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COMPARATIVE STUDY FOR OXYGENASES PRODUCED BY *Aspergillus niger*, ATCC 9642, IN SOLID-STATE AND SUBMERGED FERMENTATION

ESTUDIO COMPARATIVO DE OXIGENASAS PRODUCIDAS POR *Aspergillus niger*, ATCC 9642, EN FERMENTACIÓN EN ESTADO SÓLIDO Y SUMERGIDO

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Abstract

In recent years, solid state fermentation (SSF) has shown much promise in the development of several bioprocesses and products. Some of these applications include bioremediation and biodegradation of hazardous compounds, biological detoxification of agroindustrial residues, production of micotoxins, antibiotics, gibberellins, biopesticides, organic acids, flavour compounds, enzymes, etc. This study deals with the comparison of the oxygenases, produced by *Aspergillus niger*, ATCC 9642, under submerged fermentation (SmF) and SSF using hexadecane as inducer, in the presence and absence of glucose. In addition, the characterization of both systems is also presented here. The results show higher biomass yield (Yx/s), higher specific growth rates (μ) and higher rates of hexadecane consumption (q_s) for SSF than for SmF, when only hexadecane is used as substrate. Mineralization is higher, for both systems, when a mixture of glucose and hexadecane is used as substrate. The oxygenase produced under SSF has no specificity difference for aliphatic hydrocarbons (linear and non linear). But, it increases for aromatic substrates as the complexity of the ring increases. Oxygenase produced under SmF has lower activities, at least 7-folds, compared to SSF.

Keywords: oxygenases, solid state culture, aromatic hydrocarbons, hexadecane, polyurethane matrix support, *A. niger*.

Resumen

En los últimos años, la fermentación en estado sólido (SSF) se ha considerado como un proceso con un gran potencial, en el desarrollo de bioprocesos y de productos de alto valor agregado. Algunas de sus aplicaciones incluyen: la bioremediación y la biodegradación de compuestos peligrosos, la detoxificación de residuos agroindustriales, la producción de micotoxinas, antibióticos, giberelinas, biopesticidas, ácidos orgánicos, saborizantes, enzimas, etc. En este estudio se hace la comparación de oxigenasas producidas por *Aspergillus niger* ATCC 9642 en fermentación sumergida (SmF) y en SSF, usando hexadecano como inductor, en presencia y ausencia de glucosa. Se presenta también la caracterización de ambos sistemas. Los resultados muestran un mayor rendimiento de biomasa (Yx/s) y velocidades específicas de crecimiento (μ) y de consumo de hexadecano (q_s) más altas, para SSF que para SmF, cuando se usa hexadecano como único substrato. La oxigenasa producida en SSF no muestra diferencia de especificidad con hidrocarburos alifáticos (lineales o ramificados). Sin embargo, la especificidad para compuestos aromáticos se incrementa con la complejidad del anillo. La oxigenasa producida en SmF muestra actividades que son por lo menos 7 veces menores comparadas con las actividades de oxigenasa producida en SSF.

Palabras clave: oxigenasas, fermentación en estado sólido, hidrocarburos aromáticos, hexadecano, soporte de poliuretano, *A. niger*.

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1 Introduction

Oxygenases are a group of intracellular enzymes with significant roles in metabolism and biosynthesis. They are important in the design of pharmaceuticals and the production of specialty chemicals (Burton, 2003; Kamerbeek *et al*., 2003; Hartman *et al*., 2006; Fraatz *et al*., 2009; Torres Pazmiño *et al*., 2010; Cresnar and Petric, 2011).

Besides, oxygenases are involved in biodegradation of hydrocarbons and related compounds that, due to their improper storage, use, and disposal have been released into the environment, where they are considered environmental pollutants. Some examples of these are chlorinated biphenyls (Dmochewitz and Ballschmiter, 1988); oil spills (Van Hamme *et al*., 2003; Radwan, 2008); nitrobenzene, 2,4- and 2,6-dinitrotoluene (Lessner *et al*., 2002; Parales *et al*., 2005); polycyclic aromatic hydrocarbons (Gibson and Subramanian, 1984; Romero *et al*., 2002; Feitkenhauer *et al*., 2003; Golubev *et al*., 2009); Short chain hydrocarbons (Hamamura *et al*., 2001); medium chain hydrocarbons (Volke-Sepúlveda *et al*., 2003; Volke-Sepúlveda *et al*., 2006; Bouchez-Naïtali and Vandecasteele, 2008) and volatiles aromatics collectively indicated as BTEX (Qi *et al*., 2002; Nikolova and Nenov, 2005, Maestre *et al*., 2007; García-Peña *et al*., 2008).

Although fungi have an important role in several ecosystems and they have predominance over bacteria in soil, oxygenases have been extensively studied in liquid cultures with bacteria, and analogous data for fungi are scarce. The use of fungi instead of bacteria offers some advantages with respect to stability and activity of the population, especially under reduced water activity and low pH conditions, which often prevail in soil bioremediation and in air biofilters.

On the other hand, several works dealing with remarkable differences between solid state fermentation (SSF) and submerged fermentation (SmF) have been described (Alazard and Raimbault, 1981; Barrios-González *et al*., 1988; Solís-Pereira *et al*., 1993; Viniegra *et al*., 2003; Volke-Sepúlveda *et al*., 2003; Hölker, *et al*., 2004; Fontana *et al*., 2005; Barrios-González *et al*., 2008). It is worth stressing that several biotechnological advantages have been reported for SSF compared to SmF, such as production of extracellular enzymes with higher activities, higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, higher hydrocarbon degradation, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi, and lower demand on sterility.

Among other factors, microbial synthesis of enzymes in a SSF system can be affected by: selection of a suitable substrate; pre-treatment, particle size and water content of the substrate; relative humidity; type and size of the inoculum; fermenting matter temperature, period of cultivation; maintenance of uniformity in the environment of SSF system, and the gaseous atmosphere (Pandey *et al*., 1999; Hölker, *et al*., 2004).

Most of the works dealing with enzyme production in SSF have been carried out for extracellular enzymes, although there are several reports of hydrocarbons degradation involving oxygenases, in SSF (Holden *et al*., 2002; Qi *et al*., 2002; Volke-Sepúlveda *et al*., 2003 and 2006; García-Peña *et al*., 2008). The aim of this work is to study the factors that affect *Aspergillus niger* oxygenase production in SSF and SmF in the presence and absence of glucose and using hexadecane as inducer. And to compare the characteristics (activity, specificity, stability) of the oxygenase(s) expressed under these conditions.

2 Materials and methods

2.1 Strain and conditions for inoculums preparation

Aspergillus niger (ATCC 9642) spores were stored at −20°C in protect-cryoblocks (bead storage system, Technical Service Consultants, Heywood, UK). Inocula were prepared by transferring a cryoblock in 250 ml Erlenmeyer flasks containing 50 ml of potato dextroxe agar (PDA), incubating for 5-7 days at 30 °C. Spores were then scraped with a magnetic bar and 20 ml of sterile 0.1 % between 80 solution. Spores suspensions were used to inoculate culture media for SmF and SSF.

2.2 Cultivation systems and culture media

First of all, several fermentations were carried out, under both type of systems, SmF and SSF, where the glucose and hexadecane were used in different concentrations. The purpose of these fermentations was to assess the conditions, under which the enzyme was expressed, by relating the production of the oxygenase with the hexadecane degradation by *A. niger*.

SmF and SSF were carried out with the mineral ATCC medium 687 with 0.05% yeast extract. The medium composition, for 12 g L^{-1} of total carbon and a C/N = 12.12, is described in Table 1. All the components were adjusted as required, depending on the amount of total carbon and on the C/N desired. The carbon source, in the culture medium, was supplied by different glucose:hexadecane ratios (Glu:Hxd) as shown in Table 2.

SmF were prepared in 250 ml Erlenmeyer flasks containing 50 ml of inoculated culture medium. Liquid cultures were started by inoculation of 1×10^6 spores ml^{-1} of *A. niger*. The initial pH was adjusted to 5.5 with 1N HCl. Erlenmeyer flasks were incubated at 30 °C in an orbital shaker at 200 rev min^{-1} for at least 70 h. Every flask constituted a sample and each sample was prepared in duplicate.

SSF were carried out in 125 ml serological bottles containing approximately 1.13 g of grounded, sterilized polyurethane foam (PUF) and the inoculated culture medium described below. PUF was used as inert support to impregnate the culture medium (Zhu *et al*., 1994). Every bottle constituted a sample and each sample was prepared in duplicate.

Mineral composition of the SSF culture medium was the same used in SmF (Table 1). The carbon source, in the culture medium, was supplied by the Glu:Hxd ratios shown in Table 2. After sterilization

Table 1. Composition of mineral culture medium ATCC 687 Pontecorvo for *A. niger*.

Component	Quantity
$NaNO_3$	6 g
KH_2PO_4	1.52 g
KCl	0.52 g
$MgSO_4 \cdot 7H_2O$	0.52 g
$ZnSO_4 \cdot 7H_2O$	0.0018 g
$FeCl_3 \cdot 6H_2O$	0.0014 g
Yeast extract	0.5 g
*Trace elements solution	1 ml
Distilled water	1000 ml (qs)

*Trace Elements Solution	
Component	Quantity
$Na_2B_4O_7 \cdot 10H_2O$	100 mg
$MnCl_2 \cdot 4H_2O$	50 mg
$Na_2MoO_4 \cdot 2H_2O$	50 mg
$CuSO_4 \cdot 5H_2O$	250 mg
Distilled water	1000 ml (qs)

Table 2. Description of the carbon sources added to the mineral medium ATCC 687 used for *A. niger* oxygenases production in SmF and SSF

SUBMERGED FERMENTATION						
*Culture Medium	Glucose (g L^{-1})	Hexadecane (g L^{-1})	Carbon concentration (g L^{-1})	% C -Glu	% C- Hxd	C:N
a	0	14	12	0	100	12.12
b	15	7.1	12	50	50	12.12
c	30	5	16.24	74	26	16.41
d	30	0	12	100	0	12.12

SOLID STATE FERMENTATION								
*Culture Medium	Glu (g L^{-1})	Glu (g g^{-1} PUF)**	Hxd (g L^{-1})	Hxd (g g^{-1} PUF)	Carbon concentration (g L^{-1})	% C -Glu	% C- Hxd	C:N
e	0	0	94.16	0.344	80	0	100	12.12
f	50	0.155	23.5	0.073	40	50	50	12.12
g	100	0.311	47.08	0.147	80	50	50	12.12
h	100	0.321	0	0	40	100	0	12.12

* Culture medium volumes for SmF were 50 ml each. Culture medium volumes for SSF were (in ml) 3.5, 3.63, 4.6 and 3.63 for samples *e*, *f*, *g*, and *h*, respectively

**The amounts of PUF used were (in g) 0.96, 1.13, 1.13 and 1.3 for samples *e*, *f*, *g*, and *h* respectively.

of the serological bottles containing the PUF, culture broth was added to each bottle to have 70% humidity, and 1×10^7 spores g^{-1} of dry initial matter. The initial pH was 5.5. The bottles were sealed with rubber caps that were connected by means of syringe needles to GC equipment and to a water saturate air flow (2 ml min^{-1}). Serological bottles were incubated in a water bath at 30 °C for at least 85 h.

2.3 Kinetic parameters

Growth curves were fitted by a Maquardt "Solver" computer program (Excel, Microsoft) using logistic equation as follows:

$$X = \frac{X\max}{1 + \left(\frac{X\max - X_0}{X_0}\right)} e^{\mu t},$$

where X (g L^{-1}) represents the biomass calculated, X_{max} and X_0 (g L^{-1}) are the maximum and initial biomass values, respectively, μ (h^{-1}) is the specific growth rate, and t (h) is the culture time. Substrate consumption curves were fitted using Pirt equation as follows:

$$S(X) = S_0 - \frac{1}{Y_{X/S}}(X - X_0) - \frac{mX_{max}}{\mu_{max}} \ln\left(\frac{X_{max} - X_0}{X_{max} - X}\right)$$

where S_0 is the glucose or hexadecane concentration at $t = 0$.

The biomass yield, $Y_{X/S}$, was calculated using the next equations:

$$Y_{\frac{X}{S}} = \frac{(X_{\max} - X_0)}{S_0 - S_f},$$

$$S_0 = C_{iGlu} + C_{iHxd},$$

$$S_f = C_{fGlu} + C_{fHxd}$$

where X_{max} and X_0 (gL^{-1}) are the maximum and initial biomass values, respectively. C_{iGlu} and C_{iHxd} (gL^{-1}) are the initial carbon concentration values from glucose and hexadecane, respectively, and C_{fGlu} and C_{fHxd} (gL^{-1}) are the final carbon concentration values, from glucose and hexadecane, respectively.

The specific substrate uptake rate, q_s, is defined as follows:

$$q_S = \frac{\mu}{Y_{\frac{X}{S}}},$$

Where q_s is given as grams of carbon, from substrates consumed, per gram of biomass per hour.

2.4 Oxygenase(s) production

Once the hexadecane degradation was studied in both types of systems, culture media *c* and *f* (Table 2) were selected to produce oxygenase(s) by SmF and SSF respectively. The fermentation conditions for oxygenase(s) production were the same as described before for hexadecane degradation. *A. niger* mycelium of 48, 72 and 120 h of growth was harvest from SSF and SmF and used to detect oxygenase activity. The oxygenase activity was analyzed by measuring the dissolved oxygen uptake rates, by resting cells, in the presence of substrate (above background respiration rate) and in the absence of it (background respiration rate or endogenous respiration). These rates were normalized with the total amount of biomass in the reaction vials and reported as nanomoles of oxygen removed per second per milligram of biomass. The oxygenase activity was then calculated by subtracting the oxygen uptake rate in the absence of substrate (RE) to the uptake rate in the presence of it (RS) and reported as units of enzyme (Sun and Wood, 1997). One unit of enzyme (U) was defined as the amount of enzyme that will catalyze the consumption of 1 nmol O_2 per second.

2.5 Cell free crude enzyme extracts preparation

To study the characteristics of the induced oxygenases, cell free crude enzyme extracts were prepared from biomass growth in both types of cultures as explained above. Extract from SSF was prepared by washing the mycelium attached to the PUF (PUF-BioM), approx. 10 g, with 500 ml cold water (5 °C). Then it was suspended for 10 min in 200 ml cold (5 °C) 50 mM tris buffer, pH 7.5 with 0.1-μmol dithiotreitol ml^{-1} and 1ml protease inhibitor cocktail (Sigma P-8215) per 20 g cells wet weight. The suspension was filtered through Whatman 41. The PUF-BioM was frozen with liquid nitrogen and disrupted in a coffee grinder (Brown, 2.5 oz). Afterwards, the PUF-BioM was wrapped in cheesecloth, unfrozen (around 4 °C) and squeezed in a hydraulic press (ERCO model PH-51) at 2000 psi, around 130 kg $(cm^2)^{-1}$. The obtained suspension was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was filtered with 0.45 μm membrane and the filtrate was kept in vials (1.5 ml) at −20 °C until enzyme analyses.

Crude enzyme extract from SmF was prepared alike the extract from SSF. The only difference was that after the mycelium was washed and frozen, it was

disrupted with a mortar, suspended in the buffer and finally treated as the SSF crude extract.

2.6 Analytical procedures

Schematic flow diagrams of the sample treatment and analyses for SmF and for SSF are given in Fig. 1 and Fig. 2 respectively. Glucose was measured using an enzymatic analyser (YSI model 2700). Hexadecane was determined by gas chromatography (Perkin Elmer autosystem XL) with a flame ionization detector under the following conditions: column, PE1 of 30 m length and 0.32 mm I.D; carrier gas, helium (15 ml min^{-1}); the injector and detector temperature were constant at 250 and 300 °C respectively; the column temperature was kept initially at 100 °C for 2 min, increasing by 20 °C min^{-1} to 250 °C and then holding for 5 min. Samples of 1 μl were injected and decane was used as internal standard. Biomass concentration was determined as dry matter content.

Oxygen and carbon dioxide evolution on SSF were followed by gas chromatography (Gow Mac 580) with thermal conductivity detector. The column used was CTR8700 (Alltech), current detector 150mA, detector and injector temperature 80 °C, column temperature 50 °C. Acquisition and integration system Multivia Chroma V.30.

Oxygenase activity assays were performed, in cell free crude enzyme extracts. Two methods were used: oxygen uptake rate (oxymetry), based on a method described by Sun and Wood (1997) and a modified spectrophotometric method described by Zazueta-Sandoval *et al*. (2003).

Activity assays by oxygen consumption were done using an O_2 micro-electrode (YSI 5300 Biological Oxygen Monitor; YSI Inc., Yellow Springs, OH, U.S.A.). The reaction mixture consisted of 1.3 ml 50mM Tris pH 7.5, 0.4 mM ml^{-1} NADH and 0.3 ml crude enzyme extract. After equilibration at the desired oxygen concentration, the reaction was started by adding 3 μl of substrate (hexadecane). O_2-uptake was calculated by the difference in O_2 concentration before and after substrate addition. The oxygenase

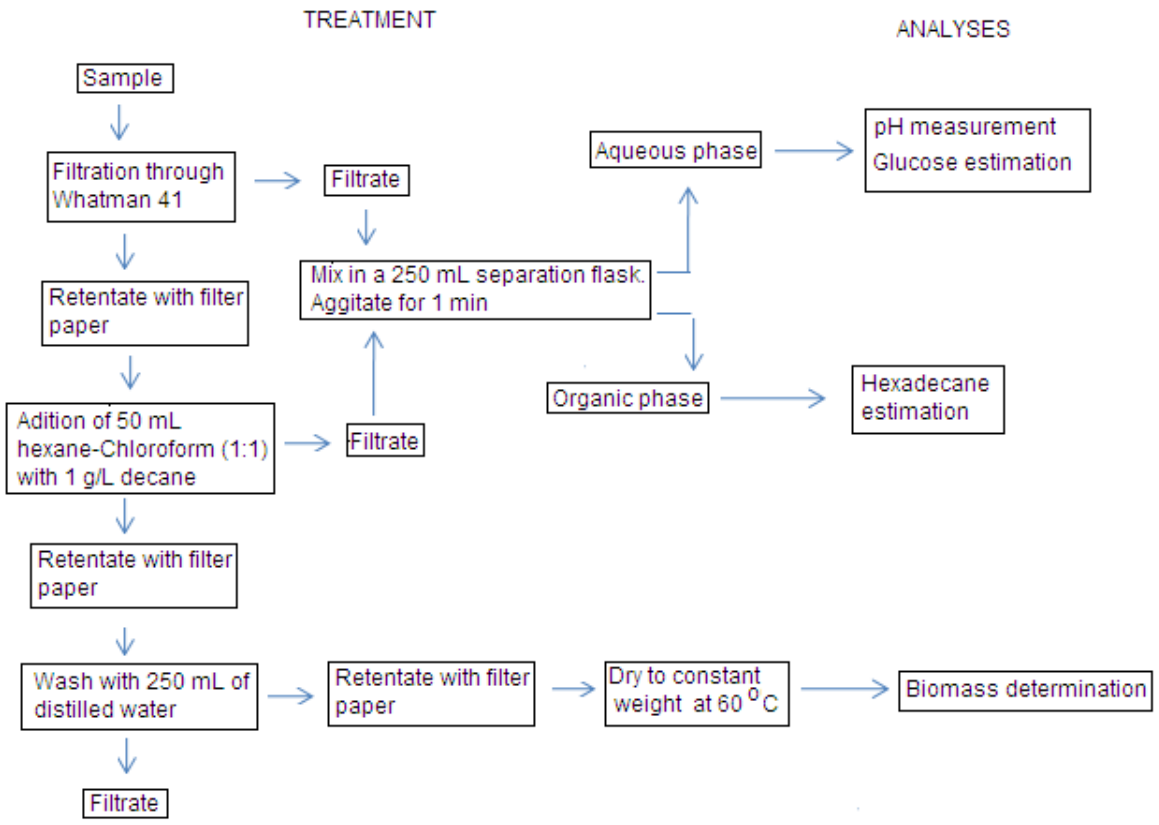

Fig. 1. Schematic diagram for treatment of the SmF samples for microbiological and physico-chemical analyses.

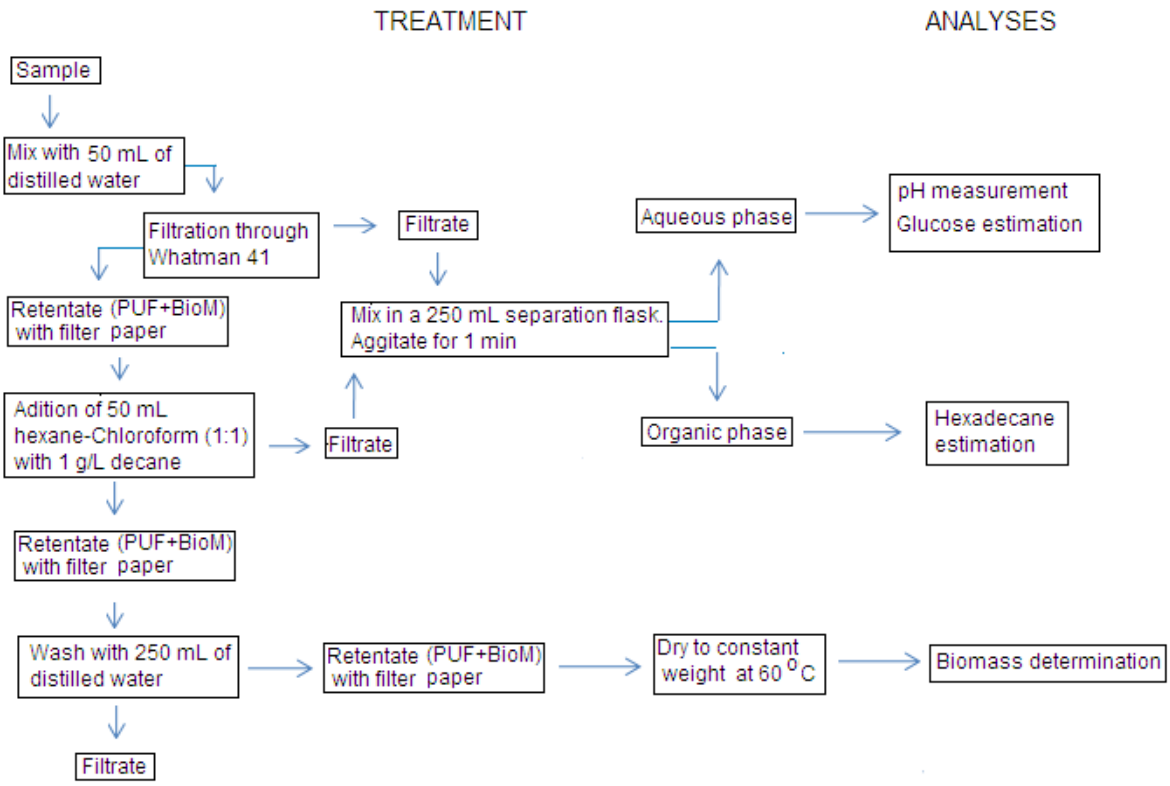

Fig. 2. Schematic diagram for treatment of the SSF samples for microbiological and physico-chemical analyses.

activity was reported as specific activity (U mg^{-1} protein). One unit of enzyme (U) was defined as the amount of enzyme that will catalyze the consumption of 1 nmol O_2 per second. Oxygenase activity measurements using the spectrophotometric method were carried out in the following way. In a 1.5 ml quartz cuvette, 1 ml of reaction mixture was added. This consisted of 300 μl of o-dianisidine dihydrochloride reagent (20 mg 3,3'-dimetoxibenzidine dissolved in 3 ml 0.025 M hydrochloride acid, added with agitation to 50 ml of 50 mM tris buffer pH 8.5 and brought up to 100 ml with the same buffer), 480 μl of 50 mM tris buffer pH 8.5 added with 0.4 μm of NADH, 20 μl of substrate and 200 μl crude enzyme extract. Measurements were performed at 30 °C by using a spectrophotometer (Shimadzu UV-160A) equipped with a thermojacketed cuvette holder and a water circulation system, absorbance at 460 nm was followed for one hour, against a blank, after the addition of substrate. The blank was prepared replacing 200 μl crude enzyme by 200 μl of buffer, besides a reference was made replacing the substrate by 20 μl of buffer.

Enzyme activity was calculated subtracting reference slope (absorbance at 460 nm vs time) from sample slope and interpolating this value on a peroxidase activity calibration curve made with different concentrations of hydrogen peroxide as substrate of the enzyme in the presence of o-dianisidine (Zazueta-Sandoval *et al.*, 2003). This method is based in the characteristics of orto-dianisidine (orto-DNS) which is a chromogenic acceptor of oxygen and in the action of oxygenase on hydrocarbon molecule and peroxidase (POD) on hydrogen peroxide, accordingly with the next two net reactions, using hexadecane as example of substrate for a monooxygenase (MOX):

$$C_{16}H_{34} + NADH + O_2 + orto - DNS \xrightarrow{MOX} C_{16}H_{33}OH + NADH + orto - DNS_{ox}$$

$$H_2O_2 + orto - DNS \xrightarrow{POD} H_2O + orto - DNS_{ox}$$

In the first reaction for each consumed oxygen molecule, one molecule of hexadecane and one molecule of orto-DNS are oxidized. Meanwhile, in the second reaction for each molecule of hydrogen peroxide reduced, one atom of oxygen is introduced

in orto-DNS molecule. Therefore, through the interpolation of the absorbance value, generated from the first reaction, in the POD calibration curve, it is possible to know the amount of oxygen consumed in the first reaction. The oxygenase activity was reported as specific activity (U mg^{-1} protein). One unit of enzyme (U) was defined as the amount of enzyme that will catalyze the consumption of 1 nmol O_2 per second.

The protein content was determined using the Bio-Rad Protein Assay, based on the method of Bradford, with bovine serum albumin as a standard.

2.7 Characterization of the oxygenase(s)

The concentration and storage effects on the enzyme activity and stability were examined by O_2-uptake rate. Crude extracts from SSF and SmF were concentrated against sucrose with a 12,000 Da membrane (Millipore tubing membrane). They were stored at −20 °C from 0 to 84 days. Samples without concentrate were also kept at the same temperature. All these samples were used for O_2-uptake analyses.

Oxygenase(s) substrate specificity was analyzed by the spectrophotometric method (Zazueta *et al*., 2003). Short (pentane, hexane, heptane), medium (decane, hexadecane) and large (eicosane) chain hydrocarbons as well as branched (2,6,10,14 tetramethyl-pentadecane) and aromatic (benzene, anthracene, phenanthrene, pyrene and toluene) compounds, were used as substrates.

The effect of temperature in oxygenase activity was measured by the spectrophotometric method. The amount of protein used in these assays were 58.60 μg per sample contained in 200 μl of crude extract. The temperatures tested were 12, 26, 30 and 35 °C. Decane was used as substrate.

2.8 Polyacrylamide gel electrophoresis (PAGE)

The degree of purification and identification of proteins with oxygenase activity was monitored on native PAGE and zymograms. Native PAGE was performed according to Laemmli (1970), with 6% resolving and 4% stacking gels. Samples were electrophoresed at 10 mA and 4 °C through 1.5 mm gel in a vertical slab gel unit Mini Protean II Electrophoresis Cells (Bio-Rad Laboratories). The gels were loaded with 35μg of protein from cell free extracts obtained from SmF and SSF. The gels were stained with Coomasie Brilliant Blue R-250. A kaleidoscope prestained standard was also loaded as molecular weight standard.

Zymograms. Nondenaturing poliacrylamide gel electrophoresis was performed with both types of crude extracts as described before. Oxygenase activity was revealed by submerging the gel in a reaction mixture composed by 10 ml of 50 mM tris buffer pH 8.5 added with 0.4 μm ml^{-1} of NADH, 6.25 ml of o-dianisidine reagent (see section 2.5) and 1 ml of substrate (decane, hexadecane or bencene) and incubating the gel at room temperature with gentle agitation until the activity bands appeared. The protein loaded was 63.5 μg per lane.

3 Results and discussion

3.1 Growth of A. niger in SmF

Fig. 3 shows glucose and hexadecane degradation profiles in SmF. From this data it was evaluated that *A. niger* degraded 66.7±1.8 % of hexadecane in the presence of glucose (*c*), in only 48 h. However, the remaining 33.3 % did not undergo any change even after 140 h. Meanwhile, a small amount (17.9±14.6) of the added hexadecane was degraded when it was used as sole substrate (*a*), during the time of analyses (75 h) and none of it was degraded when the carbon source was 50 % from glucose and 50 % from hexadecane (b).

Another remarkable result in medium *c* (Fig. 3c) was the way that hexadecane was consumed. The first 20 h, *A. niger* consumed glucose only, but around 25 h, it consumed hexadecane and glucose simultaneously. Around 50 h the glucose has been totally consumed and the hexadecane uptake stopped. The biomass production of *A. niger* ATCC 9642, in SmF, reached the highest value (14.5 ± 0.27 g L^{-1}) in the culture medium *d* (glucose alone), whereas medium *a* (hexadecane alone) gained the lowest value (0.63±0.17 g L^{-1}). Table 3 presents the values of the kinetic parameters associated with the fungal growth: the specific growth rate (μ) and the maximum growth (X_{max}).

The specific growth rate increased when glucose plus hexadecane were used as substrates (mediums *b* and *c*). The highest value for μ (0.23 h^{-1}) corresponded to medium *b*. But it had zero hexadecane consumption. Noordman *et al*. (2002) reported a μ value of 0.02 h^{-1} for *P. aeruginosa* grown in a liquid culture with hexadecane concentrations ranging from 0.1 to 2.0 g L^{-1}, this value is 2.2 times

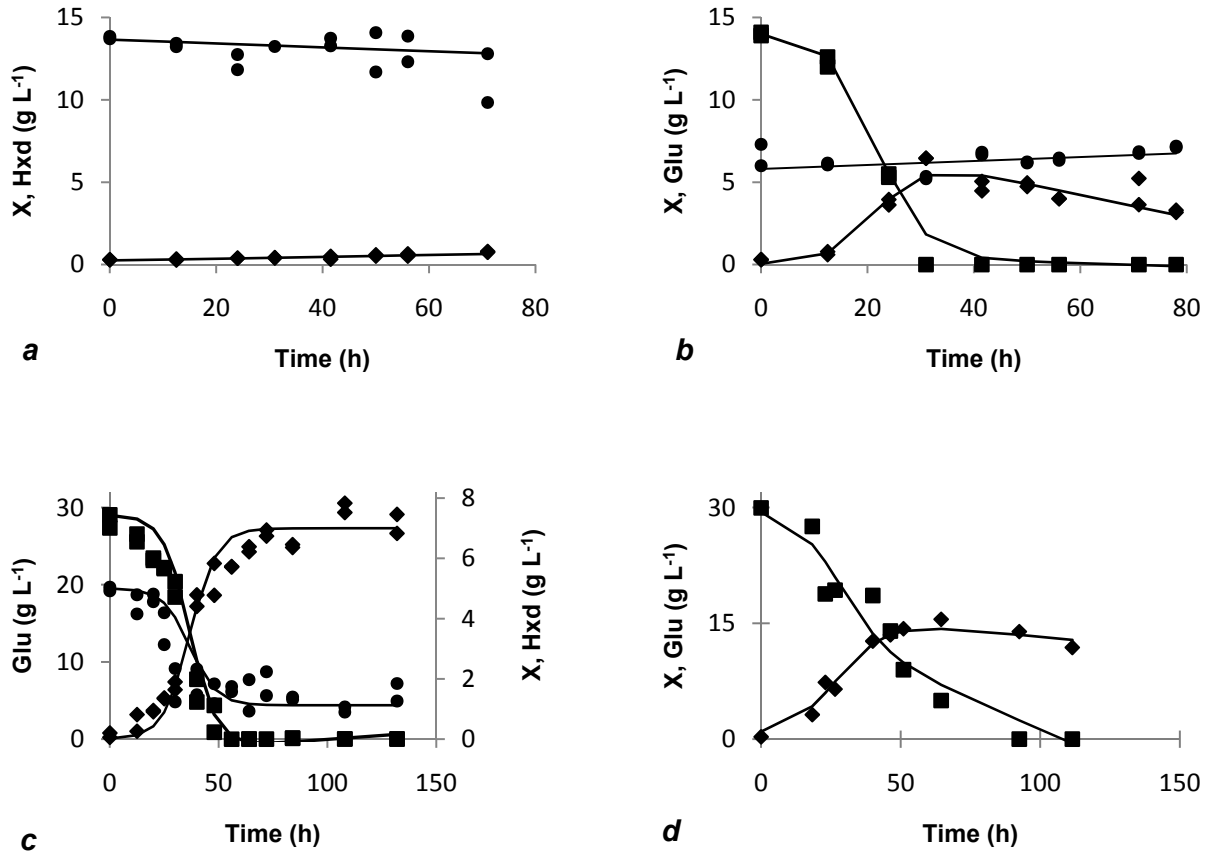

Fig. 3. Liquid culture of *A. niger* in a mineral medium ATCC 687 with 0.05% yeast extract, added with (a) 14 g L^{-1} hexadecane; (b) 15 g L^{-1} glucose, 7.1g L^{-1} hexadecane; (c) 30 g L^{-1} glucose, 5 g L^{-1} hexadecane; (d) 30 g L^{-1} glucose. ♦ X (biomass growth), •Hxd (hexadecane conversion), ■ Glu (glucose consumption). Lines represent calculated values and points are experimental values.

Table 3. Kinetic parameters associated to fungal growth in SmF and SSF

*Substrate		**Parameter		Fermentation
Glucose (gL^{-1})	Hexadecane (gL^{-1})	μ (h^{-1})	X_{max} (g L^{-1})	System
0	14	0.009	0.63 ± 0.17	SmF
15	7.1	0.23	6.15 ± 0.01	SmF
30	5	0.16	5.59 ± 0.09	SmF
30	0	0.12	14.5 ± 0.27	SmF
0	94.16	0.03	40.19 ± 0.59	FES
50	23.5	0.13	18.55 ± 0.36	FES
100	47.08	0.11	23.50 ± 0.26	FES
100	0	0.15	27.04 ± 1.27	FES

μ: Specific growth rate; X_{max}: Maximum biomass values

*The substrates correspond to culture mediums *a* to *h* from top to bottom

**This parameters were evaluated during the constant growth phase

higher than the μ value obtain in the present work for *A. niger*, under SmF, when only hexadecane was used as substrate. However, the μ values for bacteria are known to be higher than that of fungi (Volke-Sepúlveda *et al*., 2006).

Table 4 shows the kinetic parameters associated

to substrate consumption. It can be observed that substrate uptake rate (q_s) increased when mixtures of hexadecane plus glucose were used as substrates, compared with the q_s of culture medium with glucose or hexadecane alone. The biomass/substrate yields (Y_{SX}) had higher values when *A. niger* growth at the expense of glucose only and smaller values when hexadecane was consumed, either alone or in the presence of glucose. This implies a higher mineralization when hexadecane was consumed. Volke-Sepúlveda *et al.* (2003) reported biomass/oxygen and oxygen/hexadecane yields, for an initial hexadecane concentration of 20 (gL^{-1}) under SmF, of 0.29 (mg X mg^{-1} O_2) and 3.34 (mmol O_2/mmol Hxd) respectively. With these data a biomass/substrate yield (Y_{SX}) of 0.14 (mg X mg^{-1} Hxd) can be calculated. García-Peña *et al.* (2008) reported a Y_{SX} = 0.13 (mg X mg^{-1} benzene). In our study for medium a (14 g L^{-1} hexadecane) Y_{SX} = 0.2 (mg X/mg Hxd). In all these cases when the hydrocarbon was the only carbon source the biomass/substrate yields were very low. Prenafeta-Boldú *et al.* (2002) attributed a low degradation pattern of benzene by *Cladophialophora* sp to the accumulation of dead-end oxidation sub products, however

Table 4. Kinetic parameters associated to substrate uptake in SmF and SSF

*Substrate		**Parameter		Fermentation system
Glucose (gL^{-1})	Hexadecane (gL^{-1})	*Y_{SX} (gX g^{-1}Cs)	**q_S (gCs g^{-1}X h^{-1})	
0	14	0.40 ± 0.24	0.03 ± 0.02	SmF
15	7.1	0.76 ± 0.06	0.30 ± 0.03	SmF
30	5	0.40 ± 0.02	0.41 ± 0.02	SmF
30	0	1.21 ± 0.02	0.10 ± 0.00	SmF
0	94.16	0.57 ± 0.01	0.06 ± 0.00	FES
100	47.08	0.60 ± 0.02	0.18 ± 0.01	FES
50	23.5	0.68 ± 0.03	0.22 ± 0.00	FES
100	0	1.16 ± 0.01	0.13 ± 0.00	FES

*Y_{SX}: Biomass/substrate yield (grams of biomass per grams of carbon from substrate)

*q_S: Substrate uptake rate (grams of carbon from substrate per grams of biomass per hour)

**These parameters were evaluated during the phase of constant growth. Average values ±DS (n = 2) are presented

Table 5. Carbon recovered as CO_2 during SmF and SSF of hexadecane, glucose and mixtures of them by *A. niger*

Substrate (g)		**Consumed substrate (g)		**X Prod (g)	**Experimental CO_2 (g)	% C as CO_2	++RQ CO_2/O_2	FS(S*)
Hxd	Glu	Hxd	Glu					
0.70	0.00	0.12±0.1	0.00	0.024±0.00	0.38±0.13+	78	0.61(0.65)	SmF(*a*)
0.25	0.70	0.00	0.70±0.01	0.308±0.00	0.524±0.02 +	51.1	1.15(1.00)	SmF (*b*)
0.36	1.41	0.17±0.00	1.41±0.06	0.259±0.03	1.99±0.19 +	82.5	0.90(0.89)	SmF (*c*)
0.00	1.50	0.00	1.50±0.00	0.761±0.03	0.96±0.006 +	43.4	1.21(1.00)	SmF (*d*)
0.33	0.00	0.29±0.01	0.00	0.163±0.17	0.45±0.05	56.8	0.57(0.65)	SSF (*e*)
0.214	0.39	0.13±0.02	0.39±0.02	0.112±0.00	0.80±0.08	82.8	0.81(0.82)	SSF (*g*)
0.064	0.12	0.06±0.00	0.12±0.01	0.071±0.00	0.23±0.006	64.1	0.75(0.78)	SSF (*f*)
0.00	0.426	0.00	0.424±0.00	0.198±0.00	0.20±0.005	36.1	1.13(1.00)	SSF (*h*)

* FS (fermentation system), S (sample). All the samples in SmF had culture medium volumes of 50 ml each. For SSF the culture medium volumes were (in ml) 3.5, 4.6, 3.63, and 3.63 for samples *e*, *g*, *f* and *h* respectively).

** Average values ±DS (n = 2) are presented

+CO_2 values were calculated by means of an oxidation reduction balance.

++Experimental value (Theoretical value)

in the case of hexadecane the reason of low hexadecane degradation in FEL is attributed to the low hexadecane solubility in water.

From the data shown so far it can be observed that even though, the amount of carbon consumed for medium *c* was the highest under SmF and it had the highest substrate uptake rate (0.35 g C-substrate g^{-1} X h^{-1}), the X_{max} was only 5.59±0.09 g L^{-1} and the substrate yield was one of the lowest under SmF, 0.40 (gX g^{-1} C-substrate). These results suggest high mineralization, which was confirmed by a carbon balance; data is presented in Table 5.

The degree of mineralization was higher when hexadecane was consumed, either alone or in the presence of glucose, than when only glucose was uptaken. This confirms what was suggested through the analysis of Y_{SX}. The respiratory quotients (RQ) are also presented in Table 5. RQ is related to the respiratory metabolism of the microorganism and to the substrate oxidation level (Volke-Sepúlveda *et al*., 2003). The RQ for SmF varied in the opposite direction than the degree of mineralization, i.e. higher for glucose uptake and smaller for hexadecane consumption. RQ values smaller than 1 are an indication of predominant mineralization of aliphatic organic compound, having low oxygen content. Ratios greater than1are obtained when organic compounds with high content of oxygen are extensively decomposed (Dilly, 2001).

In a similar study Wang *et al*. (1996) found for *Pseudomonas putida* (ATCC 17514) that when glucose was the sole carbon and energy source, the culture utilized glucose following Monod kinetics. When phenol was the sole carbon and energy source, the culture biodegraded it following Andrews (inhibitory) kinetics. When both glucose and phenol were present in the medium, the culture used them simultaneously but with lower specific rates. They stated that reduction of the specific substrate utilization rates indicates that the two substances are involved in a cross-inhibitory pattern which can be classified as uncompetitive. García-Peña *et al*. (2008) working with Paecilomyces variotti, found that the degradation of binary mixtures of BTEX rates obtained with single substrates (BTEX) and binary combinations of them were sensibly reduced in the mixtures. The results shown here are similar to Wang *et al*. (1996) results, except that in this work higher specific substrate utilization rates were obtained with mixtures of substrates. An increment in the specific substrate utilization rate suggests a synergistic effect of glucose in hexadecane consumption in culture medium c. However, in culture medium b, glucose inhibits hexadecane consumption, that could be by glucose repression. However, once glucose was depleted the enzyme was not derepress, or at least its action was not observed.

3.2 Growth of A. niger in SSF

Fig. 4 shows glucose and hexadecane degradation profiles in SSF. It can be observed that *A. niger* degraded hexadecane in the presence and absence of glucose, regardless of concentration and type of carbon source. The highest hexadecane degradation was attained in culture medium f, 95.5±1.9 % of the initial hexadecane in less than 60 h, followed by medium e with 89.8±2.1 % and 62.7±7.2% for medium g. *A. niger* in the presence of glucose and hexadecane (Fig. 4 *f* and *g*) started consuming glucose first and once this was completely exhausted, *A. niger* consumed hexadecane. Apparently there was catabolite repression which was more severe for culture medium g which contained the double of glucose than medium *f*. In Fig. 4 (*f* and *g*) it can be seen that hexadecane consumption started around 20 h for medium *f*, immediately after all the glucose was assimilated, while it started after 50 h for *g*, although glucose was depleted in 30 h.

In SSF higher X_{max} were attained for culture mediums with glucose or hexadecane as sole carbon sources (see Table 3). In cultures mediums with glucose and hexadecane X_{max} decreased with an increase in glucose and hexadecane concentration. The specific growth rates in SSF (Table 3) were similar for all the culture mediums studied, except for the culture medium e (hexadecane as sole carbon source) that was 3.6 times slower. For *A. niger* with an initial hexadecane concentration of 360 mg g^{-1} PUF, Volke-Sepúlveda, *et al*. (2006) reported a μ value of 0.70±0.02 that is similar to the one reported here in the presence of 344 mg g^{-1} PUF of hexadecane (0.72).

Table 4 shows the kinetic parameters associated to substrate consumption in SSF. It can be seen that substrate uptake rates (q_s) had the same trend as in SmF. The biomass/substrate yields (Y_{SX}) were higher for glucose alone and had the minimum value when hexadecane is the sole source of carbone. From data reported by Volke-Sepúlveda *et al*. (2003) a Y_{SX} = 0.67(mg X mg^{-1} Hxd) was calculated for a sample with 90 g L^{-1} of inital hexadecane concentration. In the present study for medium e (94.16 g L^{-1}) Y_{SX} = 0.56 (mg X mg^{-1} Hxd).

The mineralization and the RQ for SSF are shown

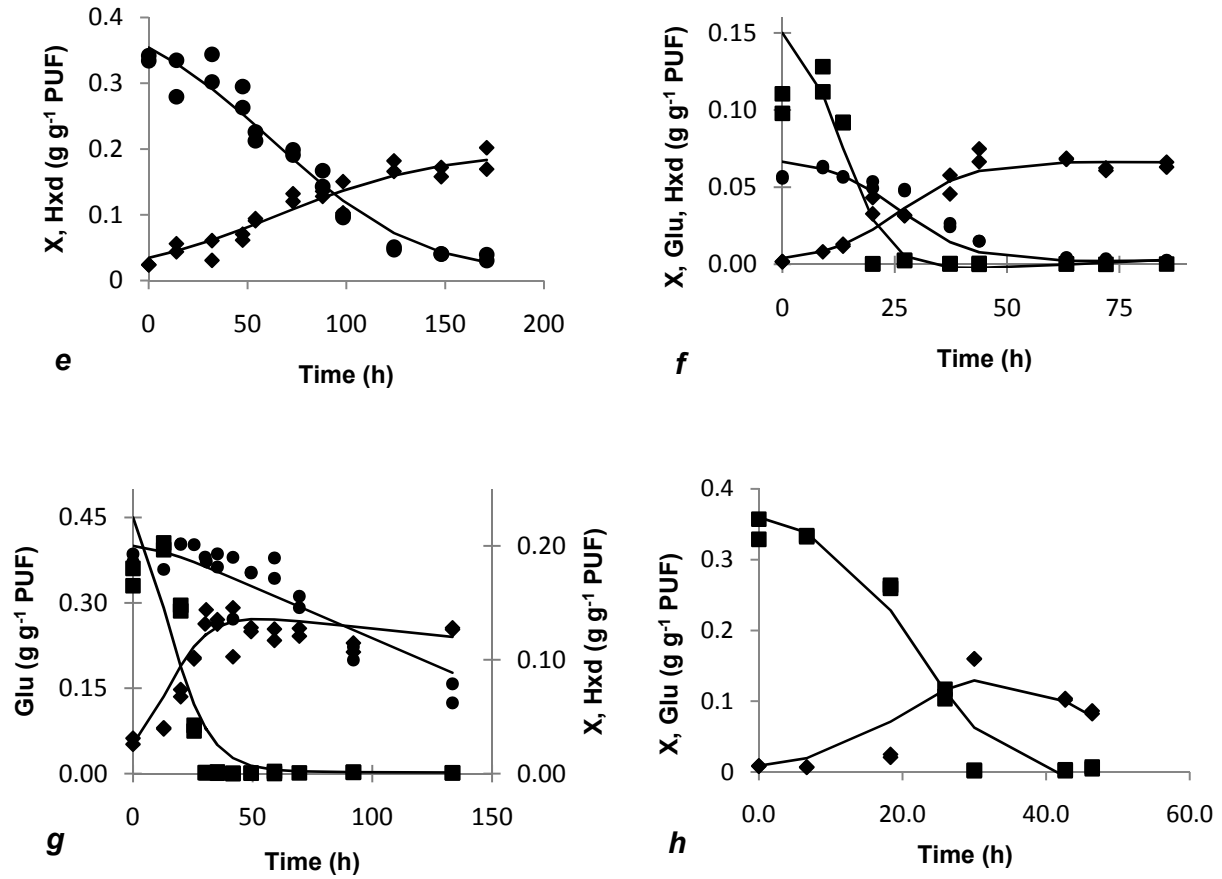

Fig. 4. Solid culture of *A. niger* in a mineral medium ATCC 687 with 0.05% yeast extract, added with (e) 94.16 g L^{-1} hexadecane; (f) 50 g L^{-1} glucose, 23.5 g L^{-1} hexadecane; (g) 100 g L^{-1} glucose, 47.08 g L^{-1} hexadecane; (h) 100 g L^{-1} glucose. ♦ X (biomass growth), •Hxd (hexadecane conversion), ■ Glu (glucose consumption). Line represents calculated values and points are experimental values.

in Table 5. As for SmF the higher mineralization was attained when glucose and hexadecane were present in the culture medium. The RQ also had the same trend as in SmF, the lowest RQ values were found for cultures mediums where only hexadecane was consumed. Volke-Sepúlveda *et al*. (2006) reported, for degradation of hexadecane in SSF with *A. niger*, that RQ values were nearly independent of the hexadecane concentration and they were higher in C/N variable (0.73 ± 0.03) than in C/N constant (0.66 ± 0.02). For hexadecane degradation under SmF Volke-Sepúlveda *et al*. (2003) reported higher and variable RQ values. In the present work, degradation of hexadecane alone under SSF or SmF, produced RQ values smaller (0.57 and 0.64 respectively) than the values reported by them. These results must be related to the higher mineralization that was attained in our case.

3.3 Solid state fermentation compared to submerged fermentation

Examination of figs. 3 and 4 shows very different profiles of substrates consumption and biomass production for liquid vs. solid cultures. Among the principal differences the following can be mentioned: 1) In SSF independently of the initial hexadecane concentration, always there was hexadecane degradation. 2) Hexadecane degradation tended to 100% in all the culture media studied in SSF. 3) When the carbon source was 50 % from glucose and 50% from hexadecane apparently there was catabolic repression under SmF as well as in SSF. However, in SSF the enzyme was derepress and hexadecane was consumed, even when high glucose concentrations were used. While, under SmF glucose was depleted, hexadecane degradation never

started and biomass concentration started decreasing. Some authors working with similar systems have reported that high hydrocarbon concentrations can be associated with heavy undispersed oils slicks in water, inhibiting biodegradation due to a nutrient and/or oxygen limitation (Leahy and Colwell, 1990). del Castillo and Ramos (2007) reported that under SmF in a minimal medium with 16 mM of glucose and 6 mM of toluene as carbon sources there was simultaneous catabolite repression, however both substrates were consumed, although with lower rates than the separated substrates. Diaz-Godinez *et al.* (2001) have shown for *A. niger* C28B25 using SmF and SSF with a mixed substrate (5 g/L pectin and 50 g/L sucrose), better pectinase productivity because of higher biomass production and lower protein breakdown in SSF. Several authors have proposed that SSF system minimizes the catabolite repression (Ramesh and Lonsane, 1991; Aguilar, *et al*., 2001; Fontana and Moura da Silveira, 2005). Generally they report lower catabolite repression with glucose concentrations ≥100 g L^{-1}, compared to SmF. In light of the findings it appears that breakdown of biomass occurred, before the enzyme is derepressed in culture medium *b* (15 g L^{-1} glucose and 7.1 g L^{-1} hexadecane) and this could be due to oxygen limitation. 4) The values for μ were similar for both systems and are higher when more glucose is consumed. Glucose had a stimulatory effect because provides easily accessible carbon source, which gives rise to an increased biomass (Östberg *et al*., 2007). 5) When the culture medium had glucose and hexadecane the q_s values were 1.36-2.27 times faster for SmF than for SSF. For culture medium with hexadecane as sole source of carbon, q_s was 2.2 times faster for SSF than for SmF, this could be due to a better hydrophilic substrate assimilation in SmF and a better hydrophobic substrate uptake in SSF.

As it was said before one of the objectives of studying the degradation of hexadecane under SmF and SSF was to assess the conditions, under which the enzyme was expressed, by relating the production of the oxygenase with the hexadecane degradation by *A. niger*. Wu *et al*. (2011) reported that an increase in P450 cytochrome monooxygenase activity was linearly correlated with hexadecane degradation. Other purpose of this type of study was to find the conditions under which a considerable amount of biomass with good characteristics, i.e. with the enzyme induced and non sporulating, was formed.

Evaluation of the parameters mentioned above resulted in the selection of medium c and f (see Table 2) to grow *A. niger*. Mycelium was harvested at 48, 72 and 120 h to evaluate the production of oxygenase(s) and to observe if the enzyme activity corresponded with the time of hexadecane consumption.

3.4 Production of oxygenase(s)

Culture media *c* and *f* were selected to produce oxygenase(s) by SmF and SSF respectively. The oxygenase activity was measured using resting cells as described in section 2.4. The results are shown in Table 6.

It can be observed in Table 6 that background respiration (RE) rate was relatively high. To avoid the interference of endogenous respiration with the oxygenase activity, two respiration inhibitors were used (NaN_3 and NaCN). The two inhibitors drastically reduced the background respiration rate. However, they also reduced the oxygenase activity. This essay did not give reproducible results for SSF (data not shown). Resting cells from SmF showed higher activity for the 48 h mycelium. It was observed that 48 h coincided with the period of steady growth of *A. niger* and with the time that hexadecane was consumed, in culture medium *c*, under SmF (Fig. 3).

Since oxygen uptake rate by *A. niger* resting cells was not an appropriate method of measuring oxygenase activity, it was decided to use a cell free crude enzyme extract to achieve this objective. The time selected to harvest biomass for crude extracts preparation was 35 h for SSF and 45 h for SmF.

3.5 Characterization of the oxygenase(s)

3.5.1 Enzyme stability

Figure 5 shows the concentration by dehydration and storage effects on the oxygenase(s) stability. First able it can be observed that oxygenase activity is at least 3.7 times higher for SSF sample than for SmF. Crude extracts from both types of fermentations had a drastic loss of activity, around 75%, when they were concentrated or stored at −20 °C for more than 7 days. Samples dehydrated and stored for 84 days at −20 °C had no further decrease of activity. The loss of activity with time of storage can be due to the disappearance

Table 6. Oxygenase activity of *A. niger* resting cells, harvest from FEL.

*SAMPLE	** $^{+}O_2$-uptake rate $nmolO_2$ $(mgX\ s)^{-1}$	$^{+}O_2$-uptake rate by oxigenase $nmolO_2(mgX\ s)^{-1}$	$^{++}$Oxygenase activity (U)
RE(48h)	0.2126±0.0155 A		
RS (48h)	0.3352±0.0051 B		
RS-RE (48 h)		0.1226±0.0144 a	0.5897±0.0693
RE(72 h)	0.1333±0.0016 C		
RS (72 h)	0.1479±0.0077 D		
RS-RE (72 h)		0.0146±0.0064 b	0.1080±0.0477
RE(120 h)	0.0713±0.0017 E		
RS (120 h)	0.0780±0.0152 C		
RS-RE (120 h)		0.0068±0.0125 b	0.0415±0.0766
$RENaN_3$ (48 h)	0.0154±0.0006 C		
$RSNaN_3$ (48 h)	0.0374±0.0006 F		
RS- $RENaN_3$(48h)		0.0220±0.0007 b	0.1072±0.0035
RENaCN (48 h)	0	0	0
RSNaCN	0	0	0

* RE (O_2-uptake in the absence of substrate), RS (O_2-uptake in the presence of substrate), RS-RE (O_2-uptake by oxygenase activity). $RENaN_3$ (O_2-uptake in the absence of substrate and in the presence of respiration inhibitor, sodium azide), $RSNaN_3$ (O_2-uptake in the presence of substrate and in the presence of respiration inhibitor NaN_3), RS- $RENaN_3$ (O_2-uptake by oxygenase activity in the presence of respiration inhibitor NaN_3), RENaCN (O_2-uptake in the absence of substrate and in the presence of respiration inhibitor NaCN), RSNaCN (O_2-uptake in the presence of substrate and in the presence of respiration inhibitor NaCN).

** O_2-uptake rate by biomass harvest from SmF at the time indicated. The units are nmol O_2 per mg of biomass per second. O_2-uptake was measured by oxymetry, with hexadecane as substrate.

$^{+}$Average values ±DS ($n = 2$) are presented. Data with the same letter are not significantly different ($\alpha = 0.05$). Uppercase and lowercase letters represent independent statistical tests.

$^{++}$One unit of enzyme (U) was defined as the amount of enzyme that will catalyze the consumption of 1 nmol O_2 per second.

of the cofactor. It is known that most of oxygenases are cofactor dependent. Among the cofactors we can cite NADH, FAD, NADPH. These cofactors are continuously forming while the microorganism is intact, however, in the extract there is a small amount of it that is lost very easily since this type of molecules are very labile. Another reason for the lost of activity it could be an adduct formation (Gray, 1989).

3.5.2 Oxygenase(s) substrate specificity

Crude extracts from SSF oxidized aliphatic and cyclic substrates (Fig. 6A). There was no significant difference ($\alpha = 0.05$) in substrate specificity for low (pentane, hexane, heptane), medium (decane, hexadecane) and high (eicosane) molecular weight aliphatic hydrocarbons, as well as for linear compared to non linear (2,6,10,14 tetramethyl-pentadecane). However, for aromatic substrates, activity was significantly higher ($\alpha = 0.05$) as the number of the rings increased (benzene< anthracene<phenanthrene<pyrene). The activity with toluene was relatively high, similar to that presented by pyrene. Substrate specificity for crude extracts obtained from SmF showed no specific pattern and the activities were lower for them, at least 7-folds compared to SSF (Fig. 6B).

van Beilen *et al*. (1994) studied the hydroxylation of a wide range of linear, branched, and cyclic alkanes and alkylbenzenes by the alkane hydroxylase system of *Pseudomonas oleovorans* GPo1 *in vivo* and *in vitro*. *In vitro* hydroxylation was determined with a reconstituted hydroxylase system consisting of AlkB (the membrane-bound catalytic monooxygenase component), AlkG (rubredoxin), and spinach ferredoxin reductase. van Beilen *et al*. (1994) found that this system was able to oxidize all types of substrates, except when a tertiary or quaternary carbon was present.

Zazueta-Sandoval *et al*. (2003) partially purified

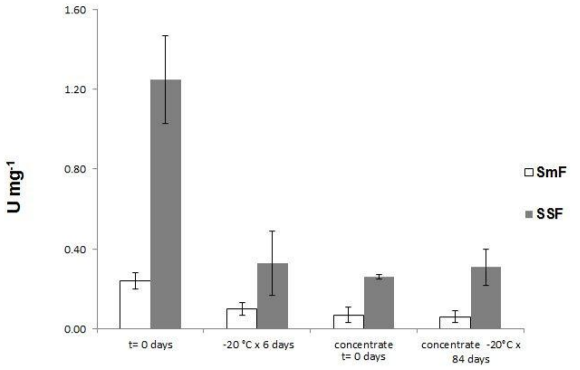

Fig. 5. Effect of concentration and time of storage of crude enzyme extract on oxygenase stability.One unit of enzyme (U) was defined as the amount of enzyme that will catalyze the consumption of 1 nmol O_2 per second.

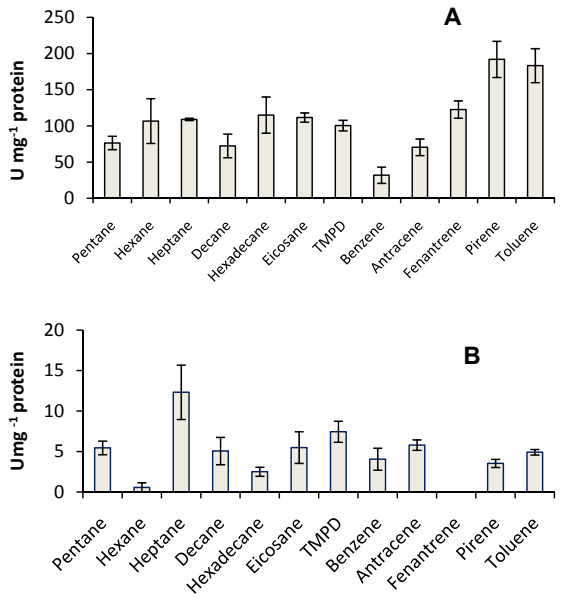

Fig. 6. Oxygenase activity from *A. niger* ATCC 9642, using the substrates shown. TMPD (2,6,10,14 tetramethyl-pentadecane). Activity was measured by a spectrophotometric assay with o-dianisidine. A) Activity of crude extract from SSF. B) Activity of crude extract from SmF. Each bar represents the average value of two replicates (± SD).

an oxygenase from an unknown fungus strain, grown in liquid culture. They reported for it a higher activity with cyclic substrates than with aliphatic ones and they found a decrease in activity as the complexity of the substrate molecule increased. They conclude the existence of one enzyme with activity as mono and dioxygenase.

Vatsyayan *et al.* (2008) found a cytochrome P450 monooxygenase (CYP) of Aspergillus terreus grown in hexadecane (cytosolic fraction) and in glucose (membrane bound). The CYP studied by them have similar specificities than the ones found in this work under SmF, albeit the activities are different. For example they report 1.28±0.011 μmol O_2 min^{-1} mg^{-1} protein for hexadecane equivalent to 21.33 nmol O_2 s^{-1} mg^{-1} protein. This is one of the highest activities reported for the enzyme extract obtained from A. terreus growth with hexadecane. The enzyme was active also with octane, tetracosane, benzene and toluene among other substrates. All the activities shown for the CYP were smaller than the ones calculated in this work under SSF, but higher than in SmF.

3.5.3 The effect of temperature on oxygenase(s) activity

There are two optimum temperatures for the oxidation of decane as shown in Fig. 7. One is at 26 °C and the other is equal or greater than 35 °C. This result together with the ones showed above suggests the presence of more than one enzyme. In addition the results show the dependence of enzyme activity with temperature. Zazueta-Sandoval *et al.* (2003) did not observe effect of incubation temperature at 28 or 37 °C, for the unknown strain they used. Faber *et al.* (2001) purified and characterized a benzoate-parahydroxylase of *A. niger*. This enzyme is a cytochrome P450 (CYP53A1). They did not report an optimum temperature. Vatsyayan *et al.* (2008)

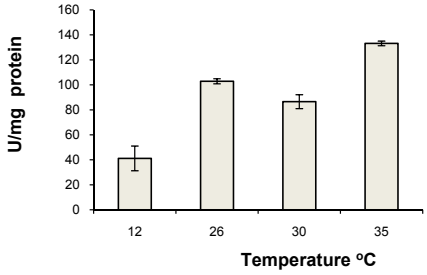

Fig. 7. Temperature effect in oxygenase activity in crude extract from SSF. Activity was measured using a spectrophotometric method with o-dianisidine. The amount of protein used was 58.6 μg per sample. Decane was used as substrate. Each bar represents the average value of two replicates (± SD). All the mean values are significantly different (α = 0.05).

observed that the optimum temperature for CYP activity was 37°C. They report that activity is rapidly lost at 40 °C and it decreases slower when the temperature is below 37 °C. They did not study the activity below 30°C.

3.6 Polyacrylamide gel electrophoresis (PAGE)

Figure 8 exhibits the results of PAGE. Lanes 1-3 were revealed for oxygenase activity (zymogram). They were loaded with cell free extract from SSF. Crude extract from SmF presented no activity with any of the substrates used even though, the amount of protein was the same for both kind of extracts or even 4 times greater for SmF(results not shown). The rest of the lanes were revealed for protein. Lane 4, was loaded with SSF sample and lane 5 with SmF extract, 35 μg of protein were used for each lane. The last lane corresponds to a molecular weight standard. It can be observed that the SSF samples show two well defined protein bands (*a*, *b*). As for SmF sample only band *a* is clear. Protein bands *a* and *b* matches with decane, hexadecane and bencene activities. Because, activity bands are too broad, it is not possible to allocate such activity to one of the protein bands a or b. Besides, both proteins have similar molecular weights, around 219 kDa. Vatsyayan *et al*. (2008) reported a CYP with a molecular weight that is half of the calculated in this work. Although, they run an SDS-PAGE so it may be a similar protein than the reported here.

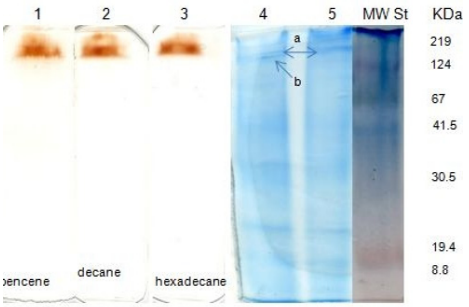

Fig. 8. Native PAGE in 6% acrylamide. Lanes 1-3 were loaded with crude extract from SSF (63.5 μg of protein per lane). Lanes 4 and 5 were loaded with crude extract from SSF and SmF respectively (35 μg per lane). Lanes 1-3 were revealed for oxygenase activity (zymogram) using the substrates indicated. Lanes 4 and 5 were developed for protein. The last lane corresponds to a molecular weight standard.

García-Peña *et al*. (2008) reported a zymogram for an oxygenase produced by *P. variotti* grown under SmF with toluene and with a mixture of toluene and glucose. The zymogram was revealed with benzene. They found that bands corresponding to the enzyme produced with toluene and glucose shown less activity, despite the fact that they did not observe catabolite repression during the toluene degradation. These results totally agree with our observations. Although in our case no bands of activity were visible for SmF.

Qi *et al*. (2002) commented that Woertz and Kinney (2000) reported that *E. Lecanii-corni* was not able to degrade benzene in liquid cultures. While they found, for the same strain, it was able to degraded benzene under solid culture.

The results shown for characterization of oxygenase(s) can be explained by the presence of a cytochrome P450 system. Faber *et al*. (2001) reported a CYP53A1and a NADH:cytochrome P450 reductase (CPR) from *A. niger*. The CYP51A showed only one band of apparent mass of 58 kDa and the CPR had also only one band of 78 kDa. However, this mass was calculated in SDS-PAGE like Vatsyayan *et al*. (2008) did.

Conclusions

The results discussed above show the importance of the type of fermentation since not only extracellular enzymes, but also intracellular enzymes, can be obtained with better characteristics by SSF. The enzymes induced by SSF had not only higher activity but also a broader specificity. Enzyme stability, of the intracellular oxygenases studied here, does not depend of the type of fermentation system. *A. niger*, ATCC 9642, produces labile oxygenases under SSF and SmF as well.

One possible reason for the higher oxygenase activity found by SSF compared with the results by SmF is the higher biomass production. Another reason could be the direct contact of the hifas of *A. niger* with the hydrocarbon when the fungus is grown by SSF, that promotes a better enzyme induction. Meanwhile in liquid culture *A. niger* grows as pellets and only a small surface is in contact with the substrate.

A. niger is a filamentous fungus that can be used to degrade hydrocarbons such as hexadecane in SSF, in the presence or absence of glucose as a cosubstrate. However, the Glu:Hxd should be carefully selected to reach the extent of degradation desired at a reasonably period of time. Or to select a Glu:Hxd depending if the objective is to have a small amount of biomass with

highly active enzyme (as for the case of biofilters) or to produce a high amount of biomass with oxygenase activity (as for the case of bioremediation).

Highly active oxigenases that can oxidize a broad spectrum of substrates (linear, non linear and aromatic hydrocarbons) can be induced in *A. niger*, under SSF using hexadecane as inducer. It is important to highlight the high activity presented by oxygenases of *A. niger* with recalcitrant compounds such as pyrene, toluene and phenanthrene.

Much work needs to be done in order to elucidate the type of enzymes that are induced. It will be desirable to do zymograms with enzyme extracts from SmF and SSF using hexadecane as sole carbon source in order to observe if the activity for SmF sample increases. It is also recommended to change the conditions for the PAGE to have a better resolution of the proteins. To further purify the enzyme(s) it will be useful to use cloning and expression techniques.

It will be interesting to look for molecules that act as signal molecules in catabolite repression. Besides to study glucose repression from the physiological and molecular points of view.

Acknowledgements

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