FUNGAL DEVELOPMENT

This chapter contains cultural informations on fungal strains, and more especially about culture of *Alternaria alternata*. Then the development of biochemical assays is described.

1. Cultural methods

The first step relating to the fungal cultivation is to determine physico-chemical parameters which promote optimum fungal development. Most if not all methods for the production of spores in culture were developed to provide a supply of inoculum for inoculation (Rotem, 1994). Fungal growth is affected by incubation temperature, nutrient media, time period of cultivation, and the type of culture conditions (i.e., stationary or shake culture). Conditions for optimal growth and sporulation were reported to differ within and among fungal species (Gupta et al., 1999). The major work performed about determination of optimal culture conditions was carried out with *Alternaria alternata*.

1.1. Culture of *Alternaria alternata*

1.1.1. Influence of culture media

A.alternata was grown in three different media at 24°C: V_8 juice agar (V_8), modified V_8 juice agar (mV_8), and modified Czapeck-Dox agar (mCD) in order to determine suitable media and conditions for optimum yield of spores and mycelial development. The mCD is a synthetic medium widely used in mycological laboratories. Many moulds produce very characteristic colonies on it and may also exude pigmented substances. Aerial growth is often suppressed and sporulation may be enhanced. Some mould, however, grows poorly on this medium and may even fail to sporulate altogether, often because of their inability to synthesize vitamins. The addition of agar to this medium makes it, in reality, a semi synthetic one (Malloch, 1997). The V_8 and mV_8 are natural media because they are partly or completely composed of natural materials. The V_8 juice used in V_8 medium probably contains many nutrients that fungi can

use, but we have a little idea what they may be. It is a medium that is used routinely in plant pathology and seems to be a good complement to Leonian's or PDA. Moulds that fail to sporulate on those media often sporulate heavily on V_8, or vice versa (Malloch, 1997).

Figure 37 shows fungal development after 9 or 14 days of incubation. The growth at 9 days of *A.alternata* in V_8 medium is characterized by a short downy grey mycelium which is dark coloured on its periphery. Culture in mV_8 medium presents an abundant white mycelium while mCD medium exhibits a much dispersed mycelium. Sporulation is highest on mCD medium, and lacking on mV_8. It is characterized by a dark ring on plate periphery after 14 days on mCD medium (Figure 37d) and appears less dense in V_8 medium after 20 days and is more located around central inoculate.

Hence, mCD medium promotes sporulation more than vegetative growth. According to Rotem (1994) all *Alternaria* species sporulate better in media poor in sugars, such as V_8 medium prepared without the addition of sugar. Such media suffice for easily sporulating *Alternaria*. Inhibition of vegetative development by starvation triggered sporulation of *A.alternata*, *A.dauci*, and *A.solani* transferred from an agar medium with nutrients to water agar (Shahin and Shepard, 1979). Spores of *A.alternata* are formed in long chain of approximately 10 spores or more, either beakless or with very short beaks.

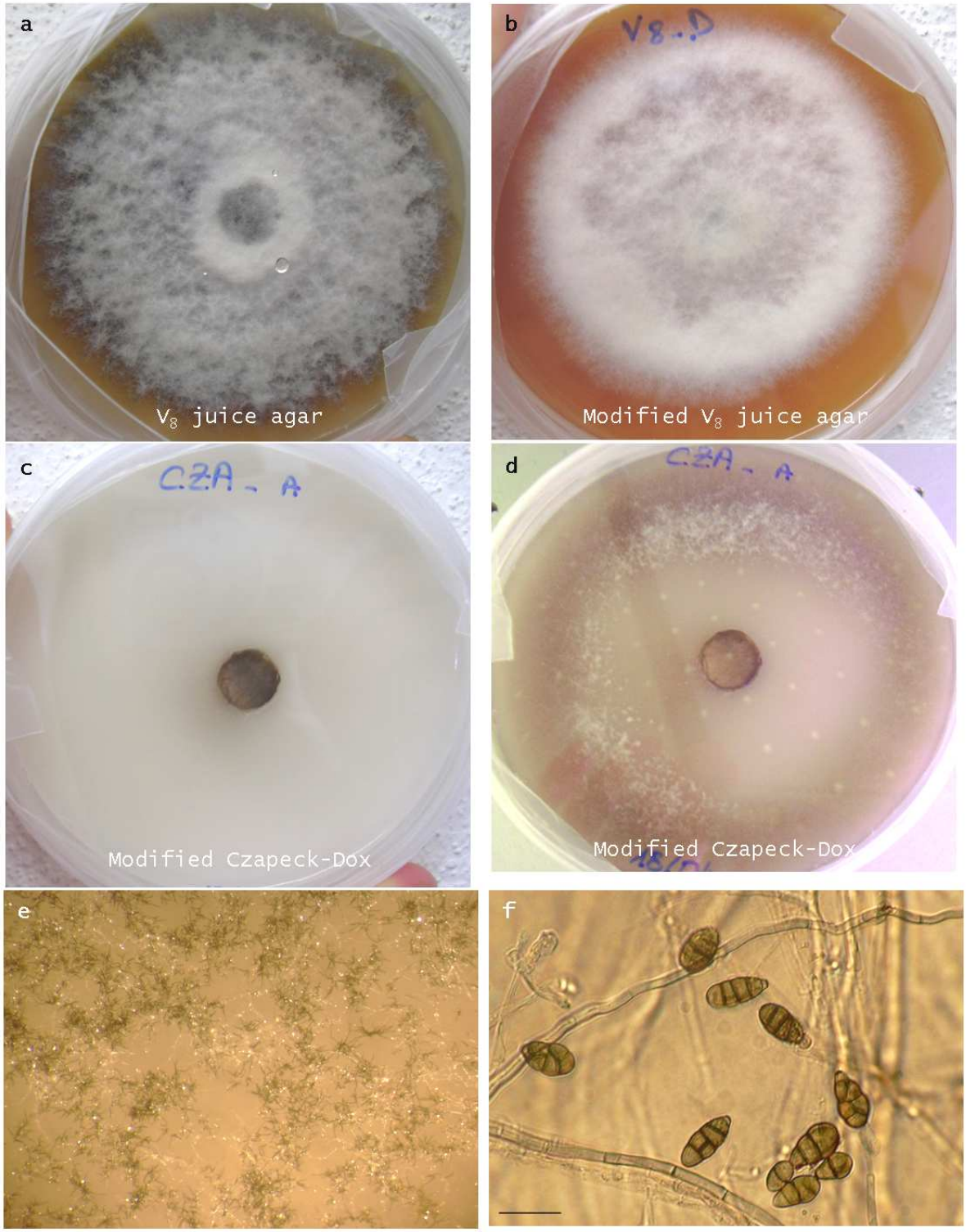

Figure 37 : *Alternaria alternata* grown on different media at 24±1°C - 90±5% RH – (a) 9 days in V_8, (b) 9 days in mV_8, (c) 9 days in mCD, (d) 14 days in mCD, (e) observation of spore chains from (d) with stereomicroscope x50, (f) observation of (e) with optic microscope (scale bare = 20 µm)

1.1.2. Influence of light on sporulation

Most species that form spores in chains sporulate easily and usually do not need triggering. Nevertheless, in some cases external induction increases sporulation in such species. Among all triggers for mass production of spores, irradiation is undoubtedly the strongest. Irradiation with UV light is the main element in most formulas for sporulation. For practical purposes it does not matter whether the source of light is a germicidal bulb with short UV wavelengths or a lamp with long UV or near ultraviolet (NUV) wavelength. It also does not matter whether glass or plastic dishes are used for the cultivation of fungi (Rotem, 1994). In species that sporulate in culture without induction, conidiophores* are obviously formed in darkness but become dark and look more typical when exposed to light (Rotem, 1994).

Figure 38 shows culture in mCD exposed or not to light after 14 days of incubation at 24±1°C and 90±5% RH. Cultures exposed to light were submitted to horticol neon (blue and red wavelengths are increased). For both sporulation is noticed but higher spore density is observed when cultures are exposed to light. According to Rotem (1994), mechanism of photosporogenesis involves two distinctive phases. The first, or inductive, phase leads to the formation of conidiophores; the second, or terminal, phase leads to the formation of conidia (spore). The temperature and light requirements for these two phases are distinct.

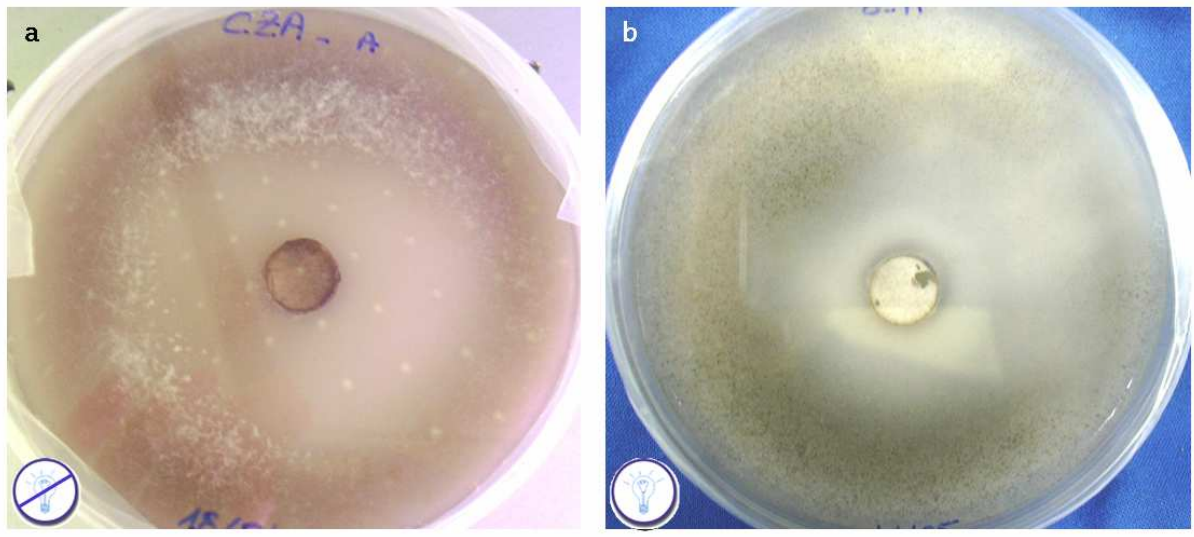

Figure 38: Influence of light exposition on sporulation for culture grown 14 days in mCD medium – (a) Not exposed to light, (b) exposed to light

* Conidiophore: It is a specialized hypha that bears conidia (spore).

The inductive phase is stimulated by NUV wavelengths in the range of 310-400 nm. The terminal phase proceeds best in darkness and is often inhibited by light (Leach, 1967).

Fungal growth is also affected by temperature, water activity (a_w), and pH. They are considered to be some of the most important factors (McMeekin and Ross, 1996). The ability to grow at high or low values of either temperature or pH increases when a_w increases. On the other hand, a decrease in a_w does not necessarily mean a decrease in pH or temperature optimum (Andersen and Frisvad, 2002). We follow DSMZ instructions relating to optimum temperature, pH and relative humidity for *A.alternata* cultivation.

The culture in mCD provides mainly spores, whereas one in V_8 favours mycelial development but permits also sporulation. Two points are important for the development of an accelerated laboratory test:

(i) Inoculation has to be reproducible in terms of concentration. For this, the use of spores suspension appeared first the most suitable method. It implies that large amounts of spores must be available. Hence, before the test *A.alternata* is grown in mCD 14 days and is daily exposed 5h to light.

(ii) To study biodeterioration of a matrix, both hyphae and spores have to be produced. At this stage of the study we plan to bring nutritive medium during the biodeterioration test, therefore we select V_8 medium to promote vegetative growth and sporulation.

1.2. Culture of *Aspergillus niger*

Aspergillus niger was grown in Potatoes Dextrose Agar (PDA) medium. Vegetative growth and sporulation were rapidly observed in these conditions (Figure 39a, Figure 39b, Figure 39c). Culture of *A.niger* yields numerous spores and promote in the same time mycelial development, which are the two main parameters for the biodeterioration test. Therefore, no further investigations about influence of parameters such as media, light exposition are carried out.

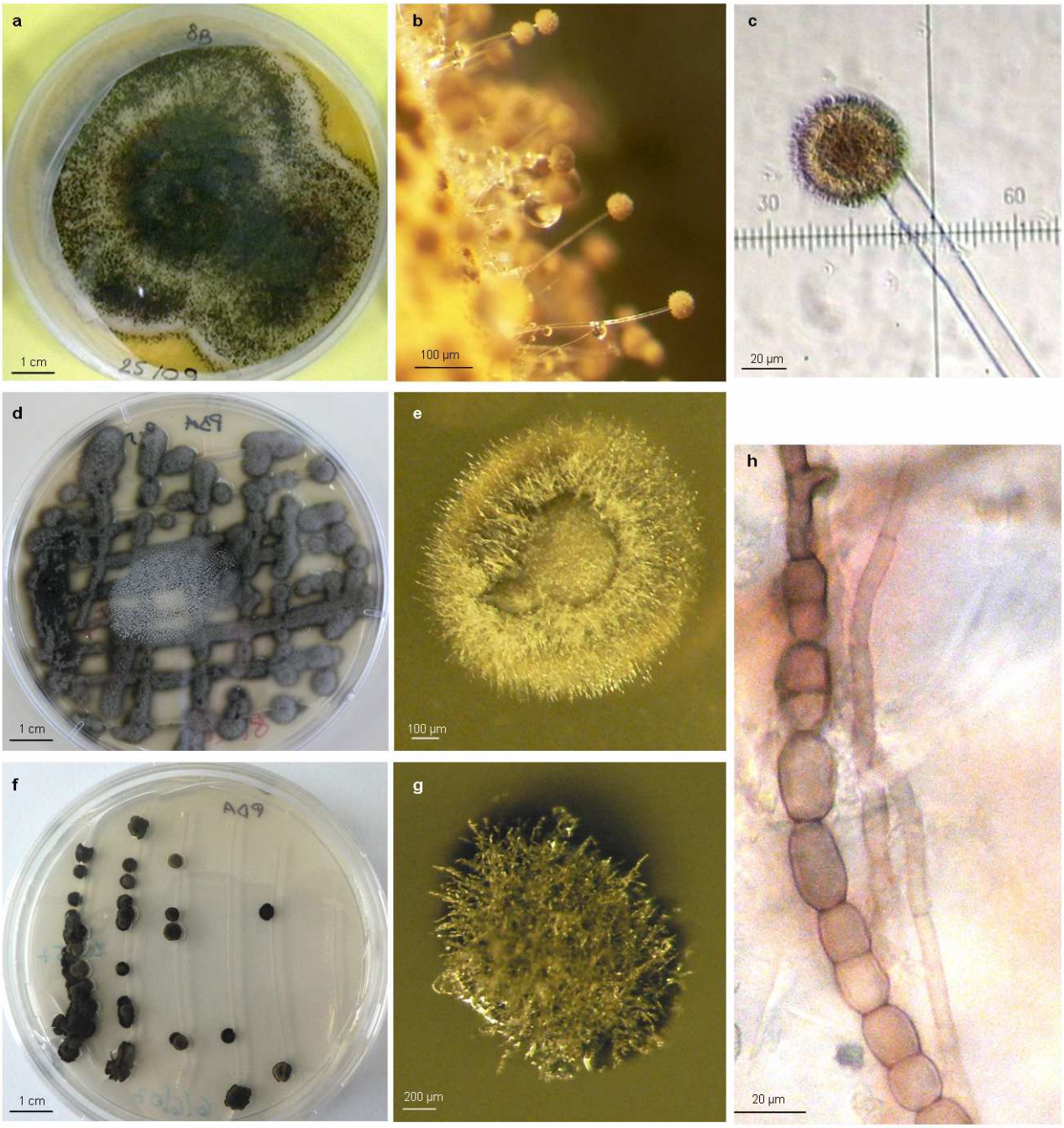

Figure 39: Observations of fungal cultures – (a) Culture in plate of *Aspergillus niger* grown 9 days in PDA, (b) observation of *A.niger* with steromicroscope, (c) observation *A.niger* with optic microscope, (d) culture in plate of *Exophiala* sp. grown 15 days in PDA, (e) observation of *Exophiala* sp. with stereomicroscope, (f) culture in plate of *Coniosporium uncinatum* grown 1 month in PDA, (g) observation of *C.uncinatum* with stereomicroscope, (h) observation of *C.uncinatum* with optic microscope

1.3. Cultures of *Exophiala* sp. and *Coniosporium uncinatum*

Both strains *Exophiala* sp. and *Coniosporium uncinatum* are involved in the final step of the study only. They are studied at the time of final optimization of biodeterioration test performed in the department of Microbiology, Genetic and Molecular sciences in Messina. We based our experiments on culture knowledge and ability of researchers working there. Therefore, no investigations on optimization of fungal growth were required. Figure 39d to 39h show culture and observations of both strains.

2. Development of assays for biomass quantification

2.1. Proteins assay

2.1.1. Bradford assay

The Coomassie Brilliant Blue protein assay established by Bradford (1976) is a spectroscopic analytical procedure used to measure the concentration of proteins in solution. The principle of the Bradford Protein Assay is based on an absorbance maximum shift from 465 nm to 595 nm for Coomassie brilliant blue G-250 (CBBG) when binding to protein occurs. The Bradford method is commonly employed since it is sensitive, fast, simple and inexpensive. The reaction between CBBG dye and protein occurs quickly and the product is stable for approximately 1h (Lü, 2006).

Some substances may interfere with the Bradford reagent during the proteins assay. Therefore to optimize the assay different solvent were tested: H_2O, methanol, physiologic solution (NaCl 0.9%), and Phosphate Buffer Saline (PBS). The performance of each assay is characterized by the limit of detection (LD) and the limit of quantification (LQ). According to the International Union of Pure and Applied Chemistry (IUPAC), the limit of detection, expressed as a concentration or quantity, is derived from the smallest measure that can be detected with reasonable certainty for a given analytical procedure (Mocak, 1997). The limit of quantification is not defined in the IUPAC. It can be defined in words that the LQ refers to the smallest concentration or the mass which can be quantitatively analysed with reasonable reliability by a given procedure (Mocak, 1997). The Table 5 presents characteristics of standard and micro-assay. The LD and LQ are calculated as follow (Recueil International des methods d'analyses – OIV, 2006):

The standard curve fit for use in the linear concentration range. It is defined by equation (1):

$$y = ax + b \quad (1)$$

The LD is the smallest concentration which can be distinguished from the blank with 0.13% of risk to keep sample holding nothing and is determined by (2) and (3):

$$y_{LD} = b + 3\,S_a \quad (2)$$
$$x_{LD} = (b + 3\,S_a) / a \quad (3)$$

With S_a = standard deviation at the intercept point of the standard curve.
The same reasoning is applied for LQ determination but with a multiplication factor of 10 (risk of 0.5‰ to conclude at the presence of the substance whereas it is absent).

$$y_{LQ} = b + 10\,S_a \quad (4)$$
$$x_{LQ} = (b + 10\,S_a) / a \quad (5)$$

Results show that the linear concentration of standard assays ranges from 0 to 500 µg/mL (Table 5) no matter the solvent used. The standard curve obtained for standard assay performed in PBS only is presented in Figure 40. The lowest LD and LQ are obtained with assay performed in PBS. Relating to the micro-assay, the standard curve is linear over all the concentration range tested, 0-10 µg/mL, with NaCl 0.9% and H_2O, whereas precipitation of proteins is noticed for assay carried out in methanol. While very low LD and LQ are reached with the micro-assay, a large volume of protein sample is used. The standard assay performed in PBS seems to be best compromise between low LD and LQ, the linear range of concentration, and the volume sample required.

2.1.2. Extraction of proteins

These assays were performed with standard proteins samples from Bovine Serum Albumin (BSA). Now, the next step is to determine the best method and protocol to extract proteins from sample that is to say to release proteins from the cell. The amount of energy that must be put into the breakage of cells depends very much on the type of organism and to some extent on the physiology of the organism. Some types of cell are broken readily by gentle treatment such as osmotic shock (e.g. animal cells and some gram-negative bacteria), whilst others are highly resistant to breakage. These include yeasts, green algae, fungal mycelia and some gram-positive bacteria which have cell wall and membrane structures capable of resisting

internal osmotic pressures of around 20 atmospheres (2 MPa) and therefore have the strength, weight for weight, of reinforced concrete (Chaplin, 2004).

Table 5: Characteristics of proteins assay performed in different solvents

Assay	Solvent	LD (µg/ml)	LQ (µg/ml)	Linear domain (µg/ml)
standard	NaCl 0,9%	12,8	14,2	0 - 500
standard	MeOH	7	7,8	0 - 500
standard	H2O	9,1	9,8	0 - 500
standard	PBS	5,3	6	0 - 500
micro	NaCl 0,9%	0,27	0,36	0 - 10
micro	MeOH	0	0	0
micro	H2O	0,2	0,252	0 - 10

LD = Limit of detection
LQ = Limit of quantification

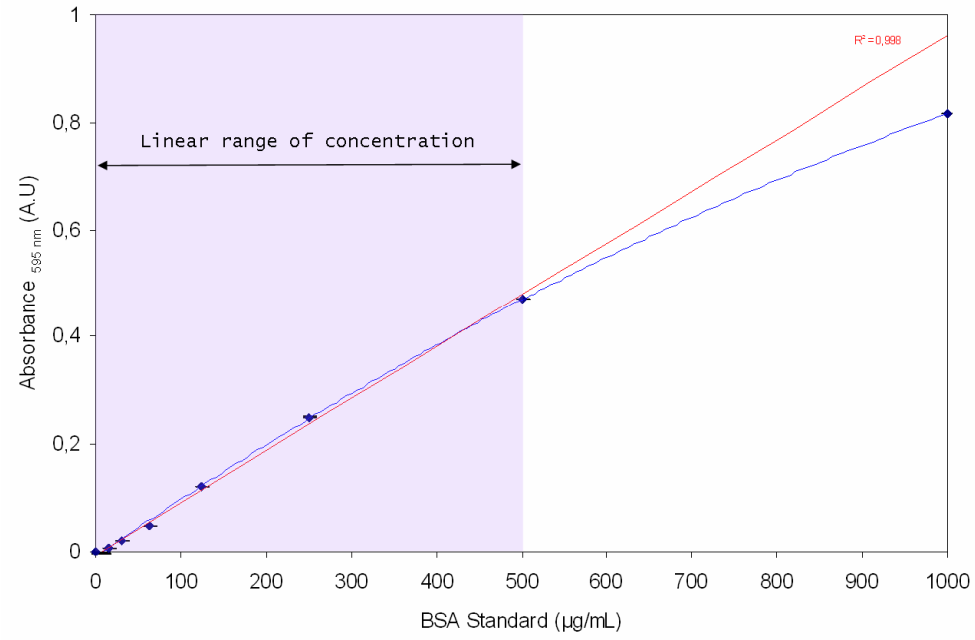

Figure 40: Standard curve produced from standard assay performed in PBS

Consequently a variety of cell disruption techniques have been developed involving solid or liquid shear or cell lysis. Ultrasonication produces cavitation phenomena when acoustic power inputs are sufficiently high to allow the multiple production of micro-bubbles at nucleation sites in the fluid. The bubbles grow during the rarefying phase of the sound wave, and then are collapsed during the compression phase. On collapse, a violent shock wave passes through the medium. The whole process of gas bubble nucleation, growth and collapse

due to the action of intense sound waves is called cavitation. The collapse of the bubbles converts sonic energy into mechanical energy in the form of shock waves equivalent to several thousand atmospheres (300 MPa) pressure. This energy imparts motions to parts of cells which disintegrate when their kinetic energy content exceeds the wall strength. Much of the energy absorbed by cell suspensions is converted to heat so effective cooling is essential.
Fungal cells were typically subjected to 7, 10, 12 cycles of sonication. After centrifugation of sonicated solution measurements of proteins content are performed on the supernatant.

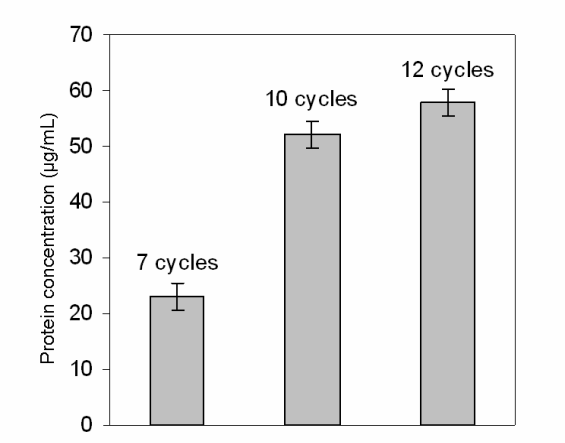

Figure 41: Influence of sonication on proteins extraction

The protein concentration increases between 7 and 10 cycles of sonication whereas similar concentrations are obtained after 10 and 12 sonication cycles (Figure 41). Although samples are kept in ice, heating of sample is noticed after 12 cycles. Ten cycles of sonication seems to be necessary to release all proteins content without heating the samples.

2.2. Ergosterol assay

Ergosterol is the most abundant sterol held in the cell membranes of most filamentous fungi, certain microalgae, protozoa and yeasts (Newell, 1992; He et al., 2000).
The ergosterol assay was developed by Seitz et al (1976), and consisted of four major steps: initial extraction; saponification; partitioning and evaporation; and HPLC determination. This basic technique has been applied with modifications (Bentham et al., 1992; Hill et al., 1993). Despite all the modifications, the analytical techniques of ergosterol determination involving saponification remain time-consuming, which may serve to limit the utility of the analysis (Ruzicka et al., 1995; de Ridder-Duine et al., 2006). Hence, the use of a simplified method of extraction is primordial to make ergosterol analysis attractive.

2.2.1. HPLC determination

The ergosterol is eluted with acetonitrile – methanol (80:20 v/v) at a flow rate of 0.5 or 1 $mL.min^{-1}$. The injected sample volume was 20 or 100 µL. The retention time of ergosterol obtained at 0.5 mL/min was 22 min with the first test. Therefore, it was decided to increase the flow rate to 1 mL/min to lower retention time. In these conditions, the ergosterol peak appears at about 16-18 min.

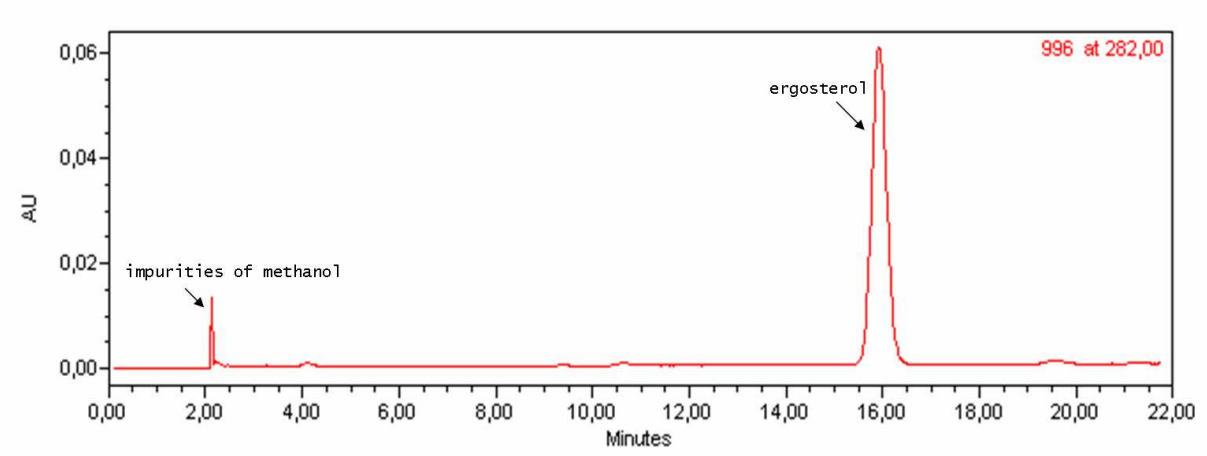

Figure 42: HPLC chromatogram of solution of commercial ergosterol eluted with acetonitrile-methanol (80:20 v/v) with a flow rate of 1 mL/min

The chromatogram obtained with a 100 µL loop is shown on Figure 42. Two peaks are observed: the first one appearing at 2.3 min remains constant no matter the dilution factor used. This peak is attributed to impurities present in methanol. The ergosterol is identified thanks its UV spectrum. Figure 43 presents the UV spectrum corresponding to the second peak, eluting at 15.9 min. A strong absorbance at 282 nm, characteristic of ergosterol is observed.

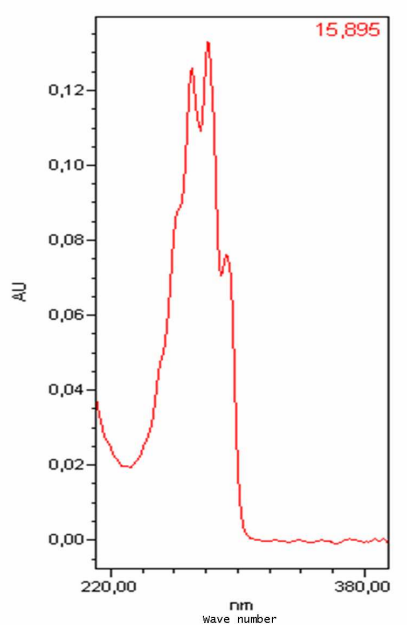

Figure 43: UV spectrum corresponding to the eluted peak at 15.895 min

The peak area at the absorption maximum of 282 nm was used for quantification with an external standard via eight point-calibrations. For a standard range of ergosterol solutions at concentrations of between 0 and 53 mg/mL, the relationships between peak surface areas and ergosterol concentrations were linear, with a coefficient of correlation, r^2, of 0.999 (Figure 44).

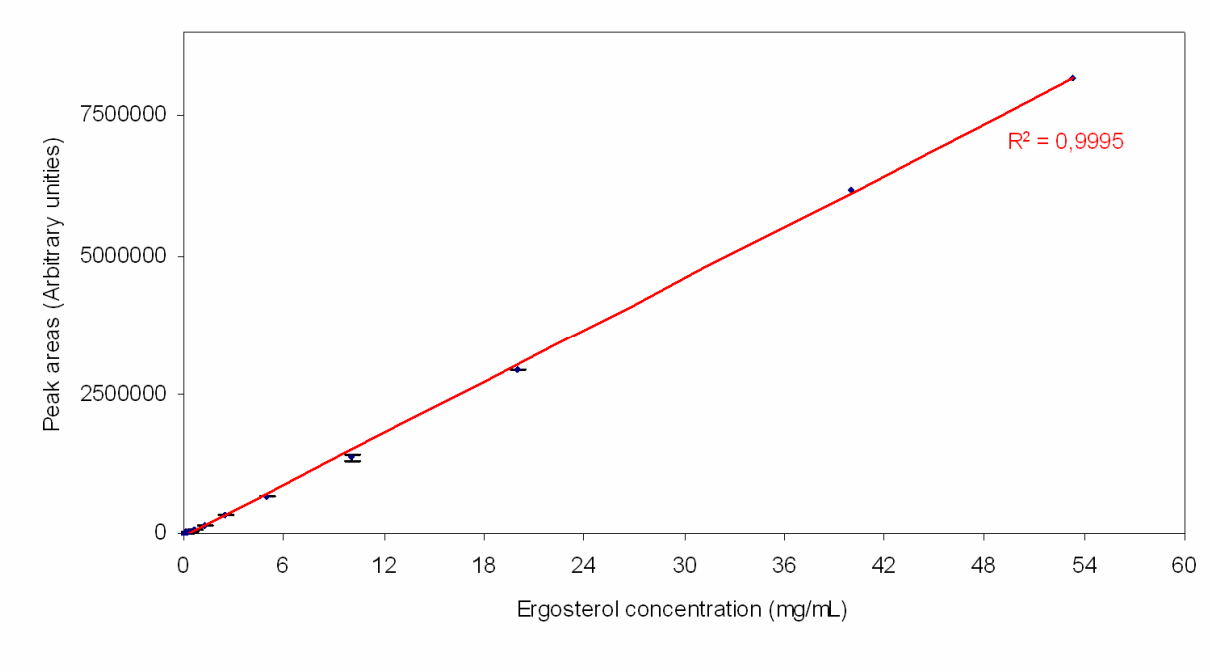

Figure 44: Standard curve of ergosterol solution

The limit of detection (LD) and quantification (LQ) are shown in Table 6. LD and LQ were determined as three and ten times the standard deviation of noise over the time range of the eluting peak, respectively.

Table 6: HPLC data for ergosterol assay

Mobile phase	Retention time	Flow rate	Injected volume	LD	LQ
v:v	min	mL/min	µL	ng/mL	ng/mL
ACN / MeOH - 80:20	22	0,5	20	-----	-----
ACN / MeOH - 80:20	17,7	1	20	120	398
ACN / MeOH - 80:20	15,9	1	100	24	79,6

LD = Limit of Detection ACN = Acetonitrile
LQ = Limit of Quantification MeOH = Methanol

LD and LQ obtained are consistent with those reported in literature (Boissier, 2003; Robine et al., 2006).

2.2.2. Extraction of ergosterol

Two fractions of ergosterol have been recognized, namely free and bound (esterified with fatty acids) (Weete, 1989). The original method consisted of an extraction of free ergosterol in methanol, followed by alkaline hydrolysis of fatty acids (saponification) (de Ridder-Duine et al., 2006). The utility of the saponification step has been under discussion (Davis and Lamar, 1992; Ruzicka et al., 1995; Djajakirana et al., 1996; Gong et al., 2001). Whereas Davis and Lamar (1992) found that more ergosterol was obtained by alkaline extraction, such differences were not found by others (Ruzicka et al., 1995; Djajakirana et al., 1996). It was argued that the saponification phase not only resulted in the release of esterified ergosterol, but also in the loss of heat-unstable free ergosterol. Therefore, Ruzicka et al. (1995) proposed a method of non-alkaline extraction in combination with ultrasonication to enhance the release of ergosterol from fungal membranes. The basic idea of this extraction is that ergosterol can be released from fungal membranes by mechanical disruption. This can replace the time- and chemical-consuming saponification step of the original method. The omission of the saponification step allowed for considerable increase in sample throughput (de Ridder-Duine et al., 2006). The Ruzicka method uses the separation of ergosterol from a polar (methanol) to a non-polar solvent (hexane: propan-2-ol).

The use of a sonic probe to extract ergosterol from sample was also reported by Boissier (2003) and Robine et al. (2006) which obtained reliable results.

The Ruzicka method appears very interesting from time consuming and simplicity point of view. Hence, extraction of ergosterol by mean of a sonic probe has been selected for this study. To validate the extraction protocol adapted to our conditions, cement specimens exposed to fungal colonization are needed. At this stage of the study, the experimental set-up relating to the biodeterioration test is not well defined. Thereby, samples are not available to test and optimize the extraction phase selected. Moreover, the method validation should also comprise a recovery study. For this, known volumes of ergosterol standard solutions should be added to cement specimens non-exposed to fungal colonization, at different level. The spiked samples should be extracted following the Ruzicka method.

2.2.3. Discussion

Before extraction varying amounts of ergosterol are lost during sample preparation and storage, with losses tending to be ordered: storage in methanol ≤ freezing followed by lyophilisation < lyophilisation ≤ freezing at -20°C << air or oven drying (West et al., 1987; Newell et al., 1988; Zelles et al., 1991; Davis and Lamar, 1992). Gessner et al. (1991) stored samples in methanol at room temperature for one week without losses of ergosterol, and extracted sample could mostly be stored at -20°C under air for 4-6 months without pronounced losses.

Moreover, knowledge of ergosterol concentration alone, does not allow conclusions about the absolute amount of fungus present. For this, appropriate factors to convert ergosterol values into biomass in terms of mycelial dry mass (or similar parameters) are necessary (Gessner and Chauvet, 1993). If valid conversion factors are to be obtained, then, culturing conditions must be chosen such that natural growth conditions are closely simulated. Indeed, the amount of ergosterol in fungal tissue is not constant. It depends among others on the fungal species, age of the culture, developmental stage (growth phase, hyphal formation and sporulation) and growth conditions (growth media, pH and temperature). Unfortunately, no clear trend relating ergosterol to any of these factors has yet been determined (Parsi and Gòrecki, 2006).

The method of ergosterol assay was developed in centre SPIN in Ecole Nationale Supérieure des Mines de Saint-Etienne. The final biodeterioration was performed with four fungal strains. While fungal growth was observed, no biomass quantification was carried out as the

experiment was not achieved in Ecole Nationale Supérieure des Mines de Saint-Etienne but in the Science University of Messina.

3. Conclusion

- Cultivation of *Alternaria alternata* on V_8 medium favoures vegetative development and allows sporulation, whereas cultivation on mCD medium promotes sporulation essentially. The exposition to light enhances sporulation phenomenon.
- Proteins assay, using the standard, method allowed obtaining a limit of quantification of 6 µg/mL.
- Ergosterol assay performed with standard solution, using the mobile phase composed of acetonitrile/methanol (80:20 v/v), permitted to quantify and characterize ergosterol at 15.9 min. The limit of quantification obtained is 79.6 ng/mL.
- More investigations about ergosterol assay must be carry out with specimens exposed to fungal colonization. Factors to convert ergosterol values into biomass must be determined for each strain.