Growth Curves of Filamentous Fungi for Utilization in Biocatalytic Reduction of Cyclohexanones

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GJSFR-B Classification : FOR Code: 100301p

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Growth Curves of Filamentous Fungi for Utilization in Biocatalytic Reduction of Cyclohexanones

Gliseida Zelayarán Melgar[α], Francisca Vanessa Souza de Assis [σ], Lenilson Coutinho da Rocha [ρ], Silmara Cristina Fanti [ω], Lara Durães Sette [¥] & André Luiz Meleiro Porto [§]

Abstract - The growth of filamentous fungal mass in malt extract culture was recorded over 10 days at 32 ºC, with the aim of utilizing the mycelium for the biocatalytic reduction of cyclohexanones1-3. Growth curves were obtained for marine fungi (*Aspergillus sydowii* CBMAI 933, *Penicillium miczynskii* CBMAI 930 and *Trichoderma* sp. CBMAI 932) and terrestrial fungi (*Rhizopus* sp. Cs1 and *Aspergillus* sp. Pd1), plotted asusing mycelialdry weight. The mycelia of *Aspergillus* sp. and *Rhizopus* sp. catalyzed the biocatalytic reduction of cyclohexanones1-3efficiently, when these substrates were added inthe exponential phase of growth at(72 h).The bioreduction of 4-methylcyclohexanone (3) by *Aspergillus* sp. and *Rhizopus* sp. yielded preferentially the *cis*-4-methylcyclohexanol (6), a thermodynamically and kinetically less stable compound. Whole cells of *Aspergillus* sp. and *Rhizopus* sp. showed good enzymatic activities in the bioreduction of ketone 1, yielding *trans*-diol 4.

Keywords : bioreduction; rhizopussp.; aspergillussp.; biocatalysis; cyclohexanones.

I. INTRODUCTION

The study of the growth of microorganisms is important in many fields, forexample clinical analysis and agriculture. Meletiades et al. used the growth curves of filamentous fungi in various nutrient media to optimize amethod for antifungal susceptibility testing [1]. Several parameters involved in testing filamentous fungi for their susceptibilities, such as inoculum preparation, incubation conditions (time and temperature) and the type of the nutrient mediumwere investigated. In addition, the use of microorganismsto improve the availability of nutrients to plants is a necessary and important practice in agriculture. Burity et al. investigated the inoculation of mycorrhizal fungi as an aid to the cultivation of *Mimosa caesalpiniifolia*. The nodulation of the plants roots was favored by appropriate inoculation of mycorrhizal fungi under specific growth conditions [2]. Growth of filamentous fungi in liquid culture is usually measured as an increase in dry weight, using either stationary or orbital shaker cultures in Erlenmeyer flasks. However, in large-scale experiments, for instance when testing the effects of various compounds on the growth of fungi, such experiments can become very space-demanding and laborious, limiting the number of treatments. Langvad developed an efficient method to measure the growth of filamentous fungi, using 96-well microtiter plates and a microplate reader [3]. In recently years, microorga-nism-shave been usedexhaustively inbiocatalytic processes [4]. Such processes require significant control and manipulation of the organisms during the growth phase.

Microorganisms are capable of growing on a wide range of substrates and can produce a broad spectrum of products. When a nutrient medium is inoculated with a microorganism, the growing culture passes through a number of the phases. Initially, growth does not occur and this period is referred to as the lag phase, while may be considered a period of adaptation. Following an interval during which the growth rate of the cells gradually increases, the cells divide at a constant, maximum rate and this period is referred to as the log or exponential phase. Exponential growth cannot continue indefinitely, because the medium is soon depleted of nutrients and enriched with metabolites. Several enzymes are synthesized in the exponential phase, while many secondary metabolites emerge in the ensuing stationary phase, when there is no net growth [5].

Microorganisms can be cultured to produce biomass that may be used to catalyze the conversion of axenobiotic compound into a structurally similar one. The enzymes obtained from microorganisms can behave as chiral catalysts with high regio-andstereo-specificity, in contrast to purelychemical catalysts, enabling the addition, removal, or modificationof functional groups at specific sites on a complex molecule, without the need for chemical protection. Enzymes generally work more effectively within the microbial cell, especially if co-factors such as reduced pyridine nucleotide need to be regenerated [5,6].

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Prochiral cycloalkanones have been shown to be convenient substratesfor asymmetric reduction to enantiomericallypure cycloalkanol derivatives [7-9]. Herein, we present a study of the growth of several species of filamentous fungi and their use in biocatalytic reduction reactions of cyclohexanones **1-3**.

II. Materials and Methods

a) General Methods

The reagents 2-(*tert*-butyldimethylsilyloxy) cyclohexanone (**1**), 3-methylcyclohexanone (**2**) and 4-methylcyclohexanone (**3**) were purchased from a commercial source (Sigma-Aldrich). All manipulations involving the fungi *Aspergillussydowii* CBMAI 933, *Aspergillus* sp. Pd1, *Penicilliummiczynskii* CBMAI 930, *Rhizopus* sp. Cs1 and *Trichoderma* sp. CBMAI 932 were carried out under sterile conditions in a Veco laminar flow cabinet. Technal TE-421 or Superohm G-25 orbital shakers were employed in the biocatalysed transformation experiments. Products of the reduction reactionscarried out with sodium borohydride were purified by column chromatography over silica gel (230–400 mesh) eluted with mixtures of *n*-hexane and EtOAc. The column effluent was monitored by TLC on pre-coated silica gel 60 F254 layers (aluminium-backed: Sorbent) eluted with *n*-hexane and EtOAc. Reaction products were analyzed with a Shimadzu model 2010 GC gas chromatograph equipped with an auto-injector AOC20i flame ionization detector (FID) and a Varian Chiral CP-Chiralsil-DEX (β-Cyclodextrin), column (25 m x 0.25 mm i.d.; 0.39 μm). The chromatographic conditions were: oven temperature initially 50 °C (3 min), rising to 185°C (5 min) at 3°C/min, giving a total run time 53 min; injector temperature 250°C; detector temperature 250°C; injector split ratio 1:20; carrier gas N_2at a pressure of 50 kPa. The retention times were: 3-methylcyclohexanone (**2**), 19.5 min; 4-methylcyclohexanone (**3**), 20.8 min; *cis*- and *trans*-cyclohexane-1,2-diol (**4**), 35.0 min; *cis*- and *trans*-3-methylcyclohexanol (**5**), 24.3min; *cis*-4-methylcyclohexanol (**6**), 25.0 min; *trans*-4-methylcyclohexanol (**6**),24.3 min.

Gas Chromatography-Mass Spectrometry (GC-MS): a Shimadzu GC 2010 Plus gas chromatography system coupled to a mass selective detector (Shimadzu MS 2010 Plus) in electron ionization (EI, 70 eV) mode was used, with a DB5 fused silica column (J&W Scientific; 30 m x 0.25 mm x 0.25). The chromatographic conditions were: oven temperature initially 50°C (0 min) rising to 270°C (10 min) at 10°C/min; run time 32.5 min; injector temperature 250°C; detector temperature 250°C; injector split ratio 1:20; carrier gas He at a pressure of 88.2 kPa. 3-methylcyclohexanone (**2**), 4.54 min; 4-methylcyclohexanone (**3**), 4.62 min; *cis*-cyclohexane-1, 2-diol (**4**), 6.48 min; *trans*-cyclohexane-1, 2-diol (**4**), 6.62 min, *cis*-

and *trans*-3-methylcy-clohexanol (**5**), 4.43 min; *cis*- and *trans*-4-methylcy-clohexanol (**6**), 4.47min.

b) Preparation of cyclohexane-1, 2-diol (*4*), 3-methylcy-clohexanol (*5*) and 4-methylcyclohexanol (*6*)

The cyclohexane-1,2-diol (**4**), 3-methylcyclohexanol (**5**) and 4-methylcyclohexanol (**6**) were synthesized by reduction of the ketones **1-3**(100 mg) withexcess of sodium borohridein methanol. The spectroscopic data (^{1}H and ^{13}C NMR and MS) of alcohols **4-6** were in agreement with those reported in the literature [10-13].

trans-cyclohexane-1, 2-diol (**4**): (GC-MS) retention time 6.62 min, ($c = 30\%$,*Aspergillus* sp.; $c = 48\%$, *Rhizopus* sp.); MS: m/z (%) 116 (M$^+$, 12), 98 (52), 83 (45), 70 (100), 57 (74);^{1}H NMR (200 MHz, CDCl$_3$, ppm)1.26-12.24 (m, 4H), 1.60-1.98 (m, 4H), 3.34 (m, 2H), 3.85 (br s, 2H); ^{13}C NMR (50 MHz, CDCl$_3$, ppm) 25.3, 34.9, 77.8.

3-methylcyclohexanol (*cis* and *trans* mixture) (**5**): (GC-FID) retention time: 24.3 min, ($c = 83\%$; *Aspergillus* sp.;$c = 99\%$, *Rhizopus* sp.); (GC-MS) retention time 4.40 min, MS: m/z (%) 114 (M$^+$, 1), 96 (51), 81 (91), 71 (100), 57 (45); ^{1}H NMR (200 MHz, CDCl$_3$, ppm) 0.82 (d, 3H), 0.7-1.9 (m, 9H), 3.48 (m, 0.7H, *cis*-CHOH), 3.95 (m, 0.3H, *trans*-CHOH), 3.9 (broad singlet, 1H); ^{13}C NMR (50 MHz, CDCl$_3$, ppm) 26.5, 31.4, 35.4, 41.5, 44.6, 66.8, 70.7.

trans-4-methylcyclohexanol (**6**): (GC-FID) retention time: 24.3 min, (35.5%*Aspergillus* sp., 55%, *Rhizopus* sp.);(GC-MS) retention time 4.50 min, MS: m/z (%)114 (M$^+$, 2), 96 (14), 81 (46), 70(21), 57 (100), 41 (48);^{1}H NMR (200 MHz, CDCl$_3$, ppm) 0.97 (d, 3H), 1.20-1.50 (m, 5H), 1.65-1.75 (m, 2H), 1.90-2.00 (m, 2H), 3.5 (tt, 1H, $J = 4.5$, 11.0 Hz).

cis-4-methylcyclohexanol (**6**):(GC-FID) retention time: 25.0 min, (42.5% *Aspergillus* sp., 42.2%, *Rhizopus* sp.); (GC-MS) retention time 4.50 min, MS: m/z (%) 114 (M$^+$, 2), 96 (14), 81 (46), 70(21), 57 (100), 41 (48); ^{1}H NMR (200 MHz, CDCl$_3$, ppm) 0.95 (d, 3H), 1.20-1.50 (m, 5H), 1.65-1.75 (m, 2H), 1.90-2.00 (m, 2H), 3.90 (q, 1H, $J = 3$ Hz).

c) Isolation and preservation of filamentous fungi

The marine fungal strains, *A. sydowii* CBMAI 933, *P. miczynskii* CBMAI 930 and *Trichoderma* sp. CBMAI 932 were isolated from the sponge, *Geodiacorticostylifera* [14]. The stock cultures of marine fungi were previously preserved at 4°C on solid media containing 2% malt extract in Petri dishes. The equivalent malt extract liquid culture medium was inoculated with stock fungal mycelium and inoculated at 32°C until 240 h, and then growth curves were constructed using dry myceliaweight in accordance with procedure described below (Figure1).

The fungi *Aspergillus* sp. Cs1 and *Rhizopus* sp. Pd1 were isolated during the germination of seeds of

Chorisiaspeciosa and *Peltophorumdubium*, collected on the soil surface. Ten seeds of *C. speciosa* and *P. dubium* were washed with distilled water (2 x 10 mL) and subsequently with solution of 0.1% sodium hypochlorite (2 x 10 mL). Then, the seeds were transferred to the Petri dishes containing a sterilized sheet of What man filter paper soaked in water, and incubated for seven days in a chamber at 37°C. After the required incubation period, from the seeds from *C. speciosa*, furry colonies of filamentous fungus were obtainedon the agar surface. The isolated fungus was identified as *Rhizopus* sp. Cs1. In addition, on the seeds of *P. dubium* colonies of a dark-sporing strain were obtained and during the growth of fungus on the agar, the seeds were completely destroyed. The purified black fungus was identified as *Aspergillus* sp. Pd1. The fungi *Rhizopus* sp. and *Aspergillus* sp. were used to inoculate liquid culture medium with 2% malt extract and cultured at 32°C for 240 h, to obtained dry weight growth curves (Figure 1). Each fungal isolation was conducted in duplicate. The fungi used in this study were identified by both conventional and molecular methods at the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) at UNICAMP, São Paulo, Brazil (http://www.cpqba.unicamp.br/) [14].

d) Preparation of culture media

Solid mediumfor the growth of fungi was prepared by adding 20 g malt extract and 10 g agar (Acumedia) to1 L distilled water and autoclaving at 121 °C for 15 min. The culture medium was dispensed into sterile Petri dishes, allowed to cool and inoculated with fungi. Liquid culture medium (100 mL) was prepared in 250 mL Erlenmeyer flasks, as the solid medium but without addition of agar.

e) Procedure to obtain the growth curves

Two small slices of solid medium (0.4×0.4 cm) bearing mycelium of each fungus (*A. sydowii* CBMAI 933, *Aspergillus* sp. Pd1, *P. miczynskii* CBMAI 930, *Rhizopus* sp. Cs1, *Trichoderma* sp. CBMAI 932) were cut from the stock solid culture and used to inoculate liquid culture medium (100 mL) contained in 250 mL Erlenmeyer flasks. The fungal cultureswere incubatedat 32°C on an orbital shaker (140 rpm). For each microorganism, 5 flasks were used. During the growth of strains in liquid medium, replicate Erlenmeyer flaskswere withdrawn from the orbital shaker one by one at 5 different times (96, 144, 192, 240 h). In addition, the mycelia were filteredon a Buchner apparatus and their dry weights determined after drying at 70°C for 24 h. The growth curves of fungi were constructed from the dry cell weights (g/100 mL) plotted *versus* incubation time (h). The experiments were conducted in triplicate (Figure1).

f) Biocatalytic conversion of cyclohexanones*1-3* by *Aspergillus* sp. and *Rhizopus* sp.

The fungi *Aspergillus* sp. Pd1 and *Rhizopus* sp. Cs1cultured in 250 mL Erlenmeyer flasks as described above. The fungal mycelia were incubated at 32°C on an orbital shaker (140 rpm) at 72 h. The cyclohexanones (**1-3**, 50 μL) previously diluted in ethanol (50 μL) were added at the time of exponential growth determined for each microorganism; in these cases, at 72 h. After a satisfactory time for, the fungal cultures were withdrawn from the orbital shaker and the mycelia were harvested by Buchner filtration and washed with ethyl acetate. Subsequently, the filtrates and washing were combined and extracted with ethyl acetate (3 x 30 mL) and the organic phase dried over Na_2SO_4, filtered and evaporated. The residues extracted were dissolved in EtOAc and analyzed by GC-FID and GC-MS (Figure 2).

III. Results and Discussion

a) Growth curves of filamentous fungi

In these studies, to optimize the use of filamentous fungi as biocatalysts for the reduction reactions of cyclohexanones**1-3**, growth curves were recorded for the dry weight of mycelia cultured over different periods of time. A total of five growth curves were obtained for the five strains of filamentous fungi (Figure 1). The exponential growth phases were characteristicofeach of the fungal strainsgrownin the malt extract liquid medium. These experiments were conducted in triplicate.
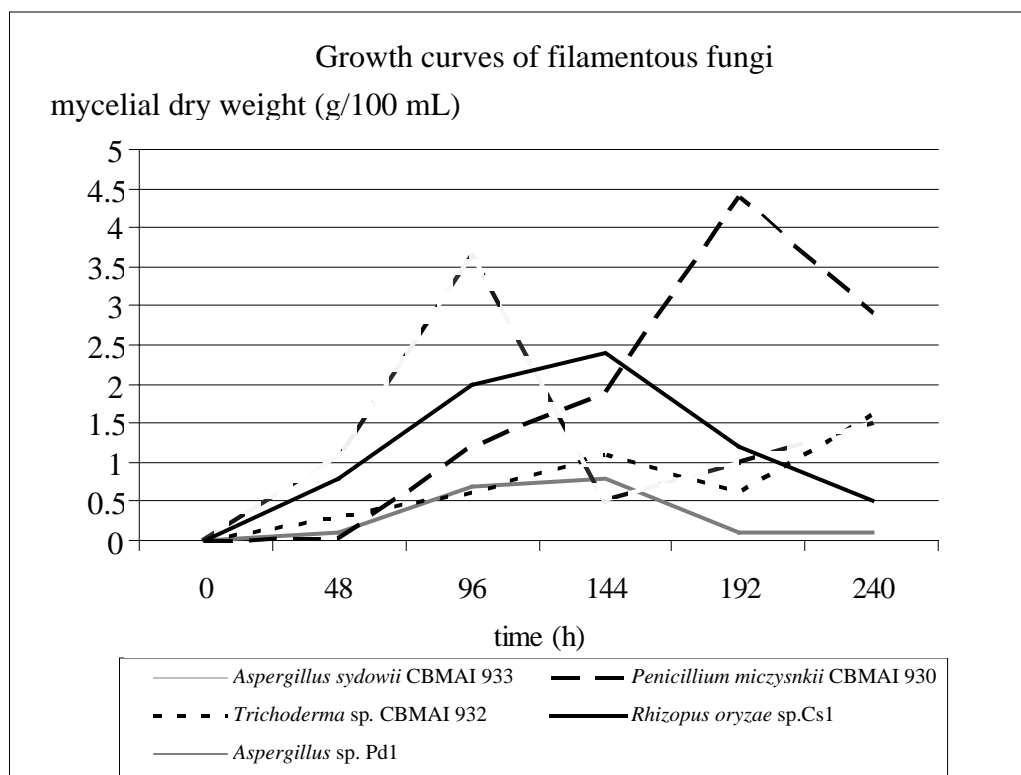
Figure 1 : Growth of filamentous fungi in 2% malt extract liquid medium at 32 °C for 10 days

For *A. sydowii* CBMAI 933, the growth curve showed a log phase that occurred in the period from 48 to 144 h. After this time, an accentuated fall in the mycelial mass until 192 h was observed, possibly because the nutrient had been consumed, leading to the death phase. *Aspergillus* sp. Pd1, a strain isolated from seeds of *Peltophorum dubium*, showed a log phase similar to that of *A. Sydowii* CBMAI 933, from 48 to 144 h. Afterwards, the massdeclined acutely from 240 h. This fungal strain showed a highermycelialproduction than *A. Sydowii* CBMAI 933 and an accelerated growth metabolism. Therefore, it was an interesting fungus to be tested in the biocatalytic process (Figure 1).

For marine fungus *P. miczynskii* CBMAI 930, the log phase in the growth curve contained to a maximum mass at 192 h, after which the growth curve had a negative slope at 240 h. In this case, also good yields of dry mycelium were obtainedin the log phase (Figure 1).

Trichoderma sp. CBMAI 932showed a log phase growth period from 48 to 144 h, which was similar to that of strains of the genus *Aspergillus*. However, following the decline phase at 192 h, a large mycelial growth occurred. Possibly, the release of metabolites produced in the primary growth phase fed a new phase of fungal growth.

The strain of *Rhizopus* sp. Cs1 showed the shortest log phase, where the highest production of mycelial mass occurred from 48 to 96 h of incubation. After this time, a decrease in mass of the myceliumwas observeduntil 144 h. When the incubation period of the fungus was prolonged, mode rate growth was observed after 144 h. However, the amount of mycelium remained significantly lower than in the log phase (Figure 1).

In conclusion, these experiments revealed a broad spectrumof behavior in the growth of filamentous fungi, which could be utilized as biocatalysts in the reduction of cyclohexanones **1-3**

b) Biocatalytic reduction of cyclohexanones **1-3**

After the growth curves of the filamentous fungi were recorded. The best strains were selected for the biocatalytic reduction reactions of cyclohexanones**1-3**. According to the results shown in (Figure 1), the fungi *Aspergillus* sp. Pd1 and *Rhizopus* sp. Cs1 were chosen for assessment of their biocatalytic reduction capacity. The strain of *Aspergillus* sp. showed a fast growth and a long exponential phase, while the *Rhizopus* sp. showed an acceleratedlog phase and a high production of dry mycelial mass.

Initially, the fungi were cultivated in 100 mL of malt extract for 72 h. After this time, the cyclohexanones**1-3** (50 μL)diluted in ethanol (50 μL) were added, during the log phase of growth, and aliquots were collected at 96 and 240 h and analyzed by chromatographic methods (GC-FID and GC-MS). The mycelia of *Aspergillus* sp. and *Rhizopus* sp. catalyzed the biotransformation of 2-(*tert*-butyldimethylsilyloxy) cyclohexanone (**1**) into cyclohexane-1,2-diol (**4**) at 240 h. GC-MSanalysis showed that ketone **1** was consumed

partially, producing the *trans*-cyclohexane-1,2-diol (**4**) in modest concentrations (*c*<50%), by both fungi (Figure 2). The cyclohexanone derivative **1** was also reduced with sodium borohydrideto yield the 2-TBS-cyclohexanol as intermediate. In this condition, the labile silyloxy group of the ketone **1** was notdeprotected. The deprotection of the TBS group was then accomplished by treatment with1M HCl in CH_2Cl_2 at room temperature for 24 h, leadingto the corresponding *cis*-diol **4**.On the other hand, the whole mycelia of *Aspergillus* sp. and *Rhizopus* sp. achieved good bioreduction of ketone **1** when it was added in the log phase of growth yielding the *trans*-diol **4**. This implied that silyloxy group was deprotected in water for 5 days. Finally, GC-MS analysis incapillary columns showed the separation of a racemic mixture of *cis*- and *trans*-cyclohexane-1,2-diol (**4**), with retention timesof 6.48 min (*cis*-**4**) and 6.62 min (*trans*-**4**).

The ketones **2-3** showed excellent bioconversion by the mycelia of *Rhizopus* sp. and *Aspergillus* sp. *Rhizopus* sp. catalyzed the reduction of**2** to yield 3-methylcyclohexanol (**5**) at a high conversion rate (*c*=99%), at 96 h. While *Aspergillus* sp. converted 83% to alcohol **5** at 96 h of incubation. The conversion of 3-methylcyclohexanol (**5**) by the fungi was measured by GC-MS analyses and compared with the amount of synthetic product **5** obtained by sodium borohydride reduction. The GC-FID and GC-MS analyses showed no separation of the stereoisomers of **5**.

Both *Rhizopus* sp. and *Aspergillus* sp. catalyzed the reduction of 4-methylcyclohexanone (**3**) to the corresponding *cis*- and *trans*-4-methylcyclohexanol

(**6**) at high conversion rates,at 96 h (Figure 2). The fungi produced a higher proportion of *cis*-diastereoisomerthan in the product of sodium borohydride reduction, which was mainly the *trans*-diastereoisomer. The ratio of *cis*- and *trans*-alcohol **6**, determined by GC-FID on the chiral chromate graphiccolumn, was 1.0: 1.3 for *Rhizopus* sp. and 1.2 : 1.0 for *Aspergillus* sp. The biocatalytic reduction produced around 50% of the *cis*-diastereoismer **6**, which is thermodynamically and kineticallyless stable than the stable *trans*-diastereoismer **6** (Figure 3). In contrast, the *cis*- and *trans*-diastereoisomers **6** were synthetized by sodium borohydride reductionin the ratio 1.0: 4.5. The biocatalyticreduction of 4-methylcyclohexanone **3** could be used for the preparation of the thermodynamically andkinetically less stable *cis*-isomer **6**.

Finally, these have been a few reports in the literature of the microbial reduction of cyclohexanones. For example, the prochiralcouponds 1, 2-cyclohexane-dione and (±)-2-hydroxycyclohexanone were reduced by, *Proteus mirabilis* bacteria leading to *trans*-cyclohexane-1,2-diol in high yields [6]. In addition, the reductionof cyclohexanone derivatives by a bacteria culture of *Serratiarubidaea* CCT 5742 and by the yeast *Saccharomyces cerevisiae* afforded cyclohexanols in good yields and selectivities [12,15]. However, these single-cell organisms are less readily separated from the culture medium than fungal mycelia.
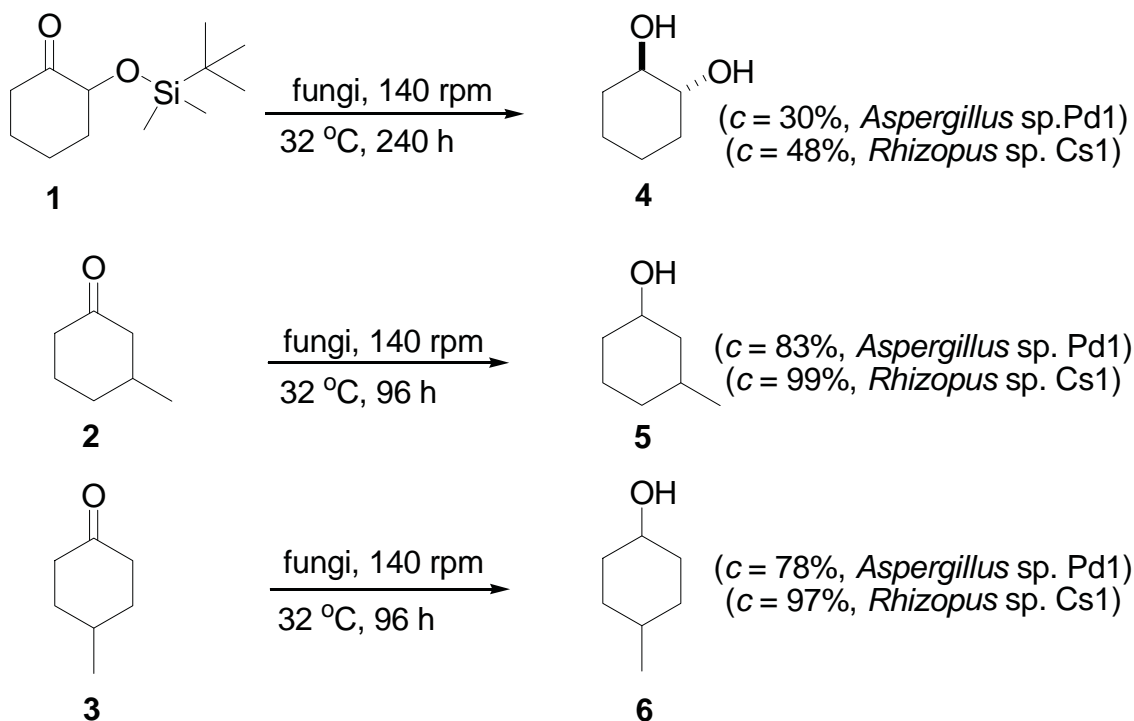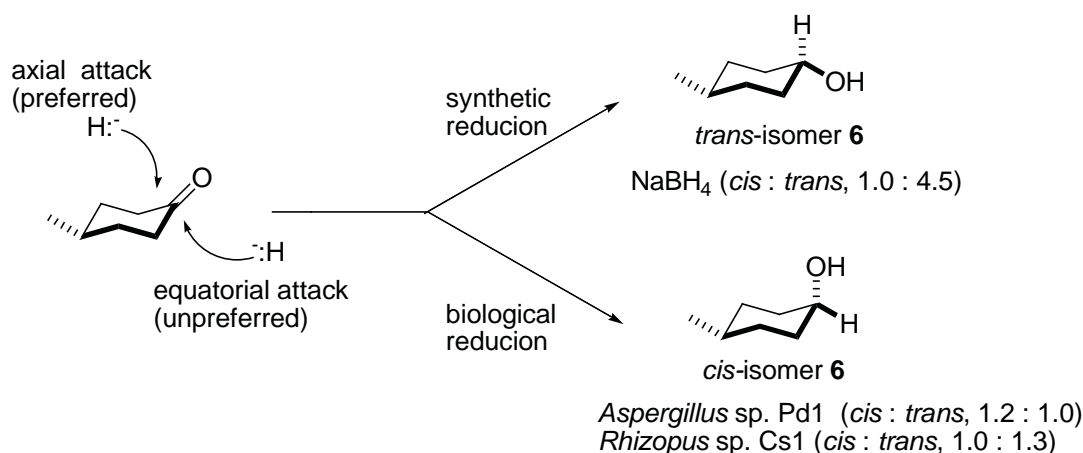

Figure 2 : Reduction of cyclohexanones **1-3** by *Aspergillus* sp. and *Rhizopus* sp. The conversions *(c)* were determined by GC-MS analyses

Figure 3 : Reduction of 4-methylcyclohexanone (**3**) by synthetic and biological methods. The ratio of *cis*- and *trans*-alcohol **6** was determined by GC-FID on a chiral chromatographic column

IV. CONCLUSION

According to the results reported here, the preliminary determination of growth curves of filamentous fungi was important to optimize the conditions for the biocatalytic reduction of cyclohexa-nones **1-3**. The addition of the substrates in the log phase of growth led to the biotransformation of cyclohexanones **1-3** to the corresponding cyclohexanols **4-6** in good yields. Therefore, during the exponential phase, enzymatic production was maximized. The bioreduction of 4-methylcyclohexanone (**3**) by *Aspergillus* sp. and *Rhizopus* sp. yielded a greaterpro-portion of *cis*-4-methylcyclohexanol (**6**), thethermody-namically and kinetically less stable isomer, thandid synthetic reduction. Finally, the bioreduction of 2- (*t*-butyldi-methylsilyloxy) cyclohexanone (**1**) by either fungus led to a higherproportion of *trans*-cyclohexane-1, 2-diol (**4**) than synthetic reduction by NaBH$_4$.

V. ACKNOWLEDGEMENTS

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