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Biotransformation of cyclohexanone using immobilized *Geotrichum candidum* NCYC49 Factors affecting the selectivity of the process

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Abstract

A screening of 416 microorganisms belonging to different taxonomical groups (bacteria, actinomycetes, yeasts and filamentous fungi) was performed looking for strains with high ketone monooxygenase activity. After that screening, the strain *Geotrichum candidum* NCYC49 was selected on the basis of the production of moderate yields of caprolactone from cyclohexanone, as well as the reproducibility of its culture conditions. Several strategies were followed in order to favor the monooxygenation reaction: growing cells, resting cells, lyophilized cells, and immobilization in different matrices (*Pterocladia* agar, calcium alginate, polyacrylamide). Interestingly, the results obtained by immobilization in polyacrylamide or in *Pterocladia* agar indicate that a complete separation of the reduction and monooxygenation activities can be achieved by controlling the oxygenation conditions. Thus, reactions carried out in high oxygenation conditions (high shaking speed or low matrix concentration) displayed a very high yield in caprolactone (90–100% conversion) without detectable production of cyclohexanol. On the contrary, reactions carried out under low oxygenation conditions clearly favored the production of cyclohexanol (reduction reaction), particularly when agar was used as a matrix.

Keywords: Geotrichum candidum; Ketone monooxygenation; Baeyer–Villiger oxidation; Whole cell immobilization

1. Introduction

The Baeyer–Villiger reaction is a well-known process that consists of the transformation of ketones into esters or lactones. The reaction is carried out using organic peroxides but low yields and poor stereoselectivities are generally observed [1]. The mechanism of this reaction proceeds in two steps. In the first step the organic peroxide attacks the carbonylic group giving the Criegee intermediate. In the second step, a rearrangement takes place to give the reaction products.

The enzyme-catalyzed Baeyer–Villiger reaction proceeds with better regioselectivity than the chemical process [2–5]. The enzyme is a NADPH dependent monooxygenase. The mechanism of the enzyme-catalyzed reaction is analogous to that of the chemical reaction. The preceding step is the hydride/proton transfer from $NADPH/H^+$ to FAD, giving $FADH_2$ [6]. This compound is the true coenzyme of the monooxygenase.

The interest in the reaction and the high regioselectivity observed in the enzyme catalyzed Baeyer–Villiger reaction are important reasons that justify the search for new microbial enzymes that could display even better activity and selectivity in this process. Several bacteria such as *Acinetobacter* sp. or *Pseudomonas putida* have been described as enzyme producers [7–13]. Also, several filamentous fungi from the *Curvularia* and *Drechslera* genera [14] have been described as catalysing this reaction. The presence of cofactors that must be regenerated and the high price of the isolated enzymes make the use of whole cells as the first

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choice when the scale up of the process is approached [15]. The main problem of the Baeyer–Villiger reaction catalyzed by whole cells is that the ester/lactone produced can be metabolized by the cells and so the product yield can dramatically decrease. Three main strategies have been described to solve this problem: (i) inhibition of cellular esterases using tetraethyl pyrophosphate (TEPP) or diethyl *p*-nitrophenylphosphate [16]; (ii) use of genetically modified strains without active esterases [17]; (iii) use of recombinant *E. coli* cells [18]. In the present paper we describe a strain of *Geotrichum candidum* that catalyses the monooxygenation and/or the reduction of cyclohexanone under controlled reaction conditions. *G. candidum* is a yeast-like filamentous fungus, that can simultaneously grow in myceliar form (filamentous fungi) and as unicellular units (yeast-like). This microorganism has traditionally been considered as a reference for reduction of both ketones and aldehydes [19].

2. Experimental

2.1. Chemicals

Cyclohexanone, cyclohexanol, ε-caprolactone, glucose and sodium alginate of low, medium and high viscosity were obtained from Sigma-Aldrich. Acrylamide, *bis*-acrylamide, and ammonium persulfate were provided by Bio-Rad. Agar from *Pterocladia* algae was gently donated by Hispanagar.

2.2. Microorganisms preservation

All the microorganisms used in this study were obtained from different public collections. Bacteria, actinomycetes, yeasts, filamentous and marine fungi were conserved as cell suspensions in a 30% glycerol solution. All these strains were stored at −80 °C in Nunc cryotubes. Basidiomycetes were also conserved as cell suspensions in 30% glycerol and stored at −150 °C in a liquid nitrogen tank.

2.3. Culture media

A preliminary screening was performed in order to select the most appropriate culture media for each type of microorganism. After that, the following culture media were selected for the different microbial groups: *Bacteria*: LB medium: Tryptone (Difco), 10 g/l; yeast extract (Difco), 5 g/l; NaCl (Merck), 5 g/l; KH_2PO_4 buffer, pH 6.5. *Actinomycetes*: ABME medium: $CaCO_3$ (Merck), 10 g/l; $FeSO_4{\cdot}7H_2O$ (Merck), 0.003 g/l; KCl (Merck), 0.5 g/l; $MgSO_4{\cdot}7H_2O$ (Merck), 0.5 g/l; meat extract (Oxoid), 5 g/l; malt extract (Difco), 40 g/l. *Yeasts*: yeast extract (Difco), 3 g/l; malt extract (Difco), 3 g/l; bactopeptone (Difco), 5 g/L; Bactodextrose (Merck), 10 g/l. *Basidiomycetes*: lyophilized potato, 22 g/L; Dextrose (Merck), 20 g/l. *Filamentous fungi*: HAGGS medium (adjust to pH 6.6): Glycine, 2 g/l; Tryptic soy broth, 6 g/l; starch, 20 g/l; mineral solution, 10 ml/l. Mineral solution: $FeSO_4{\cdot}7H_2O$, 1 g/l; $MnSO_4{\cdot}4H_2O$, 1 g/l; $CuCl_2$, 0.025 g/l; $CaCl_2$, 0.10 g/l; H_3BO_3, 0.056 g/l; $ZnSO_4{\cdot}7H_2O$, 0.2 g/l; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.019 g/l. *Marine fungi*: lyophilized potatoes, 22 g/l; Dextrose (Merck), 20 g/l, NaCl (Merck), 5 g/l.

2.4. Reaction methodologies

2.4.1. Method A: growing cells

2.4.1.1. Bacteria and yeasts. Conical flasks of 100 ml containing 20 ml of the selected culture media were inoculated with 50 μl of the microbial cell suspension in glycerol. Cultures were performed in an orbital shaker at 28 °C and 250 rpm [16,20]. After 48 h of incubation, the ketone used as substrate was added to the flasks at a 10 mM final concentration [20,21]. The reaction time was 72 h for all the microbial groups. When the reaction was finished, the content of the conical flask was transferred into a falcon tube and 5 ml of ethyl acetate (with 1 mg/ml of hexadecane as internal standard) were added. After vortexing for 10 s, the organic phase was transferred into a 2 ml Hewlett-Packard vial. All the reactions were repeated three times and the averaged results are displayed.

2.4.1.2. Other microorganisms. With the rest of microbial groups the screening process was essentially the same as described above. Culture and reaction times were 72 h for actinomycetes and filamentous fungi. For basidiomycetes and marine fungi the culture time was 120 h and the reaction time was 72 h.

2.4.2. Method B: resting cells

The culture conditions were the same as in the case of the growing cells. Once the culture time defined for each group of microorganisms was reached, the content of the conical flask was transferred into a falcon tube, and centrifuged during 15 min at 4000 rpm. Then the cells were recovered and washed for three times using 20 ml of 50 mM KH_2PO_4 buffer, pH 6.5. The cells were sedimented by centrifugation using the conditions previously described. When the cells were free of culture media they were resuspended in 20 ml of the same KH_2PO_4 buffer, in a 100 ml conical flask. Then, the substrate was added to the reaction media at a 5 mM final concentration. The flasks were shaken at 28 °C and 250 rpm in an orbital shaker (Khüner).

2.4.3. Method C: lyophilized cells

The experimental protocol followed in this case was similar to that used for the resting cells. After washing, the cells were quickly frozen at −80 °C and lyophilized for 72 h in a Lab-Conco lyophilizer. The reactions were performed using 100 mg of lyophilized cells in 5 ml of 50 mM KH_2PO_4 buffer, pH 6.5, and a 5 mM substrate concentration. Reactions were performed in non-reactive plastic flasks (pyrolysis type) at 28 °C with stirring at 100 rpm.

2.5. Immobilization

According to the published information [21–24], we decided to use the entrapment as immobilization method and six different polymers as matrix for the preliminary assays. These polymers were polyacrylamide, calcium alginate, agar, κ-carrageenan, polyvinyl alcohol and chitosan.

In order to select the most favorable immobilization conditions for each matrix we ran a factorial experiment. The design was performed using the Statgraphics Version 4 software package from Statistical Graph Corp. The analyzed variables (concentration and volume of matrix, volume of cell culture, shaking speed) were used as parameters for the statistical design, and served to achieve conclusions about the main properties of the immobilized derivatives: physical stability and mass transfer diffusion. The concentrations of the solutions were measured as % (w/v).

2.5.1. Preparation of cells

The *G. candidum* cells were cultured in 100 ml erlenmeyer flasks containing 20 ml of YM culture medium. Incubations were performed at 28 °C and 250 rpm during 48 h, using a Khüner orbital shaker. Afterwards the cells were centrifuged at 4500 rpm during 15 min using 50 ml Falcon tubes. The supernatant from the centrifugation was discarded and the pellet was washed using 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 6.5. The washing step was repeated twice.

G. candidum is a yeast-like filamentous fungi that could simultaneously grow under myceliar form (filamentous fungi) and as unicellular units (yeast like). Due to that, the quantification of the biomass was referred to the amount of cells produced per 100 ml flask containing 20 ml of culture medium.

2.5.2. Immobilization in polyacrylamide

The immobilization in polyacrylamide as matrix was performed using different concentrations of acrylamide/*bis*-acrylamide (2.5, 7.5 and 12.5%) [25].

Solution A: acrylamide 30% (w/v) and *bis*-acrylamide 0.8% (w/v) in milli-Q water; Solution B: 0.4% (w/v) Glucose in 1.5 M Tris–HCl buffer, pH 8.8; TEMED: *N*,*N*,*N*′,*N*′-tetramethyl-ethylen-diamine; 10% (w/v) ammonium persulfate solution in milli-Q water. All this solutions must be stored at 4 °C for their conservation. The ammonium persulfate solution must be daily prepared. Ammonium persulfate and TEMED were the initiator and the accelerator of the polymerization, respectively [26].

For the production of the gel used for the entrapment, the selected quantities of Solutions A and B were mixed with purified water according to the volumes shown in Table 1. Afterwards, the corresponding amounts of TEMED and ammonium persulfate were added. After mixing, the appropriate amount of *G. candidum* cells was added to the final mixture. The acrylamide-cell suspension was mixed and rapidly transferred to a flat plastic recipient. After 30 min a layer of solid polymer was formed. This solid was cut in cubic portions of 0.5 cm side.

Table 1
Experimental conditions for different polyacrylamide gel preparation

Acrylamide (%)	Solution A (ml)	Solution B (ml)	H_2O (ml)	Ammonium persulfate (μl)	TEMED (μl)
2.5	0.42	1.25	3.33	25	7
7.5	1.17	1.25	2.55	25	7
12.5	2.10	1.25	1.55	25	7

2.5.3. Immobilization in Pterocladia agar

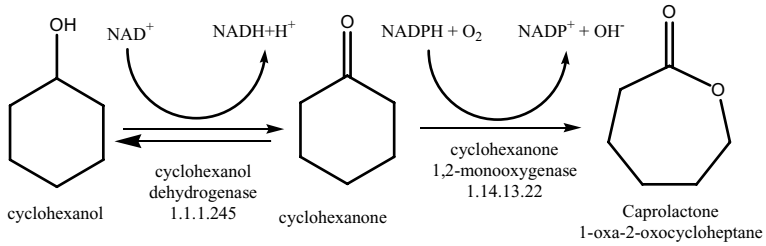
Agars from algae belonging to the genera *Gelidiella*, *Gelidium*, *Gracilaria* and *Pterocladia* have been tested in our research group. The *Pterocladia* agar has the highest degree of methoxylation, that leads to the lowest gellification temperature (39 °C) [27]. This special feature allows working at low temperatures, thus avoiding cell damage. The desired concentration of agar was prepared in 50 mM KH_2PO_4 buffer, pH 6.5, containing 5% glucose, and the mixture was sterilized for 15 min at 121 °C and 1 atm. Afterwards, the hot agar solution was introduced in a 45 °C thermostatic bath, and the temperature of the solution was monitored. When the solution reached 45 °C, the *G. candidum* cells were added with slow stirring. Then, the agar-cell suspension was poured on a plastic recipient. The mixture was allowed to solidify, thus obtaining a solid agar layer. The solid layer was cut in 0.5 cm blocks using a scalpel.

2.6. Reactions using immobilized biocatalysts

The small cubes obtained according to the immobilization conditions described above were placed into 100 ml erlenmeyer flasks containing 20 ml of 50 mM KH_2PO_4 buffer, pH 6.5. The reactions were started by adding cyclohexanone at a final concentration of 5 mM. The flasks were incubated at 28 °C and 100 rpm in an orbital shaker (Khüner). The reaction time was 72 h in all cases.

2.7. Gas chromatography analysis of the reactions

For the analysis of the samples we used a Hewlett-Packard 5890 Series II chromatograph with an automated sampler for 100 vials (Agilent Technologies). The electrolytic hydrogen generator was from Domnick Hunter (UHP-601). The GC column was a carbowax (Sugelabor SGL-1000, 60 m, 0.25 mm, 0.25 μm). According to the conditions proposed by Carnell and Willetts [14], we used the following analytical conditions for the automated screening: initial temperature, 155 °C; time, 1 min; final temperature, 175 °C; time, 10 min; rate, 4 °C/min; flow, 40 psi; split, 100 ml/min.



Scheme 1.

3. Results and discussion

3.1. Screening for cyclohexanone monooxygenation using growing cells

The search for new microorganisms able to perform cyclohexanone monooxygenation was carried out using a microbial library composed of 416 species that were selected looking for the highest biodiversity achievable. The microbial library was composed of 71 bacterial strains, 45 actinomycetes, 59 yeasts, 60 basidiomycetes, 33 marine fungi and 148 filamentous fungi. All the selected microorganisms have been described to be mesophilic, considering the requirements for their culture.

Cyclohexanone was selected as substrate for the automated screening procedure. All the reactions were repeated three times. The whole cell biotransformation of cyclohexanone [28] may lead to the formation of either cyclohexanol (reduction) [29–31] or ε-caprolactone (monooxygenation) [21], [32] as we show in Scheme 1.

In Table 2 we show that only 11 microorganisms were active in the process. From them, only six strains showed acceptable cyclohexanone monooxygenase activity (yield in lactone >10%). However, reduction of cyclohexanone was also observed in these cases. This result seems to indicate the presence of an active cyclohexanol dehydrogenase, which is more active than the cyclohexanone monooxygenase in some cases. In Table 3 we can also observe that *G. candidum* and the fungus *Hamigera striata* were the most active strains in the formation of ε-caprolactone. Nevertheless, the monooxygenation process was never the main reaction using growing cells. It is interesting to indicate that no bacteria were characterized as active strains during the screening.

Table 2
Groups and percentage of microorganisms from each microbial group that display activity in the monooxygenation of cyclohexanone

Microbial group	Initial number	Positives	Percentage referred to the initial number
Actinomycetes	45	0	0
Filamentous fungi	148	1	0.7
Bacteria	71	0	0
Marine fungi	33	0	0
Yeasts	59	10	17
Basidiomycetes	60	0	0

Table 3
Results on cyclohexanone monooxygenation: Microorganisms with yields higher than 10%

Group	Microorganisms	Reference	Monooxygenation yield (%)	Reduction yield (%)
Fungi	*G. candidum*	NCYC49	17	35
Fungi	*H. striata*	CBS 584.72	17	41
Yeast	*B. naardenensis*	NCYC 924	16	0
Yeast	*A. fermentans*	CBS 7830	15	20
Yeast	*P. fermentans*	NCYC 1657	14	16
Yeast	*S. ahearnii*	CBS 6121	13	6

The yeast *Brettanomyces naardenensis* did not show cyclohexanone reduction, but the reproducibility of the results was poor and we discarded this strain for further studies. The other strains, as the interesting yeasts *Saturnispora ahearnii* and *Pichia fermentans*, displayed lower selectivity and gave secondary products, corresponding to the degradation of cyclohexanone. The yeast *Arthroascus fermentans*, and the fungus *H. striata* did not display reproducible results and they were discarded. Therefore, *G. candidum* NCYC49 was the only selected candidate although the reduction of cyclohexanone was the main reaction in the screening conditions. This result is in agreement with previously published data [19]. In spite of this, we decided to explore this strain in order to improve its monooxygenase activity by means of modifications in the experimental conditions.

3.2. Resting and lyophilized cells

Firstly, the catalytic activity of the strain was assayed using cyclohexanone as substrate in different physiological conditions: growing cells, resting cells, and lyophilized cells. In Table 4, we can observe the yields measured for reduction and monooxygenation. Both activities were present both in

Table 4
Cyclohexanone biotransformation by *G. candidum* NCYC49 in different physiological conditions

Cell conditions	Cyclohexanone monooxygenation yield (%)	Cyclohexanone reduction yield (%)
Growing cells	17	36
Resting cells	7	23
Lyophilized cells	0	4

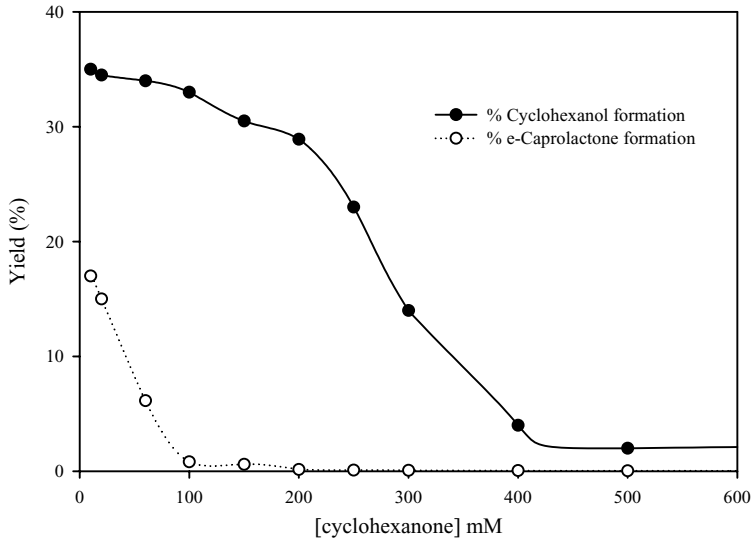


Fig. 1. Influence of the substrate concentration on the relative proportion of cyclohexanol and ε-caprolactone produced using *Geotrichum candidum* NCYC49 growing cells. Reaction conditions: $T = 28\,^{\circ}C$; stirring speed: 250 rpm (48 h of culture time); Reaction time: 72 h.

growing and in resting cells. The lyophilized cells did not present remarkable yields.

We can observe that the selectivity versus the monooxygenation was not clearly improved in any of the conditions tested. All the three conditions favored the reduction reaction versus the monooxygenation. The difference observed between the yields obtained with growing and resting cells and those obtained with lyophilized cells could be due to the fact that the diffusion of the oxygen is reduced in the last conditions, thus drastically affecting the reaction yield.

3.3. Influence of the substrate concentration on the monooxygenase activity of G. candidum growing cells

In Fig. 1 we can see that with growing cells the lower the concentration of cyclohexanone the higher the monooxygenation activity. At cyclohexanone concentrations higher than 100 mM, the reduction of cyclohexanone is the only reaction that takes place. Therefore, it seems that the substrate is more toxic for the cyclohexanone monooxygenase responsible of the Baeyer–Villiger process than for the alcohol dehydrogenases that catalyzes the reduction reaction. The tolerance of *G. candidum* against the cyclohexanone is much higher than the tolerance described in the literature for the fungus *Fusarium oxysporum* [33].

3.4. Kinetic profile of the biotransformation of cyclohexanone by G. candidum as whole cell biocatalyst

From the kinetic profile of the reaction (Fig. 2) we can observe that the reduction is the predominant reaction during the first 72 h. After that time, the monooxygenase activity substantially increases and most of the produced cyclohexanol and the remaining cyclohexanone are transformed into lactone. These reaction profiles indicate that cyclohexanol product is part of an equilibrium whereas the lactone is the final product of the Baeyer–Villiger irreversible oxidation [34]. The equilibrium between cyclohexanone and cyclohexanol is catalyzed by an NADPH-dependent alcohol dehydrogenase. In addition to that, it is well known that monooxygenases are usually part of a degradative pathway that allows the microorganisms to utilize non-carbohydrate compounds as sources of carbon and energy [11,35]. Therefore, when the energy of the cell (related to NADPH) is reduced till near zero, cyclohexanol can be oxidized to cyclohexanone producing NADPH as subproduct. Then, the cyclohexanone can be oxidized to lactone, according to Fig. 2.

3.5. Reaction with immobilized cells

The main objective of this study was to obtain an efficient system for the monooxygenation of the cyclohexanone. Since the reaction performed with growing cells produced both monooxygenation and reduction, we tried to improve the selectivity of the process by testing the reaction in the presence of immobilized *G. candidum* cells. The microorganism was immobilized by trapping in different polymers. Among the tested conditions, no remarkable results were obtained in the preliminary assays using polyvinyl alcohol, κ-carrageenan, calcium or barium alginate and chitosan as matrix.

3.5.1. Cells immobilized in polyacrylamide

We have used the factorial design of experiments to try to improve the selectivity of the monooxygenation process. Three main variables were considered: Xa = acrylamide (%); Xb = biomass used (measured as number of cell pellets obtained after centrifugation of 20 ml of culture); Xc = immobilization volume (ml). The experimental design was

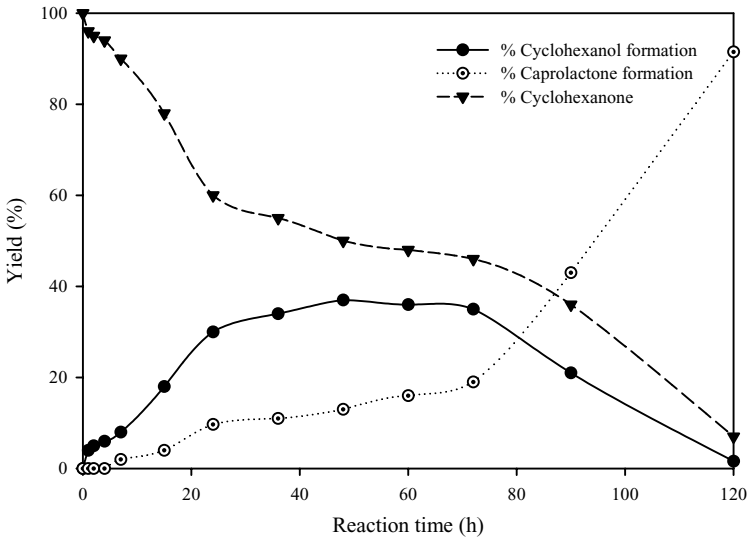


Fig. 2. Kinetic profile of the cyclohexanone biotransformation using growing cells of *Geotrichum candidum* NCYC 49. Reaction conditions: $T = 28\,^{\circ}C$; stirring speed: 250 rpm (48 h of culture); Reaction time: 72 h. The substrate concentration was 10 mM.

done with a reduced matrix of three variables (n) with 2^{n-1} experiments. The maximum (+) and minimum(−) values and the center points (0) are shown in Table 5. In the same table we show the different assay conditions (in parentheses the value + or − or 0) carried out with a polyacrylamide matrix. I-1 and I-6 were the center points. The experiments were performed with a cyclohexanone concentration of 5 mM in order to favor the monooxygenation versus the reduction of the ketone (Fig. 2) and the reaction time was 72 h, in order to improve the production of lactone (Fig. 3).

The results shown in Table 5 reveal an interesting correlation between the acrylamide concentration within the matrix and the ability to preferentially produce reduction or monooxygenation. Thus, the assays I-4 and I-5, performed in 2.5% acrylamide, led to a 99% yield in the formation of lactone without cyclohexanol formation. The assays I-1 and I-6 that were performed in 7.5% acrylamide displayed a high yield in ε-caprolactone but also an important production of cyclohexanol. Finally, the assays I-2 and I-3 that were performed in 12.5% acrylamide, displayed a small production of ε-caprolactone and most of the cyclohexanone substrate was transformed into cyclohexanol. From this experiment we can clearly deduce that the amount of cyclohexanol increases with the percentage of acrylamide within the matrix. By the contrary, the amount of ε-caprolactone decreases as we increase the amount of acrylamide. The same is observed in Fig. 3, where we show additional data using other acrylamide concentrations. It seems that the presence of high concentrations of acrylamide could lead to a polymer that presents diffusion problems and this might affect the oxygen diffusion. This effect would favor the monooxygenation reaction at low acrylamide concentrations, and, by the contrary, the reduction reaction at high acrylamide concentrations. The other variables considered in the factorial design do not seem to have a significant impact on the reaction yield, according to the data displayed in Table 5.

After performing the factorial experiments we can conclude that polyacrylamide is a very interesting immobilization matrix for *G. candidum* and that the concentration of acrylamide is the factor that determines the predominant activity (reduction or monooxygenation). The use of 2.5% acrylamide leads to impressively high yields in the monooxygenation activity. Thus, the use of immobilized cells in low acrylamide concentration allows to a selective

Table 5
Factorial design for the immobilization of *G. candidum* using polyacrylamide as matrix

Assay	Experimental design variables			Reaction yield (%)	
	Xa Acrylamide (%)	Xb biomass[a]	Xc gel volume (ml)	Cyclohexanol	ε-caprolactone
I-1	7.5 (0)	2 (0)	10 (0)	36	57
I-2	12.5 (+)	1 (−)	5 (−)	71	22
I-3	12.5 (+)	3 (+)	15 (+)	10	4
I-4	2.5 (−)	3 (+)	5 (−)	0	99
I-5	2.5 (−)	1 (−)	15 (+)	0	99
I-6	7.5 (0)	2 (0)	10 (0)	34	51

Cyclohexanone: 5 mM; reaction time: 72 h; temperature: 28 °C; shaking speed: 100 rpm.
[a] Number of cell pellets. Each pellet was obtained after centrifugation of 20 ml of cell culture (48 h culture time).

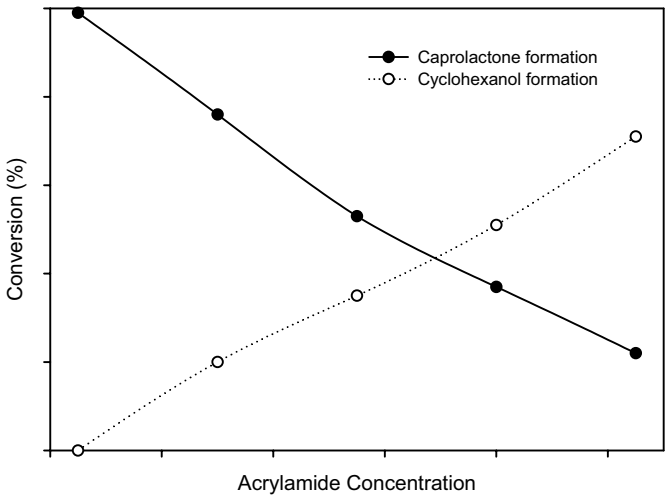


Fig. 3. Influence of the acrylamide concentration on both the monooxygenation and the reduction activities.

Table 6
Cyclohexanone monooxygenation using immobilized derivatives of *G. candidum* NCYC49 using *Pterocladia* agar as matrix

Assay	Experimental design variables			Yield (%)		
	Xa agar (%)	Xb biomass volume	Xc agar volume	Xc shaking speed (rpm)	Yield cyclohexanol (%)	Yield ϵ-caprolactone (%)
I-1	2.5 (0)	2 (0)	10 (0)	175 (0)	0	92
I-2	3.75 (+)	3 (+)	15 (+)	100 (−)	39	0
I-3	3.75 (+)	1 (−)	15 (+)	250 (+)	0	11
I-4	1.25 (−)	3 (+)	5 (−)	250 (+)	0	42
I-5	1.25 (−)	1 (−)	15 (+)	100 (−)	33	0
I-6	1.25 (−)	1 (−)	5 (−)	100 (−)	35	0
I-7	1.25 (−)	3 (+)	15 (+)	250 (+)	0	84
I-8	3.75 (+)	1 (−)	5 (−)	250 (+)	0	75
I-9	3.75 (+)	3 (+)	5 (−)	100 (−)	39	1
I-10	2.5 (0)	2 (0)	10 (0)	175 (0)	0	91

Cyclohexanone: 5 mM; reaction time: 72 h; temperature: 28 °C, 100 rpm.

monooxygenation of the cyclohexanone, with practical absence of the reduction reaction.

3.5.2. Cells immobilized in Pterocladia agar

The *G. candidum* cells were also immobilized in agar from different species of algae, following the process described above. Among the materials tested the most reproducible results were obtained with the agar from *Pterocladia*. The results obtained in the experimental design are shown in Table 6. Four variables ($n = 4$) were used and an experimental matrix of 2^{n-1} was considered.

According to the data displayed in Table 6, we can observe that the reactions that were performed at low shaking speed (100 rpm) produced moderate levels of cyclohexanol (reduction reaction) and a total absence of ε-caprolactone (monooxygenation). By the contrary, the reactions performed at higher speed (175 and 250 rpm) produced high levels of ε-caprolactone and a total absence of cyclohexanol. Thus, we can conclude that the shaking speed is an essential parameter that controls the type of cyclohexanone transformation (reduction or monooxygenation). As in the previous case, it seems that there is a correlation between the level of oxygenation produced within the reaction flask and the type of transformation. In the case of low shaking speed, where the oxygen transfer is small, the favored reaction is the reduction. However, in the case of higher shaking speeds, the oxygen transfer is better and the main reaction is the monooxygenation. These results clearly indicate the main role of oxygen diffusion in the selectivity of the reaction process.

4. Conclusions

The use of growing cells of *G. candidum* NCYC49 for cyclohexanone biotransformation produces a mixture of cyclohexanol and ε-caprolactone (reduction and monooxygenation reactions). However, the use of cells immobilized either in polyacrylamide or in agar from *Pterocladia* allows for a full control of the cyclohexanone biotransformation. Cells immobilized in 2.5% polyacrylamide constitute a specific biocatalyst for the Baeyer–Villiger reaction, and

specifically produce ε-caprolactone. When the immobilization is performed in *Pterocladia* agar the biocatalyst can act both as selective for the reduction to cyclohexanol, if the reaction is performed at low shaking speed (100 rpm), or as selective for the Baeyer–Villiger reaction if the reaction is performed at high shaking speed (250 rpm). This effect could be due to the differences in the access to oxygen for the immobilized derivatives of low and high concentration, and also for the reactions at low and high shaking speed.

In summary, the immobilization of the microorganism in both synthetic and natural matrix and the modification of particular reaction conditions allow us to get a clean and selective process for either Baeyer–Villiger oxidation or reduction of ketone to alcohol.

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