydroxyester, while freeze-dried cells afforded the (*S*)-butyrolactone (Rustoy et al. 2004). Whole cells of *Rhodotorula* have also used in asymmetric reduction, giving satisfactory results in the reduction of ketones (Yang et al. 2006), diketones (Boutoute et al. 1998), oxoalkylphosphonates (Żymańczyk-Duda et al. 2005) and hydrolysis reactions (Cinelli et al. 2006).

In this study, the biotransformation of a series of dialkyl 2-ketoglutarates by whole cells of *Rhodotorula minuta*, an imperfect stage of basidiomycetes (Urediniomycetes) (Kirk et al. 2001), was investigated in order to explore new sources of reductases acting on 2-keto esters (Figure 1).

Figure 1

2. MATERIALS AND METHODS

All solvents and reagents were reagent grade and used without purification.

2. 1. Substrates

Dialkyl esters of 2-oxoglutaric acid (**1a-f**) were prepared through reaction of 2-oxoglutaric acid and an excess of the corresponding alcohol and *Candida antarctica* lipase B at 30°C according to a previously reported protocol (Rustoy et al. 2004).

2. 2. Microorganism

Rhodotorula minuta strain was gently provided by Dr. N. Refojo from Laboratorio de Micología of the Instituto de Microbiologia Dr. C.Malbran (Buenos Aires, Argentina).

2. 3. Cultivation of Rh. minuta

Yeast cells previously grown in Sabouraud broth plus 0.3 % yeast extract at 28ºC and 160 r.p.m. were inoculated in 8.33 x volume Erlenmeyer flasks containing the same medium at an initial Optical Density of 0.025 at 630 nm. Cultures thus prepared were incubated at 28ºC for 48 h in a rotary shaker at 160 r.p.m. Then, cells were harvested by centrifugation (10,000 x g for 10 minutes), washed twice with sterilized deionized water and centrifuged again. Pellets obtained were used for the biotransformation experiments described below.

2. 4. Rh. minuta biotransformation

Biomass (2 g wet weight) was incubated with 5 ml of organic solvents such as ethyl acetate, toluene or hexane, alone or in biphasic systems mixed with 1 ml sterile water, in 25 ml sterile Erlenmeyer flasks stoppered and sealed. Water incubations were performed in 1 ml sterile water alone. The substrates were added to these systems (0.25-1 mmol for standard assays) and incubated at 30°C in a rotatory shaker at 200 r.p.m. for different times. Samples (50 µl) were taken at different times, centrifuged at 10,000 x g and the supernatants analyzed by GLC and GC-MS. When applied, water phases were extracted with ethyl acetate. The products were purified by flash chromatography (eluant: hexane: ethyl acetate, 95:5 to 75:25). All experiments were performed in

duplicate. ^{1}H NMR, ^{13}C NMR, MS and specific rotation data of compounds **2a-b** and **3c-f** are in accordance with those reported in literature (Rustoy et al. 2004; Drioli et al. 1999). Conversion of

compounds **2a**-**2b** and **3c**-**3f** obtained by *R. minuta* cells reduction are reported in Table II.

2. 5. Analytical methods

GLC analysis for determination of percentage of conversion, were obtained on Finnigan Focus GC, Thermo Electron Co. instrument, the capillary column being Carbowax 20H-022 , 30m X 0.2 mm , film thickness 0.2μm, (1 min at 80°C, 2°C/min, 200°C). For enantiomeric excess determination HP Chiraldex G-TA capillary column, 40m x 0.32 mm, (15 min at 80°C, 2°C/min, 100°C) was used and compared with corresponding racemic products. The mixture of enantiomers **2a** and **2b** was obtained through reduction with $NaBH_4$. The absolute configuration of products **2a-b** was determined by comparison with the sign of reported specific rotation using CH_3OH as solvent with a Perkin Elmer 343 polarimeter.^{1}H-NMR and ^{13}C-NMR spectra were recorded at 200 MHz using a Bruker AC-200 spectrometer with TMS as internal standard. CGMS were obtained using a Shimadzu QP5050A gas chromatograph mass spectrometer.

3. RESULTS AND DISCUSSION

2-Ketoglutarates bearing various alkoxy chains were submitted to reduction by fresh cells from *Rh. minuta* as source of reductase activity. The results are shown in Tables I and II .

3. 1. Solvent effect

Table I shows the results obtained in the yeast reduction of ethyl 2-oxoglutarate **1b** in various media.

Table I

Yeast cells were active both in organic media and in biphasic systems. The best results were obtained in a mixture hexane:water 5:1, with high enantioselectiviy of the (*S*)-enantiomer. The use of low polar organic solvents was only effective in hexane and toluene, but the degree of conversion and the stereoselectivity decreased. In the presence of more polar solvents, such as dioxane or ethyl acetate, only the starting material was recovered. We observed that the conversion decreased by increasing the polarity of the solvent and in water the substrate was totally consumed as carbon source. The (*S*)-Di-ethyl-2-hydroxyglutarate was the isolated enantiomer in all cases.

Considering that the biphasic system hexane:water 5:1 was the best medium in the case of substrate **1b**, we studied the course of its conversion and enantiomeric excess by *Rh. minuta* whole cells under these conditions. The results can be observed in Figure 2.

Figure 2

Conversion reached a maximum value after 1 h. An enantiomeric excess of around 90-98% was obtained throughout, independently of the degree of conversion.

3. 2. Ratio biomass-substrate influence

We also studied the influence of the ratio biomass/substrate on the conversion and the enantiomeric excess in the reduction of ethyl 2-oxoglutarate by *Rh. minuta* cells in hexane:water 5:1. It could be observed that conversion increased with this ratio and attained a maximal performance at 2 g of biomass/mmol substrate. Under our experimental conditions *Rh. minuta* reductases showed a better productivity than the reported previously on reductions of 2-ketoesters using baker's yeast (10 g/mmol) (Crocq et al. 1997) and *M. rouxii* (17 g/mmol) (Rustoy et al. 2004). The high productivity or the reaction and high enantiomeric purity of the products showed by *Rh. minuta* activity could overcome some of the disadvantages of yeast-mediated reductions, that complicate the downstream processing. For instance, one of the most important industrial application of yeast mediated reduction is the baker's yeast reduction of an steroidal diketone in the multi step synthesis of trimegestone, a pharmaceutical used in hormone replacement therapy. In this process, it is necessary to use more than 300 kg yeast per kg of trimegestone, which implies a very low productivity (about 100 g /mmol) (Crocq et al.1997).

3. 3. Substrate effect

Table II shows the results of yeast-catalyzed reduction of the rest of substrates **1a-f**, using a suspension of cells in hexane:water 5:1.

Table II

It can be observed that in the biotransformation of methyl 2-oxoglutarate **1a** and ethyl 2-oxoglutarate **1b** the carbonyl group of the ketoesters was chemoselectively reduced to give the corresponding hydroxyesters **2a** and **2b**, while the ester carbonyl group remained unchanged. In both cases the alcoxy chain in the ketoglutarate esters did not affect either the degree of conversion of substrates or the chemoselectivity and enantiomeric purity of the product. Table II shows that an enantiomeric excess of 97% and 98% was obtained respectively in the reduction of **1a** and **1b**.

The behavior of the cells was completely different in the reduction of the rest of substrates (**1c-1f**). So, when methyl and ethyl group of the ester moiety was replaced by a propyl (**1c**), isopropyl (**1d**), butyl (**1e**) or isobutyl group (**1f**) no hydroxyglutarates were detected as products. Only the corresponding 4-hydroxy butanoic esters (**3c-3f**)were obtained in these cases (Scheme 1).

Scheme 1

This behavior has not been previously observed in this species of yeast.

A hypothetical explanation of the production of 4-hydroxybutanoate involving a different metabolic way could be proposed according to Scheme 2.

Scheme 2

The mechanism of formation of the alkyl 4-hydroxybutanoates, enzymatically mediated, might proceed through the regioselective hydrolysis of the alkoxycarbonyl group leading to the hemiesters **4 c-f** (reaction *1*), followed by decarboxylation to the

corresponding aldehydes **5 c-f** (reaction *2*). Reduction of the formyl group would eventually furnish the products **3 e-f** (reaction *3*).

The possibility of a spontaneous reaction pathway *1* to *3* is discarded by considering the results obtained in experiments performed with substrates **1c** to **1f** in absence of this biocatalyst. We observed that the substrates were isolated unchanged from the reaction medium, so the process is indeed catalyzed by *Rhotodotorula* cells.

4. Conclusions

Whole cells of the yeast *Rh. minuta* were used to prepare enantiomerically pure dialkyl 2-hydroxyglutarates and alkyl 4-hydroxybutyrates, from several dialkyl esters of 2-oxoglutaric acid. The yeast cells, grown in aerobic conditions, were efficient in aqueous-organic two phase reaction medium. They showed a remarkable regio- and stereoselectivity in the reduction of keto carbonylic groups with a productivity ten times higher than microbial reductions of 2-oxoesters previously reported. The activity displayed by *Rh. minuta* yeast cells was dependant on the alkoxy chain length in the substrate. In the case of substrates containing methyl and ethyl groups as alkoxy moiety, the cells showed only reductase activity and produced dialkyl 2-hydroxyglutarates in high enantiomeric excess. Using substrates with longer alkoxy chains, alkyl 4-hydroxybutyrates were obtained through a combination process including hydrolysis, decarboxylation and reduction.

Acknowledgement: We thank UBA (X089), ANPCyT (PICT 06-08293) for partial financial support. AB and MAG are Research Members of CONICET. The authors gratefully acknowledge M. M. Rivero for his assistance in GC analysis.

5. References

Baiker A. 2005. Reflections on chiral metal surfaces and their potential for catalysis. Catal. Today 100:159-170.

Cinelli G, Cuomo F, Hochkoeppler A, Ceglie A, Lopez F .2006. Use of Rhodotorula minuta live cells hosted in water-in-oil macroemulsion for biotransformation reaction. Biotechnol. Prog. 22:689-695.

Coppola GM, Schuster HF .2000. Chiral α-hydroxyacids in Enantioselective Synthesis, Wiley-VCH, Weinheim.

Crocq V, Masson C, Winter J, Richard C, Lemaitre G, Lenay J, Vivat M, Buendia J, Prat D. 1997. Synthesis of trimegestone: The first industrial application of baker's yeast mediated reduction of a ketone. Org. Proc. Res. Dev 1:2-13.

Drioli S, Nitti P, Pitacco G, Tossut L, Valentin E .1999. Enantiomerically pure tetrahydro-5-oxo-2-furancarboxylic esteres from dialkyl 2-oxo-glutarates. Tetrahedron: Asymmetry 10:2713-2728.

Fernandes P, Cruz A, Angelova B, Pinheiro HM, Cabral JSM .2003. Microbial conversion of steroids compounds: recent developments. Enz. Microb. Technol. 32:688-705.

García-Urdiales E, Alfonso I, Gotor V. 2005. Enantioselective enzymatic desymmetrizations in organic synthesis. Chem Rev. 105:313-354.

Ishihara K, Yamguchi H, Hamada H, Nakajiman N, Nakamura K. 2000. Stereocontrolled reduction of α-keto esters with thermophilic actinomyecete Streptomyces thermocyaneviolaceus IFO 14271. J. Mol. Catal. B: Enzym 10:429-434.

Kaluzna IA, Matsuda T, Sewell AK, Stewart JD .2004. Systematic investigacion of

Saccharomyces cerevisiae enzymes catalyzing carbonyl reductions. J. Am. Chem. Soc. 126:12827-12832.

Mangone CP, Pereyra EN, Argimon S, Moreno S, Baldessari A. 2002. Enzyme Microb. Technol. 30:596-601.

Martin DAG, Reynolds WF, Reese PB. 2005. Stemodane skeletal rearrengement: chemistry and microbial transformation. Phytochemistry 66:901-909.

Nakamura K, Yamanaka R, Matsuda T, Harada T. 2003. Recent developments in asymmetric reduction of ketones with biocatalysts. Tetrahedron: Asymmetry 14:2659-2681.

North M. 2003. Synthesis and application of non-racemic cyanohydrins. Tetrahedron: Asymmetry 14:147-176.

Radosevich AT, Musich C, Toste FD. 2005. Vanadium catalyzed asymmetric oxidation of α-hydroxy esters using molecular oxigen as stoichiometric oxidant. J. Am. Chem. Soc. 127:1090-1091.

Ramachandran PV, Pitre S, Brown HC. 2002. Selective reductions. 59. Effective intramolecular asymmetric reductions of α-, β-, and γ-keto acids using diisopinocamphenylborane and intermolecular asymmetric reductions of the correponding esters with β-chlorodiisocamphenylborane. J. Org. Chem. 67:5315-5319.

Rathbone DA, Bruce NC. 2002. Microbial transformation of alkaloids Curr. Opin. Microbiol. 5:274-281.

Rustoy EM, Pereyra, EN, Moreno S, Baldessari A. 2004. Combination strategy using pure enzymes and whole cells as biocatalysts for the preparation of 2-hydroxyesters and lactones from 2-oxoglutaric acid. Tetrahedron: Asymmetry 15:3763-3768.

Yang W, Xu JH, Xie Y, Xu Y, Zhao G, Lin GQ. 2006. Asymmetric reduction of

ketones by employing Rhodotorula sp. AS2.2241 and synthesis of the β-blocker (*R*)-nifenalol. Tetrahedron: Asymmetry 17:1769-1774.

Zhu D, Mukherjee C, Rozzell JD, Kambourakis S, Hua L. 2006. A recombinant ketoreductase tool-box. Assessing the substrate selectivity and stereoselectivity towards the reduction of β-ketoesters. Tetrahedron 62:901-905.

Żymańczyk-Duda E, Klimek-Ochab M, Kafarski P, Lejczak B. 2005. Stereochemical control of biocatalytic asymmetric reduction of diethyl 2-oxopropylphosphonate emplying yeasts. J. Organomet. Chem. 690:2593-2596.

Table I. Solvent effect on ethyl 2-oxoglutarate 1b reduction by *Rh. minuta.*

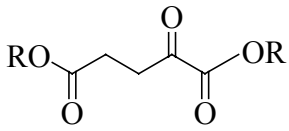
Solvent	Time (h)	Conversion (%)	ee[a] (%)
water	12	100	-[b]
hexane	12	60	83 (*S*)
hexane-water[c]	4	100	78 (*S*)
hexane-water[d]	4	100	98 (*S*)
toluene	24	43	65 (*S*)
toluene-water[d]	24	56	58 (*S*)
dioxane	36	0	-
dioxane-water[d]	36	0	-
ethyl acetate	36	5	-
ethyl acetate-water[d]	36	7	-

Reaction conditions are described in the Experimental.
Biomass/mmol substrate ratio was 2.
[a] ethyl 2-hydroxyglutarate.
[b] is completely utilized as carbon source
[c] 1:1
[d] 5:1

Table II. *Rh. minuta* cells catalyzed reduction of alkyl 2-oxoglutarates

Substrate	Product	Time (h)	Conversion (%)	ee (%)
1[a]	**2**[a]	2	100	97 (*S*)
1b	**2b**	2	100	98 (*S*)
1c	**3c**	2	100	--
1d	**3d**	2	98	--
1e	**3e**	8	71	--
1f	**3f**	8	46	--

Reaction conditions are described in the Experimental.Biomass/mmol substrate ratio was 2. Solvent: hexane:water 5:1.



1 a-f

a: R = CH_3- **;** **b**: R = CH_3CH_2- ; **c**: R = $CH_3CH_2CH_2$- ; **d**: R = $(CH_3)_2CH$- ;
e: R = $CH_3CH_2CH_2CH_2$- **;** **f** : R = $(CH_3)_2CHCH_2$-

Figure 1

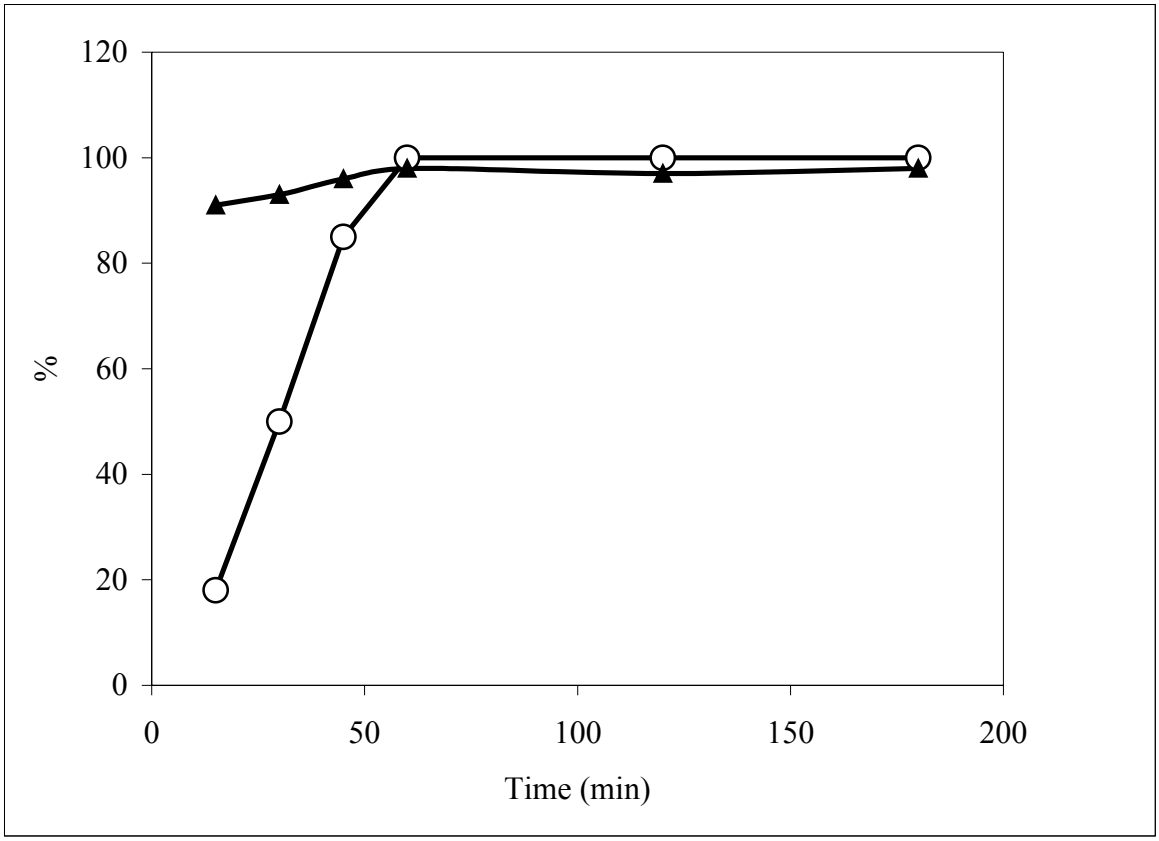
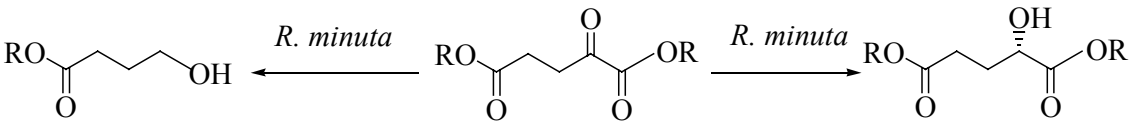


Figure. 2.

Figure captions

Figure 1. 2-Ketoesters

Figure. 2. Time course *Rh. minuta* cells catalyzed reduction of ethyl 2-oxoglutarate in hexane:water 5:1 at a biomass ratio of 2 g/mmol. Symbol (▲) denotes enantiomeric excess and (○) conversion (%).

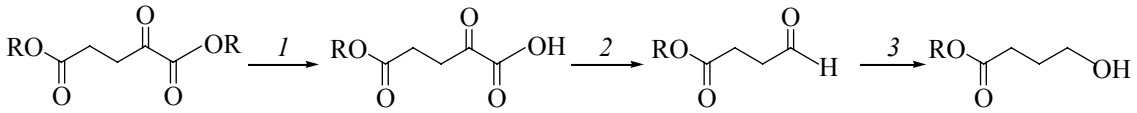


3 c-f **1 a-f** **2 a -b**

c: R = $CH_3CH_2CH_2$-
d: R = $(CH_3)_2CH$-
e: R = $CH_3CH_2CH_2CH_2$-
f : R = $(CH_3)_2CHCH_2$-

a: R = CH_3-
b: R = CH_3CH_2-

Scheme 1



1 c-f **4 c-f** **5 c-f** **3 c-f**

c: R = $CH_3CH_2CH_2$-; **d**: R = $(CH_3)_2CH$-; **e**: R = $CH_3CH_2CH_2CH_2$-**; f** : R = $(CH_3)_2CHCH_2$-

Scheme 2. Proposed reactions of *Rh. minuta* production of 4-hydroxyalkanoates from dialkyl 2-oxoglutarate (**1c-f**): *1*. hydrolysis, *2*. decarboxylation, *3*. reduction.