

## Chiral Enzymatic Activity in Cell Cultures of *Taxus* Species

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**Abstract.** Plant cell suspension cultures of *Taxus brevifolia* and *Taxus globosa* were used as biocatalysts on an exogenous racemic substrate in order to determine the influence of several parameters on its configurational deracemization bioactivity. *Taxus brevifolia* demonstrated a directly proportional relationship between the enantiomeric ratio (er) [1] and the time of reaction, yielding a mixture of 90(S):10(R) after 7 days. Pure enantiomers were tested and the results showed that the (S)-enantiomer remained intact, whereas the (R)-enantiomer was biotransformed. The buffer solution did not present any effect. Sucrose and temperature modified the reaction time and the er. The pH was a critical variable in relation to the age of the suspension cultures. *Taxus globosa* showed a low efficiency for distinguishing between the enantiomers.

**Key words:** *Taxus brevifolia*, *Taxus globosa*, Cell Cultures, Desracemization, Chirality.

**Resumen.** Cultivos de células en suspensión de *Taxus brevifolia* y *Taxus globosa* se utilizaron como biocatalizadores para determinar, bajo diferentes condiciones, la actividad catalítica de desracemización configuracional ejercida sobre el racemato y enantiómeros puros de un sustrato exógeno. *Taxus brevifolia* presentó una relación directamente proporcional entre la relación enantiomérica (er) [1] y el tiempo de reacción, dando una mezcla de 90(S): 10(R) después de 7 días. La biotransformación de los enantiómeros puros demostró que sólo el isómero (R) fue biotransformado por resolución cinética y, una fracción, mediante estereoinversión en su enantiómero opuesto, mientras que el isómero (S) se mantuvo intacto. Las soluciones reguladoras no presentaron ningún efecto, sin embargo la adición de sacarosa y la temperatura sí modificaron los tiempos de reacción y la er. En relación con la edad de los cultivos y la variación del pH el efecto fue crítico. *Taxus globosa* presentó una baja eficiencia en su actividad catalítica configuracional.

**Palabras clave:** *Taxus brevifolia*, *Taxus globosa*, cultivos celulares, desracemización, quiralidad.

### Introduction

Plant cell cultures are an important tool in applied studies in several areas. Many plants produce secondary metabolites whose chemical synthesis is not usually feasible economically due to their highly complex structures and specific stereochemistry [2]. Although the use of synthetic techniques for production of medically relevant compounds and for general synthesis has increased substantially, plant cell cultures have provided an alternative and environmentally friendly protocol. Not only has the plant cell culture protocol been used for producing secondary metabolites, it has also served as a biocatalyst for transforming endogenous and exogenous substrates [3-6].

*Taxus* species have been extensively investigated by several research groups [7-10]. These species produce a family of taxanes including paclitaxel (Taxol<sup>®</sup>), which has been an extremely successful anticancer drug. Research in regard to the biosynthetic pathway of paclitaxel revealed 19 enzymatic steps, which could offer a remarkable enzymatic arsenal for selective biotransformation processes [11].

With the aim of exploring the possibility of advantageously using the amount and types of enzymes available, we recently reported that cell suspension cultures of *Taxus globosa* Schltdl. and *Taxus brevifolia* Nutt. were able to cyclize and to reduce chemo- and stereoselectively exogenous substrates under several experimental conditions [12], following our prior

research with these species. Here we report their enzymatic deracemization activity on racemic ethyl-3-hydroxybutanoate by transesterification in response to the influence of different reaction parameters: pH, buffer solution, sucrose concentration, temperature, substrate concentration and age of the suspension cultures.

Enantiomers of ethyl-3-hydroxybutanoate and its  $\beta$ -hydroxyacid are reported as chiral starting materials for the production of numerous biologically active compounds of commercial interest [13-18].

### Results and discussion

Whole cells were used in the context to obtain one pure enantiomer from racemic ethyl-3-hydroxybutanoate as an exogenous substrate. In all cases, analysis of blanks or standards did not show deracemization of the racemic ethyl-3-hydroxybutanoate or racemization of pure enantiomers, which indicates that the results observed in the biotransformations were solely due to enzymatic activity.

#### Biotransformation with *T. brevifolia* Nutt

At pH ranging from 4.0 to 8.0, the results showed a directly proportional relationship between enantiomeric ratio (er) and

the reaction time with a straight behavior and with the enantioselectivity maintained during the reaction course. High enantiomeric ratio was obtained at pH 4 after 7 days by adding 10% m/v of sucrose using citrate-phosphate (cP) as buffer solution (Fig. 1).

Similar activities at pH 6.0 and 7.0 were obtained using cP or phosphates (P), indicating that the type of buffer solution did not affect the er. The (*S*)-enantiomer was identified as the main constituent in the enantiomeric mixture.

It has been reported that temperature may be a factor in increasing or decreasing the efficiency of enzymatic processes [19]. In order to test this effect, biotransformations were incubated at different temperatures for 7 days (Fig. 2).

The highest activity was observed at 20 °C (74% er). At temperatures >30 °C, the activity decreased dramatically, resulting in 0-2% er on day 7. When the biotransformation was performed at 10 °C, we obtained only 34% er, with a continuous increase to 65% er when the reaction time was extended to day 11 (data not shown). At 20 °C and 30 °C, similar rates of reaction were measured although the reaction course showed instability after 3 days of incubation, resulting in 50% er by day 7.

Many enzymatic processes, and thus their resultant yields, are known to be limited by the substrate concentration, e.g.,

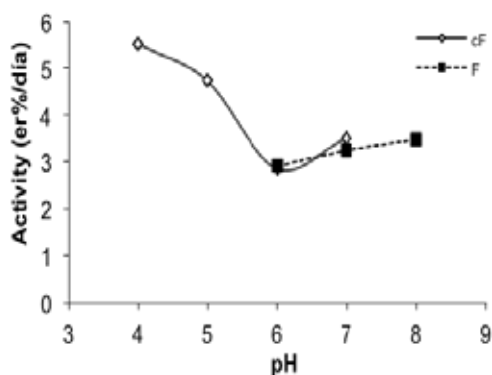


Fig. 1. Effect of pH on biotransformation of racemic ethyl-3-hydroxybutanoate in *T. brevifolia* Nutt.

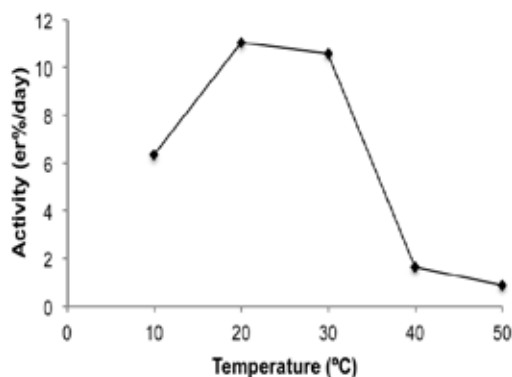


Fig. 2. Influence of temperature on biotransformation in *T. brevifolia* Nutt. after 7 days.

enantioselectivity was affected when cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* was used to biotransform, through an enantiodivergent oxidation, racemic bicycle[3.2.0]hept-2-en-6-one into lactones [20]. In view of the above statements, different substrate concentrations were tested to determine any modifications in the er and/or enantioselectivity. The results showed that the enantioselectivity did not change, and in all cases the (*S*)-enantiomer was obtained (Fig. 3).

However, the er showed an inversely proportional relationship with the substrate concentration with higher er being obtained at 0.05% v/v (71% er by day 5) compared to 0.2% and 0.6% v/v with 26% and 4% er, respectively.

In order to investigate the enantiomer behavior, pure (*R*)- and (*S*)-enantiomers were used independently with whole cells of *T. brevifolia* Nutt. in 0.1% m/v and 0.2% m/v of substrate to eliminate any enzymatic saturation. As shown in figure 4, pure (*S*)-enantiomer was totally recovered in both concentrations, whereas only 10% of chemical yield was obtained as a mixture in the proportion 10(*S*):90(*R*) when pure (*R*)-enantiomer was inoculated (*R* 0.1% and *R* 0.2%). At the same time, racemic-ethyl 3-hydroxybutanoate was used under the same

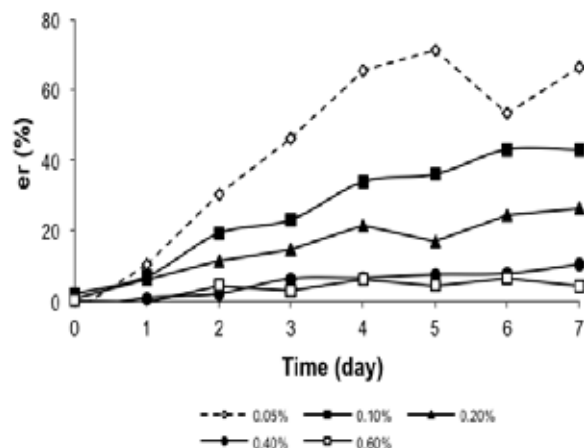


Fig. 3. Enantiomer ratio dependence of racemic substrate concentration in *T. brevifolia*.

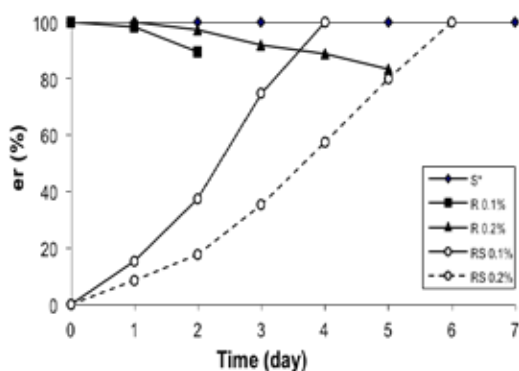


Fig. 4. Reaction course using racemic and enantiomeric ethyl-3-hydroxybutanoate in *T. brevifolia*. *S*\*: 0.1% and 0.2% v/v.

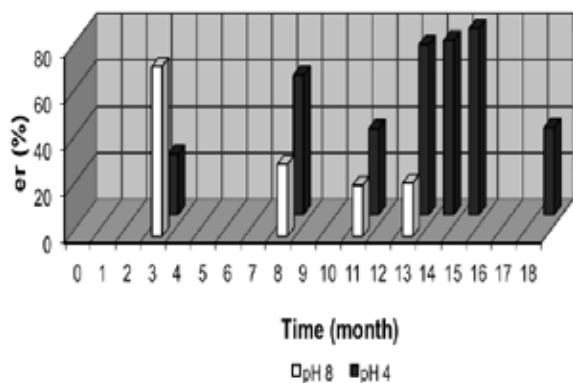
conditions and concentrations with the results showing that *er* was increased with prolonged reaction times resulting in the same mixture proportion of 90(*S*):10(*R*). These results revealed that the (*S*)-enantiomer remained intact in the reaction media during the reaction time and that only 10% of the pure (*R*)-enantiomer was changed into the opposite enantiomer by stereoinversion, according to one of the mechanisms of chiral resolution [21] (Fig. 4).

In the case of racemic ethyl-3-hydroxybutanoate, production of an *er* was possible because the (*R*)-enantiomer was biotransformed to (*S*)-enantiomer by a kinetic resolution mechanism [22] confirmed when we obtained a chemical yield range of 30 to 56%.

Finally, when different pH values were tested with 10% v/v of sucrose at 20°C and with 0.2% v/v of substrate concentration, higher values of *er* were found at pH 8.0 and pH 4.0 than at intermediate pHs. In order to better understand these results, and because several assays were performed during different stages of continuous cultivation, we compared the influence of suspension culture age with the influence of other parameters such as pH.

Suspension cultures of *Taxus brevifolia* Nutt. were maintained for 1 year and 9 months. The results presented in Figure 5 showed that, 3 months after generation of the suspension cultures, greater activity was achieved at pH 8.0 (73% *er*) than at pH 4.0 (26% *er*). However, 5 months later the *er* was elevated at pH 4.0 (60% *er*), whereas at pH 8.0 it decreased to 22% *er*. A consistent level of activity was observed during months 13-15 at pH 4.0 (80% *er*), whereas activity was decreased during suspension culture maintenance at pH 8.0 (Fig. 5).

Production of secondary metabolites in several plant cell cultures decreased with culture age [2]. In *Taxus* species, paclitaxel production is not constant but rather is influenced by season *in vivo* [23-25]. Additionally, paclitaxel production is also influenced by the age of the suspension *in vitro* [9]. Difficulties encountered when establishing cell cultures such as cessation of cell growth/production of the secondary metabolites after a period of time in suspension can be attributed to the changes in genomic structures [26]. Metabolic changes associated with genomic instability may affect cellular metabolic activity and



**Fig. 5.** Influence of age of the suspension cultures on the optimum pH in the biotransformation of racemic ethyl-3-hydroxybutanoate using *T. brevifolia*.

thus may modify the pH conditions in which kinetic resolution results in one pure enantiomer. The GC chromatograms showed a similar pattern at both pH values across culture ages, and new signals were not observed. This is the first report showing that reaction conditions must be modified according to the age of the suspension culture in order to biotransform an exogenous substrate such as ethyl-3-hydroxybutanoate.

### Biotransformation with *Taxus globosa* Schltdl

Previous studies mentioned that most enzymatic processes operate under neutral aqueous conditions [27, 28]; therefore, we performed the biotransformation under standard conditions (pH 6.5 and 7.0) to obtain one pure enantiomer. As a result, we obtained the (*S*)-enantiomer with values of 1% and 3% of *er* on day 7. When several pH values (4.0-8.0) were tested with daily sample collection, we observed a 16% *er* for the (*S*)-enantiomer and chemical yields ranging from 89% to 92% within the same time frame.

The presence of sucrose favored *er* with an approximate 50% increase being observed. The reaction courses for all pH treatments are presented in Table 2.

The results showed an oscillatory behavior between *er* and reaction time, resulting in enantioselectivity changes according to pH conditions within 1 week. In the course of 1 day, we observed an increase of *er* in all pH treatments, but by the second day the opposite was observed with the exception of the treatment at pH 6.5 and 8.0. By the third day, a decrease in *er* was observed at pH 6.5, whereas a continuous increase was observed at pH 8.0. These results exhibited no periodic dependence between *er* and reaction time. Both enantiomers were identified as the main product in the enantiomeric mixture. At pH values of 4.0 and 6.0, the oscillatory behavior was also present, but the opposite enantioselectivity was observed during the reaction course because the (*R*)-enantiomer was mainly obtained. Enantioselective changes were previously reported when racemic benzoin was deracemized by *Rhizopus oryzae* at pH 7.5-8.0 and pH 4.0-5.0 [29].

The present results suggest that *Taxus globosa* Schltdl. cells are capable of transforming one enantiomer, depending on the pH. When racemic ethyl-3-hydroxybutanoate was used, the cells could produce (*S*)- or (*R*)-enantiomers, causing an oscillatory behavior related to pH changes in the reaction media or within the cells. The result is an inefficient process for obtaining a pure enantiomer. Racemic ethyl-3-hydroxybutanoate was inoculated in the reaction media without cells as a control experiment and was recovered with no change.

## Experimental procedures

### Plant material

Callus tissues of *Taxus brevifolia* Nutt. were induced from young stems. These were subcultured for 2 years using the experimental conditions previously reported [12], whereas callus

**Table 1.** Enantiomeric ratio on the biotransformation with whole cells of *T. globosa* Schltdl. after 7 days

pH	[er (%)]
4	3.5 ( <i>R</i> )
5	6 ( <i>S</i> )
6	0
6.5	1 ( <i>S</i> )
7	3 ( <i>S</i> )
8	16 ( <i>S</i> )
8 <sup>a</sup>	7 ( <i>S</i> )

<sup>a</sup>Without sucrose.  
er, enantiomeric ratio.

**Table 2.** Enantiomeric ratio (er) dependence (%) on the reaction time at several values of pH using *T. globosa* Schltdl.

Reaction time (days)	er (%) in several values of pH (main enantiomer)					
	4 ( <i>R</i> )	5 ( <i>S</i> )	6 ( <i>R</i> )	6.5 ( <i>S</i> )	7 ( <i>S</i> )	8 ( <i>S</i> )
0	0	0	0	0	0	0
1	0.5	2	2	1	1.5	1
2	2	0	0	4	0	2
3	2	1	nd	1	5	5
4	3	2.5	6	6	2	7
5	2.5	1.5	nd	5	2	8
6	4	3	nd	2	1.5	13
7	3.5	6	0	1	3	16

nd, not determined.

tissues of *Taxus globosa* Schltdl. were induced from mature shoots and maintained by subculturing for 1 year. *T. brevifolia* Nutt. callus was transferred to Erlenmeyer flasks (250 mL) containing B5NB liquid medium supplemented with 2% m/v of sucrose and 1% m/v of PVP, regulating at pH 5.5 after sterilization. *Taxus globosa* Schltdl. callus were transferred to Erlenmeyer flasks (250 mL) with SH liquid medium containing 1 mg/L of NAA, 0.5 mg/L of 2,4-D, 0.05 mg/L of BA, 1.5% m/v sucrose and 1% m/v PVP at pH 5.6, adjusted after sterilization. Both plant cell cultures were maintained under dark conditions on a rotary shaker (100 rpm) at 20 °C, replacing old media with fresh media every 15 days for 5 weeks. From week 6, biotransformations were performed and subsequent experiments were carried out with suspensions of cells in continuous culture.

#### Biotransformation with *Taxus brevifolia* Nutt

After filtration, 14 g fresh weight of cells were resuspended in Erlenmeyer flask (250 mL) containing 40 mL of buffer solution under dark conditions on a rotary shaker (100 rpm) at 20 °C (except for the experiment) to determine temperature dependence. Racemic ethyl-3-hydroxybutanoate (0.2% v/v)

and sucrose (10% m/v) were added to the biotransformation reaction mixture.

The influence of pH was determined by measuring the er at several pH values. Buffer solutions used were citrate (50 μM)-phosphate (100 μM) (pH 4.0-7.0) and phosphates (200 μM) (pH 6.0-8.0). The influence of sucrose concentration was tested in its absence at pH 8.0. To determine the influence of temperature, 7-day incubation was performed at temperatures ranging from 10 °C to 50 °C (pH 4.0) with 10% sucrose.

Different concentrations (v/v) of racemic ethyl-3-hydroxybutanoate were tested to determine the influence of concentration on activity (Fig. 3). Biotransformation was performed by adding 10% m/v sucrose at pH 4.0. Analysis of each pure enantiomer during its biotransformation was independently carried out with 0.1% and 0.2% v/v of (*R*)-ethyl-3-hydroxybutanoate and (*S*)-ethyl-3-hydroxybutanoate. Racemic ethyl-3-hydroxybutanoate was used as a control experiment at the same concentrations. Appropriate control experiments with each type of biotransformation were tested by mixing all the components except i) racemic ethyl-3-hydroxybutanoate or each pure enantiomer and ii) whole cells. The final enantiomeric excess and chemical yield were determined.

#### Biotransformation with *Taxus globosa* Schltdl

The cells were harvested by filtration and 14 g fresh cell weight were suspended in a 250 mL Erlenmeyer flask containing 40 mL of buffer solution with the addition of 10% m/v sucrose and 0.2% v/v racemic ethyl-3-hydroxybutanoate. The biotransformation reaction was incubated under dark conditions on a rotary shaker (100 rpm) at 20 °C for 7 days while monitoring the er. Several values of pH were tested. Buffer solutions used were as follows: citrate 50 μM-phosphate 100 μM (pH 4.0-7.0) and phosphates 200 μM (pH 6.0-8.0). To determine the influence of sucrose, biotransformation was performed in its absence at pH 8.0.

Control experiments were performed with i) whole cells and ii) racemic ethyl-3-hydroxybutanoate in each buffer solution with or without 10% m/v of sucrose.

#### General methods

<sup>1</sup>H NMR spectra were recorded with a Varian Unity Detector at 300 MHz using CDCl<sub>3</sub> as the solvent and Me<sub>4</sub>Si (TMS) as the internal reference. Biotransformations were monitored by GC in an Agilent 6890 chromatograph using hydrogen as a carrier gas. The yield was determined with an Alltech ATTM-AquaWax column (30 m × 0.25 mm ID × 0.25 μm) with the following conditions: constant flow rate of 2 mL/min, oven temperature of 130°C, injector and FID detector temperature of 230 °C and split of 200. Retention times were 32.2 min for (*S*)-ethyl-3-hydroxybutanoate and 33.3 min for (*R*)-ethyl-3-hydroxybutanoate. The er was determined with a Restek RT-γDEXsa column (30 m × 0.32 mm ID × 0.25 μm) using a constant flow rate of 2 mL/min, oven temperature of 90 °C, injector and FID detector temperature of 200 °C and split

of 20. Under these conditions, retention times were 3.2 min for *n*-hexanol and 5.3 min for racemic ethyl 3-hydroxybutanoate.

### Racemic ethyl-3-hydroxybutanoate

The exogenous substrate was obtained by reduction of ethyl acetoacetate (25 g, 0.19 mol) with sodium borohydride (1.85 g, 0.04 mol) in ethanol (500 mL) under magnetic stirring for 4 h. A saturated solution of NaCl (30 mL) was added, extracted with ethyl ether (3 × 90 mL), and the organic phase dried over Na<sub>2</sub>SO<sub>4</sub> was concentrated. The residue was distilled (74 °C, 20 mmHg) obtaining a colorless liquid. <sup>1</sup>H NMR spectrum was in agreement with the literature and the purity was determined by GC/FID using an Alltech ATTM-AquaWax column.

### Determination of enantiomeric ratio and yield

The progress of er development was monitored every 24 h by collecting 1 mL samples. These samples were centrifuged (11,500 rpm, 5 min) and the supernatant was extracted with ethyl ether (2 × 400 μL). The organic phase was analyzed by chiral GC/FID. The yield was determined by collecting 1 mL samples at the end of the biotransformation (7 days). These samples were centrifuged (11,500 rpm, 5 min) and the supernatant was analyzed by GC/FID using *n*-hexanol as the internal standard.

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