Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 99 (2008) 4590–4596
BIORESOURCE TECHNOLOGY

Production of *trans*-2-methyl-5-isopropylhexa-2,5-dienoic acid by *Pseudomonas rhodesiae* CIP 107491

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Received 2 December 2006; received in revised form 17 July 2007; accepted 18 July 2007
Available online 12 September 2007

Abstract

The feasibility of *trans*-2-methyl-5-isopropylhexa-2,5-dienoic acid (novalic acid) accumulation using the α-pinene degradation pathway of *Pseudomonas rhodesiae* CIP 107491 was studied. This appeared possible by using concentrated living bacterial cells produced under oxygen limitation with α-pinene as sole carbon source. The second step of the process, the bioconversion itself, had to be performed without oxygen limitation due to the need for cofactor regeneration. Results showed that a not yet reported cofactor-dependent enzymatic isomerization of isonovalal into novalal was likely to occur and that both aldehyde isomers could be oxidized to the corresponding acid. Precursors tested, α-pinene oxide and isonovalal had a strong permeabilization effect on bacterial cells. This effect, which increased from the oxide to the aldehyde, led to an inactivation of the respiratory chain and to acids synthesis stop. Present results allowed to obtain about 12 g/L acids (80% novalic acid) with an average yield close to 50% after 12 h reaction in a biphasic system using α-pinene oxide as precursor.

Keywords: Terpenoid; Biphasic medium; Bioconversion; α-Pinene oxide; Novalic acid

1. Introduction

Terpenes and terpenoids are the primary flavour and fragrance impact molecules found in the essential oils of higher plants. They are naturally branched chain C-10 hydrocarbons formed from isoprene units and are widely distributed in nature. Terpenes are traditionally isolated from essential oils. Biotransformations of these compounds remain of great commercial interest for the food and perfume industry since one of the main advantages in using biotechnological methods for the production of flavour and fragrances is to avoid drawbacks associated to natural extraction processes. Several comprehensive reviews (Krasnobajew, 1984; Schrader and Berger, 2001; de Carvalho and da Fonseca, 2006) focussed on many transformations of terpenoids for the flavour and fragrance industry.

Nevertheless, these compounds are generally both volatile and lipophilic, which poses several problems. Their low water solubility often leads to the presence of an excess substrate that gives an organic layer. They also often exhibit a rather low chemical stability when poured into an aqueous environment, and undergo spontaneous autooxidation processes. Their high volatility can additionally give rise to loss by air stripping, especially when the reaction is performed aerobically (Grivel, 1999; Grivel and Larroche, 2001). Another difficulty, encountered when whole cells are used as biocatalyst, is their high toxicity. This feature is generally attributed to a high concentration in the cytoplasmic membrane, where hydrophobic compounds preferentially accumulate (Berger et al., 1999; Sikkema et al., 1992, 1994, 1995). As a consequence, only a few biotransformation processes have been proposed up to now at an industrial scale in the area of terpenes.

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0960-8524/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2007.07.029

Most abundant terpene in nature is α-pinene. It has always constituted important resources in aroma industry. Many works led to the characterisation of different metabolic pathways allowing bioconversion of this substrate by both fungi and bacteria. Among them, a microbial catabolic pathway leading to acyclic "nova" compounds was described (http://www.genome.jp/kegg/pathway/map/map00903.html) and could be globally interpreted as follows. The route started with the oxidation of α-pinene in α-pinene oxide by monoxygenase NADH-dependant reaction (Best et al., 1987). α-pinene oxide formed was decyclized by α-pinene oxide lyase without cofactors need, conducing to *cis*-2-methyl-5-isopropylhexa-2,5-dienal (isonovalal). *Trans*-2-methyl-5-isopropylhexa-2,5-dienal (novalal) production additionally appeared in fresh cell culture. Aldehydes were then probably degraded by NAD^+-dependent dehydrogenase(s) in *cis* and *trans*-2-methyl-5-isopropylhexa-2,5-dienoic acids (Griffiths et al., 1987a). Acids were finally metabolized in acetyl-CoA and propanoyl-CoA by a presumed β-oxidation-like catabolism pathway accumulating 3,4-dimethylpentanoic acid (DMPA) as an intermediary metabolite (Zorn et al., 2004).

A highly efficient bioconversion process of α-pinene oxide into isonovalal by a new strain, *Pseudomonas rhodesiae* CIP107491, in a biphasic medium has been reported (Fontanille et al., 2002; Fontanille and Larroche, 2003). Several key parameters have been evidenced. Among them, the need for a cell permeabilization by freezing and further contacting the thawed material by an organic solvent such as chloroform, toluene or diethyl ether has to be pointed out. This operation allows both an enzyme release in the aqueous phase outside cells and an improvement of transport properties of substrate and product across the cell membrane, strongly increasing reaction rates. It also inactivates other enzymes of the precursor degradation pathway, enabling isonovalal to be an end-product obtained with high yields.

Trans-2-methyl-5-isopropylhexa-2,5-dienoic acid (novalic acid) presents a potential interest in perfumes and industrial aroma production. It is thought that its esterification or carboxylation would lead to the formation of compounds with specific aroma properties not found in plants (Berlitz et al., 2004).

This compound is obtained in the following step in this α-pinene degradation pathway which involves oxidases using NAD^+ as acceptor (see above). As a result, accumulation of such compounds implies the use of active whole cells exhibiting a functional respiratory chain to re-oxidise cofactors. Preliminary studies with *Pseudomonas rhodesiae* CIP 107491 have shown that use of viable cells actually allowed to produce novalic acid with α-pinene oxide as substrate. A two step process was then designed, comprising a biomass production step (by cultivation) and a second one, called bioconversion, using concentrated resting cells in biphasic conditions. We present here some results obtained with this system.

2. Methods

2.1. Microorganism

Pseudomonas rhodesiae CIP107491 (Fontanille et al., 2002) was maintained on Nutrient Agar (Difco) poured in Petri dishes held at 30 °C. Successive replications were performed every 3 weeks. A stock material was also prepared using the Protect Bacterial Preserver system (Technical Service Consultants Ltd., Heywood, Lancashire, UK) which is made of porous ceramic beads immersed in a cryopreservative fluid stored at −75 °C (Felthman et al., 1978).

2.2. Biomass production

2.2.1. Preculture

Precultures were prepared in Erlenmeyer flask of 500 mL volume filled with 250 mL "*Pseudomonas* Basal Medium" (Cohen-Bazire et al., 1957) and with 8 g/L of sodium lactate as carbon source (Pequignot et al., 1998). Flasks were inoculated with colonies picked out an agar plate (see above). After 24 h growth at 30 °C in a rotary shaker operated at 200 rpm the optical density at 600 nm (OD_{600}) of the media was close to 3.

2.2.2. Culture in Erlenmeyer flask

Forty milliliter of preculture were used to inoculate Erlenmeyer flasks of 2 L. The medium was made of 1 L "*Pseudomonas* Basal Medium" with different carbon sources according experiments. Addition of 50 mL of hexadecane has been done in presence of α-pinene or α-pinene oxide. The stirring rate was maintained at 200 rpm, the temperature at 30 °C.

2.2.3. Culture in bioreactor

Two hundred fifty milliliter of preculture were used to inoculate a bioreacor (Biostat MD, B. Braun, Melsungen, Germany) of 5 L working volume equipped with a partial pressure in oxygen (pO_2) sensor (Mettler-Toledo, Switzerland). The medium was made of 4.3 L *Pseudomonas* Basal Medium and 200 mL of an organic solution made of 30 mL α-pinene in hexadecane (150 g of α-pinene per liter of hexadecane). Aeration flow was controlled at 9 or 30 L/h according to the experiments. Measurement of O_2 consumption and CO_2 production rates were done by a gas analyzer Servomex Xentra 4100 (Servomex Company Inc., Nerwood, USA) connected to the gas exhaust of the reactor. Decrease in pH was limited to 6.0 using 0.5 M NaOH solution. The stirring rate was maintained at 500 rpm and the temperature at 30 °C.

2.2.4. Concentration of biomass

The resulting biomass was centrifuged for 10 min and 5000*g* at ambient temperature. The pelleted cells obtained were resuspended in an appropriate volume of KH_2PO_4/K_2HPO_4 buffer (20 mM, pH 7.5) to yield a biomass concentration close to 7 g/L (OD_{600} close to 15.5). This con-

centrated biomass was immediately used for bioconversion assays.

2.3. Novalic acid production (bioconversion)

Ability of the concentrated biomass to produce 2-methyl-5-isopropylhexa-2,5-dienoic acids was tested by performing bioconversions in biphasic liquid/liquid conditions. Organic layer, made of substrate in organic solvent (hexadecane), was added to the resting cells concentrated in phosphate buffer. Acids production was then routinely followed by injecting organic phase in the gas chromatography (GC) apparatus. Acids at pH values in the range 6–8 were found to highly partition towards the organic phase, allowing to neglect their concentration in the aqueous layer. Presence of acids in organic phase at pH 6 to 8 was controlled as highly predominant. "Activity" of the biomass was assessed by three variables, initial rate of acid production (Vi in g/L h), maximal metabolite concentration (C_{max} in g/L) and mass production yield (Y in %). Concentrations were expressed in g per liter of hexadecane.

2.3.1. Bioconversion in Erlenmeyer flask

The reaction medium was a two-phase system comprising an organic phase made of an organic solution of different substrates (α-pinene, α-pinene oxide, isonovalal or novalal according to experiments) in 25 mL of hexadecane. Aqueous phase was composed of concentrated biomass in phosphate buffer. The phase volume ratio was 1:1 (aqueous/organic phase), and reaction temperature was 30 °C. Erlenmeyer flasks of 250 mL total volume were placed in a rotary shaker (200 rpm).

2.3.2. Bioconversion in reactor

A 1.5 L working volume reactor (Applikon Dependable Instruments), containing 300 mL of concentrated biomass in phosphate buffer and an organic phase comprising 4 mL of α-pinene oxide in 200 mL hexadecane (20 g of α-pinene oxide per liter of hexadecane) was used. The aeration was controlled as described in experiments. Temperature and stirring rate were regulated at 30 °C and 200 rpm, respectively.

2.4. Acids extraction

Acids were first extracted in an aqueous alkali phase (NaOH 0.25 M) then in a fresh organic phase (hexane) by decreasing pH with sulfuric acid. This procedure allowed to discard neutral products such as residual α-pinene oxide. Sample was concentrated by solvent evaporation using a rotating evaporator (Büchi) fitted with a refrigerated condenser and operated under controlled vacuum (−900 mBar).

2.5. Identification and quantitation of metabolites

2.5.1. Gas chromatography

Quantification of substrate consumed and metabolites produced was done by gas chromatography (GC) analyses. 1 μL of organic phase was directly injected into the apparatus. The chromatograph (HP 6890, Agilent Technologies, Palo Alto, CA, USA) was fitted with an apolar SPB-5 (Supelco Inc., Bellefonte, PA) capillary column (30 m × 0.32 mm i.d., film thickness 0.25 μm) and a flame ionization detector. The carrier gas was nitrogen, and the oven temperature was kept at 80 °C for 5 min, them raised to 200 °C at 20 °C/min. The injector and detector temperatures were 250 °C for both; the split ratio for injection was 1:5. Concentrations were calculated by assuming that all compounds had the same response factor as α-pinene oxide. Internal standard was heptadecane (1 g per liter of hexadecane).

2.5.2. GC–MS

Identification of produced metabolites was done by GC–Mass Spectral (MS) analyses performed on an apolar HP-5MS (Agilent Technologies) capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The carrier gas was helium, other operating parameters were as above. The gas chromatograph was an Agilent 6890-plus, which was coupled to a 5973 N MS-engine. MS conditions were electron-impact ionization energy 70 eV, accelerating voltage 1.1 kV, emission current 35 μA, and quadrupole, ion source and interface (line transfer) temperature 150, 230 and 280 °C, respectively. Library searches were performed using the NIST/EPA/NIH mass spectral library (version 1.7a, 2000).

2.5.3. NMR spectrometry

Identification of produced acids was done by proton and ^{13}C nuclear magnetic resonance (NMR). Spectra were obtained with a Bruker AC-400 spectrometer at 400 MHz in ^{1}H and 100 MHz in ^{13}C. About 10 mg of sample were diluted in 0.5 mL $CDCl_3$. Chemical shifts (δ) were expressed in ppm with tetramethylsilane (TMS) as reference.

2.6. Viability test

Two hundred microliter of aqueous phase were mixed with 800 μL of methylene blue solution (NaCl: 0.9 g, KCl: 0.042 g, $CaCl_2$: 0.048 g, $NaHCO_3$: 0.02 g, glucose: 1 g, methylene blue: 0.025 g for 100 mL distilled water). After 10 min of staining, cells were observed with microscope (×1000). Uncolored cells were considered as viable whereas blue cells were considered as permeabilized.

3. Results

3.1. Identification of acids produced

3.1.1. GC–MS analyses

Acids produced in the organic phase during bioconversion of α-pinene oxide by *Pseudomonas rhodesiae* CIP 107491 resting cells were extracted in hexane and concentrated by evaporation. GC–MS analysis of the samples gave a recurrent chromatogram with a major peak at

Rt = 10.2 min. Mass spectra of the 10.2 min peak was assigned as 2-methyl-5-isopropylhexa-2,5-dienoic acid. GC–MS analyses did not allow for specific identification of *cis* and *trans* isomers.

3.1.2. RMN analyses

^{13}C RMN spectrum of the same sample gave the following major peaks: RMN ^{13}C ($CDCl_3$) δ (ppm): 173.6 (C_1); 152.3 (C_2); 143.0 (C_3); 127.9 (C_5); 108.7 (C_6); 34.2 (C_8); 33.6 (C_4); 21.6 (C_9 et C_9'); 11,9 (C_7) corresponding to those observed by Griffiths et al., (1987a) during bioconversion of α-pinene oxide by *Nocardia* sp. strain P18.3 and by Tudroszen et al. (1977) during RMN analyses of novalic and isonovalic standards (Table 1). The carbon 7 signal was found specific of the isomer; it was at 20.5 ppm and 12 ppm for isonovalic acid and novalic acid, respectively. Results showed clearly that the major acid produced was novalic acid.

^{1}H RMN spectrum gave the following major peaks: RMN ^{1}H ($CDCl_3$) δ (ppm): 6.97 (t, 1H, $J = 7.5$ Hz, H_3); 4,83 (s, 1 H, H_6a); 4,69 (s, 1H, H_6b); 2.92 (d, 2H, $J = 7.5$ Hz, H_4); 2.26 (m, 1H, $J = 7.0$ Hz, H_8); 1.86 (s, 3H, H_7); 1.05 (d, 6H, $J = 6.5$ Hz, H_9) corresponding to novalic acid. Other minor peaks corresponding to isonovalic acid were also detected (data not shown). Comparison of integrated peak areas at 2.92 ppm (novalic acid) and 3.28 ppm (isonovalic acid) allowed to calculate novalic/isonovalic ratio at about 5/1, i.e. novalic acid accounted for ca. 80% of the mixture.

The following study was carried out assuming that the novalic acid concentration in a sample obtained during a bioconversion process was proportional to the area of the peak eluted from 10 min in GC analysis. It was referred to as "acids" production and was, in fact, a mixture where the *trans* isomer was highly predominant.

Table 1
Identification of acids produced during bioconversion of α-pinene oxide by *Pseudomonas rhodesiae* CIP 107491 resting cells

Carbon N°	^{13}C NMR peaks (ppm)				
	Reaction products[a]		2-Methyl-5-isopropylhexa-2,5-dienoic acids standards[b]		Reaction products[c]
	Major	Minor	*Cis* (isonovalic acid)	*Trans* (novalic acid)	Major
1	171.6	171.8	173.7	173.7	173.6
2	154.2	152.3	154.1	154.1	152.3
3	144.1	142.9	144.6	144.6	143.0
5	126.6	127.6	126.9	126.9	127.9
6	107.9	108.7	107.9	107.9	108.7
8	34.6	33.6	35.2	35.2	34.2
4	34.2	29.7	34.6	34.6	33.6
9 and 9′	21.6	21.6	21.6	21.6	21.6
7	**20.6**	**12.0**	**20.5**	**12.1**	**11.9**

[a] Values reported by Griffiths et al. (1987a,b).
[b] Values reported by Tudroszen et al. (1977).
[c] Values observed in our study.

3.2. Influence of conditions for biomass production on further bioconversion activity

3.2.1. Carbon source(s)

Several bacterial cultivations were carried out in Erlenmeyer flasks with α-pinene, α-pinene oxide, lactate and glucose as carbon sources, sodium lactate being the substrate used in the precultivations. Results showed that biomass obtained by culture with α-pinene as sole carbon source allowed to achieve the best acids production (Table 2). A significant Cmax of 15.1 g/L associated to a yield of 53% and an initial rate Vi of 1.6 g/L h were achieved in these conditions.

3.2.2. Use of a bioreactor for cell production

Cultures in Erlenmeyer flasks are simple to use but control of some culture parameters (pH, aeration) is difficult. This behaviour is even worse when cultures involve biphasic conditions. Preliminary study on the use of a stirred aerated tank reactor allowed a 21-fold increase of the biomass concentration obtained (1.8 g/L biomass in reactor against 0.36 g/L in Erlenmeyer flasks) (Table 3). Nevertheless, biomass obtained in reactor showed lower activity than that obtained in Erlenmeyer flasks. Hence, the maximal acid concentration obtained was decreased to 13 g/L with a yield of 31% while Vi was roughly the same (1.55 g/L h). The major difference between the two processes was the cultivation duration (29 h in Erlenmeyer flask against 19 h in reactor), which probably indicated a strong oxygen limitation in flasks.

3.2.3. Duration of cultivation and oxygen limitation

A first set of experiments, allowing biomass recovery after different cultivation times (9, 15, 19 and 23 h), was

Table 2
Influence of carbon sources used for bacterial growth on resulting biomass activity, described by the initial rate Vi, the maximal product concentration C_{max}, and the molar yield Y, for production of acids from α-pinene oxide

Carbon source(s) used for biomass production[a]	Activity of biomass[b]		
	Vi (g/L h)	C_{max} (g/L)	Y (%)
α-Pinene oxide and glucose	0	0	0
α-Pinene oxide	0.1	1.1	3.6
Lactate and α-pinene	0.25	1.6	6
Glucose and α-pinene	0.28	3	24.5
Lactate	0.37	2.07	31
α-Pinene	**1.6**	**15.1**	**53**

[a] Cultivations were performed in 2 L Erlenmeyer flasks filled 1 L of MBP medium and 40 mL of inoculum coming from preculture on lactate. Initial concentrations of carbon sources were: α-pinene 76.8 g/L in organic phase; lactate 2 g/L or glucose 10 g/L in aqueous phase. Stirring rate 200 rpm, temperature 30 °C.
[b] These values were measured for bioconversions carried out in 250 mL Erlenmeyer flasks with 25 mL of biomass concentrated at 8 g/L and 25 mL of an organic phase made of a solution of α-pinene oxide in hexadecane. Initial substrate concentration 30 g/L, stirring rate 200 rpm, temperature 30 °C.

Table 3
Influence of cultivation duration and oxygen limitation on the activity of the biomass in bioconversion processes carried out in Erlenmeyer flasks with α-pinene oxide as precursor

Cultivation duration (h)[a]	Time elapsed since oxygen limitation (h)[b]	Activity of biomass[c]		
		Vi (g/L h)	C_{max} (g/L)	Y (%)
9	0	1.1	9	15
15	1	0.7	8	27
19	5	1.6	13	31
23	9	1.6	14	34
15[d]	–	0.8	7	8
35[e]	26	1.6	14	47

[a] Cultures carried out in a 5 L bioreactor filled with 4.5 L of MBP medium, 40 mL of inoculum (grown on lactate), α-pinene being used as the sole carbon source and incorporated under the form of 200 mL of a 150 g/L solution in hexadecane. Stirring rate 500 rpm, temperature 30 °C, pH = 6, aeration rate 30 L/h (0.11 VVM).
[b] Oxygen limitation was considered as taking place when the dissolved oxygen tension became equal to zero.
[c] See Table b legend for parameter definitions.
[d] Oxygen limitation was avoided by increasing the stirring rate at 12 h (500–600 rpm) and 14 h (600–800 rpm).
[e] Oxygen limitation occurred earlier due to the lowered aeration rate used, 9 L/h (0.03 VVM).

carried out in the reactor. Data in Table 3 showed that the activities were improved by lengthening the oxygen limitation period rather than by the cultivation time itself. This finding was supported by a second set of experiments, where two cultivations were carried out with opposite aeration strategies. In the first, oxygen limitation was avoided by increasing the stirring rate when necessary while in the second, an early oxygen limitation took place, due to the lowering of the aeration rate from 30 to 9 L/h. The second approach allowed to reach the results obtained with Erlenmeyer flasks (Table 3). Theses results showed clearly that oxygen limitation during biocatalyst production allowed later increasing of acid production yields and rates.

3.3. Bioconversion conditions

3.3.1. Nature of the precursor

Biomass was produced in the bioreactor with α-pinene as sole carbon source and an aeration rate of 9 L/h (0.03 VVM) ensuring an early oxygen limitation. After recovery and concentration (see Section 2), this material was used in Erlenmeyer flasks for bioconversion processes using several precursors. They were the different intermediates of the pathway leading to the acids, i.e. α-pinene, -pinene oxide, isonovalal, and novalal. This last aldehyde was obtained by chemical isomerization of an hexadecane solution of isonovalal poured in an aqueous NaOH-glycine buffer 1 mol/L, pH = 11 for 48 h at 30 °C (Fig. 1).

Results in Table 4 showed that surprisingly α-pinene was not consumed in bioconversion conditions while it served as sole carbon source during biomass production. Oxidation of α-pinene to α-pinene oxide is carried out by α-pinene monooxygenase, a NADH-dependent reaction

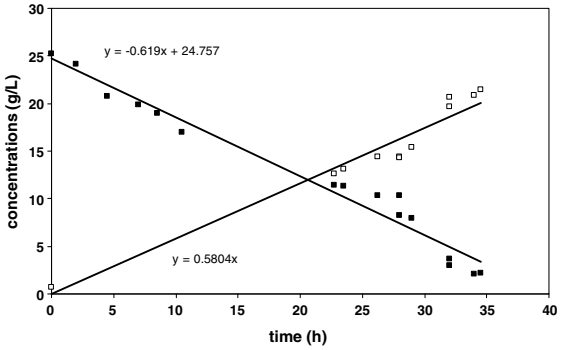

Fig. 1. Typical time-course of isomerization of isonovalal (■–■) into novalal (□–□). Twenty milliliter of aqueous buffer NaOH-glycine 1 mol/L pH = 11 contacted with 20 mL of a 25 g/L solution of isonovalal in hexadecane in a 200 mL Erlenmeyer flask maintained at 30 °C and stirred at 200 rpm. Data corresponding to novalal during the first 10 h revealed that quantitation of low amounts of this isomer in the presence of high concentrations of isonovalal gave over-estimated values and were not reported on the figure.

Table 4
Influence of the nature of the precursor on acids accumulation during bioconversion reactions

Substrate[a]	Vc (g/L h)[b]	Activity[c]		
		Vi (g/L h)	C_{max} (g/L)	Y (%)
α-Pinene	0	0	0	0
α-Pinene oxide	3.36	1.6	14	47
Isonovalal	0.37	0.2	2	**55**
Novalal[d]	0.60	0.2	5	50

[a] Biomass production was carried out in the 5 L bioreactor filled with 4.5 L of MBP medium with α-pinene as sole carbon source (76.8 g/L of organic phase). Stirring rate 500 rpm, temperature 30 °C, pH = 6, aeration rate 9 L/h.
[b] Average substrate consumption rate.
[c] Initial concentrations of α-pinene, α-pinene oxide, isonovalal and novalal were 30, 30, 7 and 11.8 g/L organic phase, respectively. See legend of Table 2 for parameter definitions.
[d] Novalal was chemically obtained from isonovalal in the presence of glycine at basic pH (see Fig. 1).

(Best et al., 1987). It could be assumed that the biomass treatment (centrifugation, suspension in buffer) prior to carry out a bioconversion led to a dramatic decrease of this monooxygenase activity. Omitting this step by direct use of α-pinene oxide as substrate allowed a high acids accumulation (14 g/L). In the same way, bioconversion with isonovalal and novalal as precursors gave high acids production yields (Table 4).

3.3.2. Cell viability

A typical time course of a bioconversion using α-pinene oxide as precursor is given in Fig. 2. It shows that the beginning of a process took place with acids being the sole compounds detected in significant amounts. Aldehydes (isonovalal and novalal) became significant after only 8 h of reaction, novalal being the first accumulated. Cell viability tests using methylene blue staining (see Section 2)

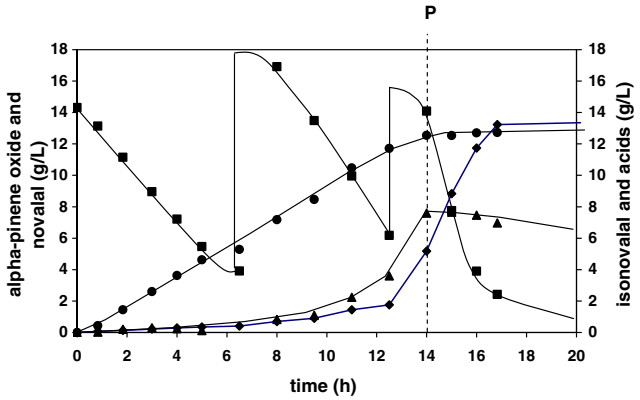

Fig. 2. Time course of of α -pinene oxide (■–■), isonovalal (◆–◆), novalal (▲–▲) and acids (●–●)concentrations during a bioconversion process carried out in an Erlenmeyer flask. Twenty five milliliter of fresh concentrated biomass (7 g/L) was added to an organic solution of α-pinene oxide in 25 mL of hexadecane. Sequential additions of α-pinene oxide were performed to avoid substrate limitation. Time of permeabilization is represented by dashed line (P). Temperature 30 °C, stirring rate 200 rpm.

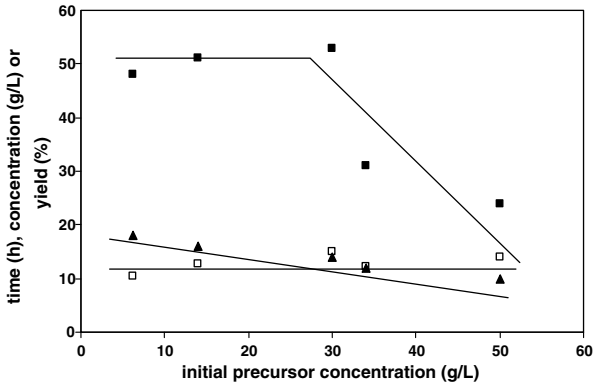

Fig. 3. Toxic effect of α-pinene oxide on living *P. rhodesiae* cells, expressed as the time elapsed before cell permeabilization (▲–▲), the final concentration of acidic metabolites (□–□) and the final yield of acids (■–■) during the course of experiments carried out in Erlenmeyer flasks. Sequential precursor feedings were performed when necessary in order to avoid any substrate limitation.

performed during this kind of experiments revealed that bacteria were permeabilized after 15 h of process. This time corresponded to the end of both acids and novalal accumulation while isonovalal production continued with a high yield (80%). This last feature was consistent with that observed during degradation of α-pinene oxide in isonovalal by permeabilized biomass (Fontanille and Larroche, 2003). It could thus be considered that α-pinene oxide had a permeabilizing effect on *Pseudomonas rhodesiae* cells, giving rise to a gradual increase in the precursor penetration rate inside the cells. This, in turn, could give an increase in intracellular precursor concentration allowing an improvement in the aldehydes synthesis rates that could become higher than the acids synthesis process.

This permeabilizing effect finally led to an inactivation of the bacterial respiratory chain, thus preventing oxido-reduction phenomena. This hypothesis would lead to consider that novalal biosynthesis took place through such a cofactor-dependent process, using probably isonovalal as precursor.

3.3.3. Toxicity of precursors

Since cell viability appeared as an important limitation factor in our system, it was important to study the toxic effect of the different precursors. A preliminary study of *Pseudomonas rhodesiae* CIP 107491 cells poured in bioconversion conditions, but without substrate revealed that cells remained intact for at least 24 h. This feature confirmed that the biphasic medium used for bioconversion experiments did not affect cell integrity. Increasing initial concentrations of α-pinene oxide led to a shortening of the active period for acids synthesis due to a faster cell permeabilization (Fig. 3). The maximal acid concentration achieved remained almost constant, close to 12 g/L while the bioconversion yield exhibited a plateau near 52% for initial precursor concentrations lower than 30 g/L. A rapid decrease in this parameter was evidenced for higher concentrations. Isonovalal exhibited a much more marked toxic effect and 30 g/L of isonovalal lead to instant cell permeabilization (Table 5). Data in Table 5 revealed that it was necessary to use very low isonovalal concentrations, less that 2.5 g/L, to achieve valuable acids synthesis. This situation will imply to perform fed-batch feedings with short time intervals between two addings.

3.3.4. Feasibility of the bioconversion in a bioreactor

Fig. 4 shows the time course of a process performed using α-pinene oxide as precursor. Data confirmed unambiguously that acids accumulation needed aeration of the medium, since only aldehydes were produced at the beginning of the reaction, carried out without aeration. Here again, novalal was the first aldehyde accumulated, isonovalal becoming important at the end of the cultivation, when acid accumulation stopped i.e. when cells became permeabilized. This result was still consistent with the fact that isonovalal synthesis is known to be cofactor independent (Fontanille and Larroche, 2003). A final concentration of 8 g/L acids was obtained, value slightly lower than that achieved in Erlenmeyer flasks. This demonstrated the need for further studies on the aeration strategy to be used during this process.

Table 5
Toxicity of isonovalal to *P. rhodesiae cells*

Initial concentration of isonovalal (g/L)	Vi[a] (g/L h)	C_{max}[a] (g/L)	Y[a] (%)	Permeabilization time (h)[b]
3.2	0.22	1.5	61	10
32.5	0.017	0.6	32	2
38	0	0	0	1

[a] Values were measured as in Table 2.
[b] Cells permeabilization was evidenced by methylene blue staining test as described in "Methods" section.

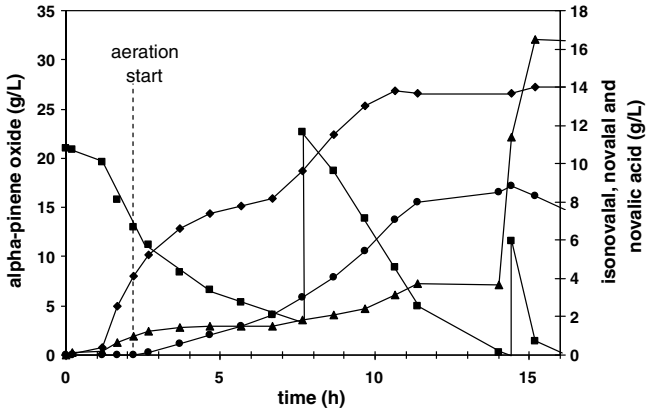

Fig. 4. Time course of α-pinene oxide (■–■), isonovalal (◆–◆), novalal (▲–▲) and acids (●–●) concentrations during bioconversion process carried out in the bioreactor. The biocatalyst consisted of 300 mL fresh concentred biomass, aeration (12 L/h) started at 2.5 h (dashed line), temperature 30 °C, stirring rate 200 rpm.

4. Conclusions

This study has shown the feasibility of novalic acid production by *P. rhodesiae* CIP 107491. Biomass has to be produced with α-pinene as sole carbon source and under oxygen limitation. The production of the enzymatic material needed for acids synthesis from α-pinene oxide seemed thus to be induced both by α-pinene and by the intracellular redox potential. Conditions needed for acid production were completely different from those used for isonovalal synthesis. Hence, aldehyde was in fact produced by a crude enzymatic extract (Fontanille et al., 2002; Fontanille and Larroche, 2003) while acid accumulation implied the use of intact whole cells of the same bacterial strain. This approach, which deals with the so-called metabolic engineering area, allowed to evidence new features. Among them, the well known toxic effect of terpenoids on bacterial cells was once again demonstrated. In the case of *P. rhodesiae* this effect was mostly due to a cell permeabilization leading to intracellular compounds release and inactivation of metabolic pathways such as the electron transport chain. However, isonovalal, an intermediate of α-pinene degradation, appeared more toxic than the precursor itself. This behaviour was unusual since organic compounds breakdown is generally considered as a detoxifying mechanism. The pathway for isonovalal breakdown involves a series of acidic compounds (Best et al., 1987; Zorn et al., 2004). Results obtained in this work allowed to demonstrate that the main compound accumulated by *P. rhodesiae* was novalic acid, even if the direct oxidation compound, isonovalic acid, was present. It indicated that a *cis-trans* isomerization took place probably at the aldehyde level. Also, synthesis of novalal from isonovalal was necessarily an enzymatic process, and some evidences that it was cofactor dependent have been provided. However, more studies on this point would be needed. Present results demonstrated the feasibility of novalic acid production by living cells of *P. rhodesiae* CIP 107491. Simplest conditions involved α-pinene oxide as precursor and about 12 g/L acids (80% novalic acid) were obtained after 12 h reaction with an average yield close to 50%. A careful optimization of precursor addition strategy would allow to significantly improve these results.

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