Monascus kaoliang CBS 302.78 immobilized in tailor-made agars as catalyst for reduction of ketones: On the quest for a green biocatalyst

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ABSTRACT

After a hierarchical screening Monascus kaoliang CBS 302.78 was selected due to its high activity in the reduction of cyclic ketones. Several tailor made agars and agaroses were used in the immobilization of the fungus. The best-immobilized biocatalyst was obtained after immobilization in low methoxylated agar (A27/03) at 2.5% (w/v). The biocatalyst shape was optimized as microplates (1.27 cm x 1.27 cm x 0.31 cm). The observable modulus for external mass transfer, \( \Omega \ll 1 \), indicates the absence of external mass transfer phenomena. The internal effectiveness factor \( \eta < 1 \) and the high value of the Thiele module (\( \phi > 3 \)) indicates that there is an intra-particle mass transfer restriction according to the Weisz criteria. As consequence, the kinetics of the reduction of ketones follows a formal first order kinetic model. The catalysts deactivation follows a simple exponential decay expression. Due to the renewal characteristics of the agar, the biocatalysts can be considered as green catalysts.

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1. Introduction

The filamentous fungi Monascus kaoliang has been widely used in the orient for food coloring [1] and medical therapy [2,3]. Recently, M. kaoliang CBS 302.78 was characterized by our group as an active strain for the reduction of ketones [4] but it showed many problems in the culture and handling.

The stereoselective reduction of ketones has been described with yeasts [5,6] or bacteria [7]. Nevertheless, the oxidoreductases from filamentous fungi have not been involved in industrial organic synthesis or marketed. These cells contain a variety of enzymes with alcohol dehydrogenase activity that is able to accept non-natural substrates. In addition, the cell contains all the necessary cofactors and the metabolic pathways for their regeneration. Therefore, the cofactor regeneration can be omitted since it is automatically done by the cell [8,9] and so, immobilized whole cells are used in order to achieve a reusable biocatalyst [10,11]. Bacteria and yeasts have been entrapped in alginate, agarose or agar beads [12–15] obtaining very active and reusable biocatalysts. Mycelium forming microorganisms such as filamentous fungi, present problems for immobilization due to their amorphous growing shape, but these problems are not insurmountable. The entrapment of filamentous fungi has been performed in several different polymers, i.e. polyurethane foams [16], alginate gels [17,18], CM-cellulose [18], polyvinyl alcohol [19], agar and agarose [20].

In the present paper we describe a new optimum growing condition and an easy procedure for the immobilization of M. kaoliang CBS 302.78 in agar or in agarose using two different methodologies in order to obtain green catalysts. Different tailor-made agars and agaroses—from different specific algae genera showing different gelling temperatures were tested. The immobilized catalysts were active in the reduction of cyclohexanone as reaction test.

2. Materials and methods

2.1. Microbial strains and chemicals

M. kaoliang was obtained from the Fungal Biodiversity Centre, Utrecht, The Netherlands (CBS). All the substrates were obtained from Sigma–Aldrich. The culture media components were supplied by Difco and Merck.

2.2. Growing conditions

M. kaoliang was cultured in HAGGS medium: (adjusted to pH 6.5) glycine 2 g/l; tryptic soy broth 6 g/l; starch 20 g/l; trace element solution ml/l. Trace element solution: FeSO4·7H2O 1 g/l; MnSO4·4H2O 1 g/l; CuCl2 0.025 g/l; CaCl2 0.10 g/l; H2BO3 0.056 g/l; ZnSO4·7H2O 0.2 g/l; (NH4)6Mo7O24·4H2O 0.019 g/l.
Conical flasks of 250 ml containing 50 ml of the selected culture media were inoculated with fresh microbial cells of *M. kaoliang* to determine the time of suitable growth. Culture was performed in an orbital shaker (Adolf Kühner AG, Switzerland) at 28 °C and 200 rpm. Samples were picked up at different culture times. After 2, 3, 5, 7, 9 or 11 days, the content of the conical flasks was transferred into a falcon tube, and centrifuged during 15 min at 4000 rpm. The cells were washed three times using 50 ml of distilled water and deposited by centrifugation using the conditions previously described. The experimental method used to evaluate the fungal cell density was the measurement of the dry weight, obtained after incubation at 80 °C.

2.3. Immobilization of whole cells

Three tailor made agars (HISPANAGAR S.A, Burgos, Spain) from different algae genera were used for the immobilization: (i) highly methoxylated agar A28/03 from *Gracilaria* (melting point 40 ± 2 °C); (ii) medium methoxylated agar A-90 from *Gelidium* (mp 36 ± 2 °C); (iii) low methoxylated agar A27/03 from *Pterocladia* (mp 34 ± 2 °C). Finally one agarose D5 (lot: F-5912) was tested, too.

(a) Immobilization in coated spheres of agar and agarose: *M. kaoliang* was cultured in liquid medium for 7 days, at 200 rpm. The red spheroids of biomass were filtered and introduced in the sterilized suspension of agar or agarose at 45 °C. Then, the coated spheroids particles were introduced in sunflower oil at 25 °C in order to solidify the thermoglul around the fungal particles. These particles were then washed in cool H2O to remove the excess of sunflower oil and stored at 4 °C till use.

(b) Immobilization in small plates of agar: This process was tested using the different matrices achieving the best results with the special *Pterocladia* agar A27/03 of low methoxylatiation degree at the lowest gelling temperature. This special feature allows working at low temperatures avoiding enzyme denaturation and cell damage. Agar solutions – different concentrations were prepared in 50 mM KH2PO4/K2HPO4 buffer (pH 6.5), and the mixture was sterilized for 15 min at 121 °C and 1 atm. Then, the hot agar solution was introduced in a 45 °C thermostatic bath, monitoring the temperature of the solution. As soon as the solution solidifies at 45 °C, *M. kaoliang* biomass (cultured for 7 days at 28 °C and 200 rpm) were added with slow stirring. Subsequently the agar cells suspension was poured on to a plastic recipient. The mixture was allowed to solidify, thus obtaining a solid agar layer. The solid layer was cut into regular blocks (1.27 cm × 1.27 cm × 0.31 cm) using a scalpel and stored at 4 °C till use.

2.4. Reduction of cyclohexanone using resting cells

Conical flasks of 250 ml containing 50 ml of the selected culture media were inoculated with *M. kaoliang* biomass. Cultures were performed in an orbital shaker at 28 °C and 200 rpm. After 7 days of incubation, the content of the conical flask was transferred into a falcon tube, and centrifuged during 15 min at 4000 rpm. The supernatant from the centrifugation was discarded and the cells were recovered and washed for three times using 50 mM KH2PO4/K2HPO4 buffer, pH 6.5. When the cells were free of culture media they were re-suspended in 50 ml of the same buffer in a 250 ml conical flask. The reaction was started by adding the reaction media cyclohexanone until 10 mM final concentration as well as glucose 0.5% as auxiliary substrate. The flask was shaken at 28 °C and 200 rpm in an orbital shaker. Samples were picked up (1 ml) at different reaction times and 2 ml of ethyl acetate were added. After vortexing for 10 s, the organic phase was transferred into a 2 ml Hewlett-Packard GC vial.

2.5. Reduction of ketones using immobilized whole cells

The small parallelepiped blocks (1.27 cm × 1.27 cm × 0.31 cm) were introduced in a 250 ml erlenmeyer flask containing 100 ml of 50 mM KH2PO4/K2HPO4 buffer, pH 6.5. The reduction of ketones using coated spheres of fungus were performed in the same conditions as described for resting cells.

2.6. Gas chromatography analysis

The analysis of the samples were performed using a Varian 3400 CX gas chromatograph equipped with an auto-sampler and a DMetPeBETA-P5086 capillary column (25 m, 0.25 mm, 0.25 μm). Calculation of the yield was performed by dividing the peak area of product by that corresponding to the standard extraction value of the substrate in the same conditions. Gas chromatographic analysis of the reduction of the different substrates was performed using a column SGL-1000 (60 m × 0.25 mm and 0.25 μm) (Sugelabor, Spain) and the following analytical parameters. The reduction of carvones was followed by chiral GC as previously reported [21].

2.7. Toxicity assays

The toxicity of cyclohexanone – as model substrate – was analyzed adding different concentrations of cyclohexanone (5, 10, 30, 50, 100, 250 and 500 mM) to the culture medium in the reactions conditions described above. The yield in cyclohexanol was followed by GC as previously described.

2.8. Diffusivity experiments

The diffusion assays were performed with agar A27/03 plates (2.5% and 3.75%, pH v) without cells, using cyclohexanone and cyclohexanol. The preparation of small plates was the same as described (Section 2.3.b). The decreasing concentration of cyclohexanone and cyclohexanol in the medium – due to diffusion into the agar gel – was followed by GC. The diffusion curves obtained were fitted to a single exponential decay.

2.9. Deactivation experiments

It is well known that biocatalyst activity decreases when reused many times. Frequently, a first order model is adequate to represent enzyme deactivation kinetics. However, the real mechanism of whole cells biocatalyst deactivation is difficult to know due to its complexity, as the complete metabolism of the microorganism is potentially involved.

In this work, the biocatalyst deactivation was performed with *M. kaoliang* biomass immobilized in agar A27/03 at 2.5% (w/v) in the cyclohexanone reduction. The catalyst was used immobilized as cubic agar blocks. The increase in concentration of cyclohexanol was followed by GC as described before. At the end of the reaction (1st cycle) the small blocks were filtered and placed into a new 250 ml erlenmeyer flask containing 100 ml of 50 mM KH2PO4/K2HPO4 buffer, pH 6.5 in order to start a new reaction in the same conditions. The same method was followed until biocatalyst deactivation during “n” cycles. The maximum activity in each cycle was calculated. At the end of the assay, a plot showing a/b0 (a0 catalytic activity in the first cycle, a catalytic activity in the cycle “n”), in Y-axis and the total reaction time in the X-axis to calculate the biocatalyst deactivation constant.

2.10. Kinetic runs

The reduction of cyclohexanone was monitored. The yield (%) was calculated according to the expression: product area/substrate area × product area. The initial reaction rate “V0” and kinetic constant “k” was determined using SIMFIT (W.G. Bardsley, University of Manchester).

3. Results and discussion

3.1. Catalytic activity in the reduction of ketones

*M. kaoliang* was grown in the experimental conditions described above. The rinsed biomass, after several days of culture, was dried at 120 °C and the dry cell weight was determined in each case.

Fig. 1 shows that the stationary phase is achieved after 7 days. The maximum biomass growing rate after 3 days approximately (dry biomass (g)/t (days)). In order to determine when the intracellular enzymes are in the highest concentration and/or activity, cyclohexanone reduction was performed using whole cells harvested at different culture times. In Fig. 2 we compare the cyclohexanol production (%) using the mycelium harvested at 3
days or at 7 days culture time. The fungus cells harvested after 3 days need longer reaction time (>72 h) to achieve the maximum yield (>90%), than when harvested after 7 days (>24 h; 92%). These data qualitatively indicate to us that the maximum ADH activity is achieved after 7 days culture time. As consequence, we selected 7 days as the optimum culture time for this fungus.

*M. kaoliang* mycelium was assayed in different physiological conditions – growing cells (fermentor conditions), resting cells and lyophilized mycelium – to determine if the physiological state of the microorganism may affect the reduction process as described with other fungi [4,21]. The lyophilized mycelium carries to a low activity (36% maximum yield to obtain cyclohexanol, 72 h).

From Fig. 3 it may be concluded that the fungi achieved the same yield (%) in cyclohexanol in both conditions–growing or resting cells. Nevertheless, resting cells conditions are more effective, giving the maximum yield at lower reaction time (≈12 h) than using growing cell (≈24 h). This behaviour may be due to the absence of secondary reactions and biomass generation in the non-growing conditions. Non-growing cell (resting cells) conditions have been previously recommended by us and other authors [4,19,21]. The catalytic activity in the reduction of cyclohexanone using recycled free mycelium (resting cells conditions) was tested (Fig. 4). From these results, we deduce that the mycelium can be reused only twice at near 100% activity. After seven cycles, 50% of the initial maximum yield is achieved.

Therefore, seven cycles can be considered as the half-life of the free mycelium (resting cells conditions). In addition the mycelium is difficult to handle and to separate from the reaction products and/ or substrates. Consequently, some immobilization methodologies were performed to improve the possibilities of this biocatalyst beyond the properties of the free cells.

Finally, using the batch fermenting biomass – after 7 days – different concentrations of cyclohexanone were added to evaluate the toxicity of the substrate. In this way, we evaluate the maximum yield (%) achieved after 72 h (contact time cyclohexanone-resting cells). Concentrations of cyclohexanone greater than 100 mM, dramatically reduce the yield in cyclohexanol achieved at 72 h (<15%). Contrarily, concentrations lower than 25 mM are excellent substrate concentrations to obtain maximum yields and productivities. These values are higher than those traditionally described by other authors using other fungi (<20 mM) [4].

### 3.2. Immobilization of *Monascus kaoliang* cells in coated spheres

The first immobilization of *M. kaoliang* was performed by means of immersion of the spheres of the fungus in suspensions of tailor-made agars or agaroses from HISPANAGAR S.A. These coated spheres produced were directly used in the reduction of different ketones (Table 1).

The reduction of cyclohexanone only gives cyclohexanol and similar yields were obtained with free mycelium (resting cells conditions) and with coated spheres in agar. There is not influence of the methoxylation degree of agar because the same yields in cyclohexanol were achieved using high (A28/03); medium (A-90)

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Cyclohexanone reduction yield (%)</th>
<th>4S-Carvone reduction yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting cells conditions</td>
<td>90</td>
<td>83</td>
</tr>
<tr>
<td>Immobilized in agar A28/03</td>
<td>100</td>
<td>48</td>
</tr>
<tr>
<td>Immobilized in agar A27/03</td>
<td>100</td>
<td>46</td>
</tr>
<tr>
<td>Immobilized in agar A-90</td>
<td>100</td>
<td>46</td>
</tr>
<tr>
<td>Immobilized in agarose D5</td>
<td>74</td>
<td>25</td>
</tr>
</tbody>
</table>

[1] = 2.5 mM; T = 28 °C, 250 rpm, 72 h contact time.

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**Table 1**

Yield (%) in the reduction of different ketones using *M. kaoliang* immobilized in coated spheres, prepared with different thermogels at 2.5% (w/v)

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**Fig. 2.** Kinetic profile of cyclohexanone reduction using *Monascus kaoliang* batch fermenting cells at different culture times (3 days or 7 days, 28°C, 200 rpm). [cyclohexanone] = 10 mM.

**Fig. 3.** Kinetic profile of the cyclohexanone reduction using *Monascus kaoliang* batch fermenting and resting cells. [cyclohexanone] = 10 mM. Culture conditions: 7 days at 28°C and 200 rpm.

**Fig. 4.** Kinetic profile of the cyclohexanone reduction using *Monascus kaoliang* resting cells in different cycles. [cyclohexanone] = 10 mM. Culture conditions: 7 days at 28°C and 200 rpm.
or low methoxylation degree (A27/03) agars to make the coated spheres. Finally, low yield was achieved with agarose D5 (lot F-5912). A27/03 was selected due to its low cost.

Using coated spheres or free mycelium (resting cell conditions), the reduction of 4S-carvone gave a mixture of products with 28,4S-carveol as major product (Table 1). The yields achieved with 4S-carvone as substrate were lower than those achieved with cyclohexanone. This result must be associated to the higher log \( P \) of 4S-carvone (log \( P = 2.55 \)) compared to that of cyclohexanone (log \( P = 1.53 \)) that restricts the diffusion of the hydrophobic molecule through the hydrophilic matrixes.

The immobilized biocatalysts gave lower yields in the reduction of 4S-carvone than resting cells. The low yield observed with the mycelium immobilized in agarose must be related to the more rigid structure of agarose-gel compared to agar-gel structure. This fact will produce a strong mass transfer restriction in the agarose-biocatalyst. In Section 3.5, we show that the intra-particle mass diffusion is the rate-controlling step as previously described by our group for other agar biocatalysts [22]. In addition, this immobilization methodology presents two main problems: (i) the biocatalysts are not homogeneous because the obtained biomass spheres have not similar sizes (0.5 cm < diameter < 1.5 cm). Therefore, it is very difficult to control the reagents and/or products diffusion and so, the biocatalyst behaviour is not reproducible. (ii) In addition the mechanic properties of the coated spheres are also very poor.

### 3.3. Immobilization of Monascus kaoliang cells in plates

In order to overcome these problems the immobilization of the fungus was performed in plates of agar or of agarose. This immobilization was possible when the fungus was cultured at moderated stirring speed (200 rpm) producing small red spheres of biomass (mean diameter ≤0.5 cm). The red spheres of biomass were filtered and rinsed with phosphate buffer and added to a sterilized solution of agar or agarose (\( T = 45 \) °C) in a plastic recipient. The mixture was allowed to solidify, obtaining a solid agar layer. The solid layer was cut into blocks (1.27 cm × 1.27 cm × 0.31 cm) using a scalpel and stored at 4 °C till use.

The immobilized biocatalysts were used in the reduction of cyclohexanone as the reaction test. The best results were obtained with the plates of agar Ref A27/03. Similar maximum yields in cyclohexanol were obtained with suspensions with 2.5% or 3.75% of agar. Lower percentages of agar than 2.5% gave soft plates and higher than 3.75% gave rigid plates with high diffusion restrictions. The addition of glucose (0.5%, w/v) – NADH regenerator – was positive for the catalytic activity. This percentage was previously optimized using a gradient concentration of saccharose or of glucose 0.1–1.0%. This positive effect should be associated to NADH optimized using a gradient concentration of saccharose or of sucrose. The reaction product (one to two mmols) is obtained with suspensions with 2.5% or 3.75% cyclohexanol were obtained with suspensions with 2.5% or 3.75% cyclohexanone as the reaction test. The best results were obtained with the plates of agar Ref A27/03.

#### 3.4. Catalyst deactivation

The deactivation curves (Fig. 5) – using the reduction of cyclohexanone and M. kaoliang, immobilized in agar A27/03 plates – were fitted to a simple exponential decay curve Eq. (1):

\[
\text{Act} = A \exp(-k_d t) + C
\]

Activity (Act) was measured as the ratio between the maximum yield achieved in the cycle “n” (\( a \)) and the yield in cyclohexanol obtained in the first cycle (\( a_0 \)). The results are summarized in Table 2. Remarkably, the apparent deactivation constant (\( k_d \)) is lower in the immobilized biocatalysts compared to free cells, indicating the stabilization achieved after the immobilization that increases the half-life of the immobilized biocatalysts. Nevertheless, the immobilized biocatalysts carry to lower yields at long reaction times indicating the presence of diffusion problems as described in the following sections. Finally, the high proportion of water in the agar catalyst oblige to us to add near 64 g of wet biocatalyst in order to have a similar amount of biomass than in the case of free mycelium catalyzed reactions. As consequence, the productivity dramatically decreases (Table 2).

#### 3.5. Kinetic runs

The kinetic runs were fitted to different kinetic models. The immobilized fungal cells in agar 27/03 showed an “induction time” (\( t_{\text{ind}} \)). The induction time is the gap observed between the initial reaction time and the time when the reaction product to be detected in the reaction medium. This gap between these moments (\( t_{\text{ind}} \)) is related to reaction product diffusion restrictions by the hydrophilic matrix of agar. Indeed, the greater the percentage of agar (polysaccharide) the longer the delay-time (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Half life (h/cycles)</th>
<th>( k_d ) (h(^{-1}))</th>
<th>Yield (%)</th>
<th>( t_{\text{max}} ) (h)</th>
<th>( W_{\text{cat}} ) (g)</th>
<th>Prod. ( \times 10^2 ) mmol/g</th>
<th>Cat. \</th>
<th>( \times 10^2 ) mmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free mycelium</td>
<td>119/9/6</td>
<td>8.0 \times 10^{-3}</td>
<td>92</td>
<td>20</td>
<td>6.9</td>
<td>5.90</td>
<td>Free</td>
<td>cat (g)</td>
</tr>
<tr>
<td>Immob. in agar</td>
<td>703/8/7</td>
<td>1.0 \times 10^{-3}</td>
<td>84</td>
<td>113</td>
<td>64.1</td>
<td>0.12</td>
<td>Immob.</td>
<td>cat (g)</td>
</tr>
<tr>
<td>2.5%</td>
<td>Immob. in agar</td>
<td>553/5/7</td>
<td>1.5 \times 10^{-3}</td>
<td>79</td>
<td>63.2</td>
<td>0.13</td>
<td>Immob.</td>
<td>cat (g)</td>
</tr>
<tr>
<td>3.75%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reduction of cyclohexanone to cyclohexanol. \( T = 28 \) °C, glucose 0.5% (w/v). 
\( V = 100 \) ml of phosphate buffer (pH 6.5), 200 rpm.

\( ^a \) Maximum yield in cyclohexanol in the first cycle.

\( ^b \) Reaction time where the maximum yield in cyclohexanol is achieved (first cycle).

\( ^c \) Mass transfer control in the process. The immobilization of the small red spheres of \( M. \) kaoliang in agar plates is justified because the spheres are very difficult to handle in a bath reactor and completely useless to be use in flux reactor type.

### Table 3

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>( W_{\text{cat}} ) (g)</th>
<th>( t_{\text{ind}} ) (h)</th>
<th>( r_0 ) (mM/h)</th>
<th>( k ) (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cells</td>
<td>6.9</td>
<td>0</td>
<td>2.22</td>
<td>0.247</td>
</tr>
<tr>
<td>Agar A27/03 2.5%</td>
<td>64.1</td>
<td>2</td>
<td>0.39</td>
<td>0.047</td>
</tr>
<tr>
<td>Agar A27/03 3.75%</td>
<td>63.2</td>
<td>4</td>
<td>0.44</td>
<td>0.056</td>
</tr>
</tbody>
</table>

\( M. \) kaoliang: free mycelium cells and immobilized cells in agar plates 27/03, cyclohexanone = 10 mM; \( T = 28 \) °C, 200 rpm, glucose 0.5% (w/v).

\( ^a \) \( t_{\text{ind}} \) = induction time.
In order to determine the kinetic order experiments with different cyclohexanone concentrations (between 5 and 10 mM) were performed. The initial reaction rate was calculated at each cyclohexanone concentration and the reaction order, \(n\), was experimentally determined using the following equation:

\[
N = \frac{\ln(r_1/r_2)}{\ln[S_1]/S_2}
\]  

(2)

\(r_1\) is the initial reaction rate at \([\text{cyclohexanone}] = S_1\); \(r_2\) is the initial reaction rate at \([\text{cyclohexanone}] = S_2\).

The mean value calculated was \(n = 1.06 \pm 0.05\). Therefore, the reduction of cyclohexanone catalyzed by free mycelium is a pseudo-first order kinetic process.

In the case of agar plates, the kinetic data could only be fitted to a first order kinetic model according to a mass transfer control process Eq. (3):

\[
y(t) = B[1 - \exp(-kt)]
\]  

(3)

The initial reaction rate (\(r_0\)) and the first order kinetic constant (\(k\)) were lower for immobilized cells than for free mycelium (non-growing conditions) (Table 3). Besides the maximum yield (\(X_{\text{max}}\)) is achieved at longer reaction times (\(t_{\text{max}}\)) with immobilized biocatalysts than in the case of free mycelium (Table 2). These data indicate to us that the rate-controlling step is associated to mass transfer restrictions. The same conclusions have been described for immobilized cells or enzymes in different matrix and different substrates [8,22,23].

3.6. Diffusion assays of cyclohexanone and cyclohexanol towards the agar plates

The diffusion assays of cyclohexanone or cyclohexanol were performed with agar A27/03 plates (2.5% and 3.75%, w/v) without cells (Fig. 6). The diffusion curves were fitted to a simple exponential decay Eq. (4):

\[
S(\text{mM}) = A\exp(-k_\text{ap}t) + C
\]  

(4)

where \(S\) is the cyclohexanone or cyclohexanol concentration in the medium at different times and \(k_\text{ap}\) is the apparent diffusion rate.

The mass-transfer coefficient (\(k_c\)) was calculated from \(k_c = (k_\text{ap}/\alpha)\), where “\(\alpha\)” is the effective interfacial mass-transfer area per unit volume in the plates. In our small plates, \(\alpha = 9.6 \text{ cm}^{-1}\). The initial mass-transfer rate (\(N_0\)) was calculated from the first derivate of the exponential decay function for cyclohexanone and cyclohexanol. An increase in the percentage of agar slightly increases the time to reach the equilibrium (\(T_{\text{eq}}\)) and decreases \(k_c\) (Table 4). Nevertheless the effects are not dramatic. The initial mass transfer rates (\(N_0\) (Table 4) are greater than \(r_0\) (Table 3). Therefore, the external mass transfer towards the immobilized biocatalyst in...
4. Conclusions

M. kaoliang was grown in optimal conditions obtaining small red beads that have been immobilized in small plates of tailormade agars and agaroses. The fungus is active in the reduction of cyclohexanone and the maximum alcohol dehydration activity is achieved after 7 days culture time. The immobilization in tailormade agars gave better results than in agarose. The methoxylation degree of the agar does not affect the catalytic activity. The internal effectiveness factor, \( \eta \ll 1 \) indicates that the internal mass transfer restriction is the rate-controlling step. This fact explains why the reduction process is a pseudo-first order kinetics and the presence of the \( t_{\text{ind}} \) in the immobilized biocatalysts.

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