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Final Report on Fungi Compartment Studies:
Liquid-state Fungi Reactor - Initial Concept

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Executive Summary

The contents of this research report relate to study results from secondary research activity.

The primary result from this research survey is an elucidation of the options available for evaluation of a liquid cultivation system. A second research result is a corresponding survey of the available control design options, which is developed in full detail in the appendix to this report by Adersa.

The principal research results are developed summaries of the relevant technologies required to commence the investigation for the conceptual design, *e.g.* a summary of state of the art agitation technologies is supplied in sections 3, 4 and 5. In addition, auxiliary information that puts the study in context is supplied, *e.g.* the introduction in section 1 and the overview of liquid cultivation systems found in section 2.

Cultivation conditions in general are discussed in section 6 but without a final fungal selection, the details supplied is necessarily brief and general. This is also true of sections 7 and 8, which discuss the feedback options available and reactor morphology, respectively.

Due to the very preliminary nature of this study a number of open questions remain to be addressed. A full list of these open questions is provided in section 9. In particular, the question of the selected fungus may still remain open. Most importantly, no technology option has been presented that cannot be scaled either up or down. Then, a summary discussion in presented in section 10.

Finally, a review of state-of-the art material was undertaken to provide a basis for presenting particular technology options. This is evidenced by the references that justify the technology selections shown. Details of the references are provided at the end of the report.

Perhaps the most salient item uncovered in the research study was that a liquid cultivation system has been developed for the production of a lignin degrading enzyme system used in the paper pulp processing industry. The enzyme system is harvested from a fungus, for sale under the trademark “Lignozym®”. The lab. scale version of this system produced the most impressive results found in the study. Depending on the pulp and pre-treatment, delignification was in the range 33.7-61.9%, with a “retention time” range of 1-4 hours (Call & Mücke, 1997)

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1. Introduction

At the start of this project, in January 2002, it was envisaged that the fungi reactor would be based on a solid-state substrate. Consequently, fungi reactor design was approached with this material phase in mind. However, following the release of uncertain results by ATO in early 2003, the solid-state substrate assumption was revised. Quantitative lignin degradation results at a petri-dish scale from ATO in July 2003 led EPAS to propose a shift in work focus as it appeared likely that a liquid, rather than solid state, fungi reactor was required for the MELISSA loop. The outcome of the liquid state reactor investigation is described in this initial supplementary report (TN6.4b).

This initial report is a supplement to the main solid-state report, TN6.4a, delivered in respect of ESA study contract 15689/01/NL/ND in September 2003. There is an associated contract between EPAS and the National University of Ireland, Maynooth (NUIM). The combination of the two reports fulfils the deliverables obligation on NUIM in respect of this contract. This initial report (TN6.4b) is focussed on the growth of fungi and/or mushrooms in a liquid-state reactor, whereas the companion final report (TN6.4a) is focussed on the growth of fungi in a solid-state reactor.

It is emphasised that, this report (TN6.4b) is a supplement to the companion final report (TN6.4a) as it deals only with those aspects of liquid bioreactor design that are different to solid-state reactor design. Common aspects, *e.g.*: an overview of lignin degradation, supply air filtration, room air conditioning, *etc*. are dealt with in the companion final report (TN6.4a).

This initial report details the outcome of the studies on the design of a liquid-state fungi compartment bioreactor, up to the end of December 2003. The report is composed of a main body authored by NUIM and a control appendix authored by a sub-contractor, Adersa.

In addition to desk studies, five extensive interviews with experts in their fields were undertaken. The first interview was with a leading researcher into shaken culture technology, Prof. Jochen Büchs, Head of Biochemical Engineering in the University of Aachen. This first interview was conducted at the research laboratories of the faculty in September 2003. The second interview was with a leading researcher into fermentation engineering, Dr. Donal O'Shea, School of Biotechnology in Dublin City University. This second interview was conducted at the research laboratories of the School in October 2003. The third and fourth interviews were repeats of two previous interviews, undertaken for TN6.4a, with two mycologists in the Department of Biology, NUIM, Prof. Martin Downes and Dr. Kevin Kavanagh. These latter repeat interviews were undertaken in August 2003. The final interview was with Dr. Hans Call, of Bioscreen E.K., developer of the Lignozym® lignin-degrading enzyme system. This interview was conducted at Bioscreen in Ubach-Palenberg in November 2003.

This report deals with the three study areas of work-package 7 (that deal with a liquid-state design) that were defined in the associated contract between the National University of Ireland, Maynooth (NUIM) and EPAS, as:

1. Study of different possible fungi compartments including instrumentation and control.
2. Study of material handling, substrate preparation, harvesting and recycling.
3. Study of interactions and interfaces with other compartments.

Each of the liquid-state system alternatives identified, and the associated subsystems, involved in achieving the required conditions for a liquid cultivation system are discussed individually in the following sections. Then some open questions are introduced and where possible, a preliminary discussion regarding the salient fungal reactor design issues is provided, and the references made are listed.

2. Liquid culture cultivation systems

Due to its multi-disciplinary nature, literature in this field tends to describe fragments of a whole system. Hence, a wide range of diverse source material has to be examined to obtain a picture of the many issues that have to be addressed in designing a liquid cultivation system.

To the knowledge of the author, no gas or plasma cultivation systems have been developed for the cultivation of fungi. Therefore, the fungal cultivation system state choices are limited to either solid-state systems or liquid-state systems. As the final report's companion (TN6.4a) is focussed on the growth of fungi in a solid-state reactor, this liquid-state system report completes the elucidation of the fungal cultivation system choices available.

Cultivation of fungi in a liquid culture is not necessarily an improvement over a corresponding solid-state system. This is because the advantages of solid-state reactors over liquid-state reactors for the production of fungal enzymes has been explained and shown (Viniegra-Gonzalez *et al*., 2003). However, due to the nature of the MELISSA loop a liquid-state fungal reactor is necessary (Demey, 2003), hence, the necessity for this initial investigative study report.

Whether grown on the surface, or submerged in the cultivation medium, both fungal mycelium and/or the mushroom fruiting body can, and have been, cultivated in liquid cultivation systems. Note that the term 'liquid cultivation' is used here in an analogous sense to the solid-state term 'substrate'. In contrast to a mushroom solid-state system, cultivation on the surface (only) is volumetrically inefficient. Therefore, this report has a principal focus on submerged cultivation systems.

In the context of dietary variety as opposed to nutritional optimality (Lasseur, 2002), it should be noted that for both shaken and stirred cultures, mushrooms can be grown in submerged conditions (*e.g.*: *Pleurotus* - Bukhalo & Solomko, 1978; *A. Bisporus* – Grappelli *et al*., 1978). In addition to mushrooms themselves, several edible mushroom mycelia have been produced using a liquid cultivation system, *e.g. Volvariella bombycina*, *Lyophyllum ulmarius*, and *Pleurotus citrinopileatus* (Cheung, 1997). Furthermore, *Morell* mycelial pellets that retain the flavour of the ascocarp have been produced in a liquid culture (Smith & Berry, 1974). Moreover, a commonly available myco-protein, Quorn®, has been produced through the growth of a filamentous fungus, *Fusarium graminearum*. This fungus is grown on a commercial scale in an air-lift fermenter, followed by RNA-reduction using a heat-shock treatment (Trinci, 1992), as illustrated in Figure 2.1, from Trinci (1992). However, as the primary purpose of a fungi bioreactor in the MELISSA loop is to degrade the lignin content of the substrate, further discussion on mushroom or mycelium growth for food production in liquid cultivation is not the principal focus here.

Regardless of the medium in which a fungus is grown, it is expected that the primary aerobic metabolic pathway is the same for liquid and solid cultivation systems. The primary assimilative metabolic model was introduced in TN6.4a, using Loeffen and Martin's (2001) assumption that the heat liberated can be approximated by the exothermic oxidation of glucose ($C_6H_{12}O_6$) as described by Equation (2.1).

$$C_6H_{12}O_6 + 6O_2 \text{ (+ fungus)} \rightarrow 6CO_2 + 6H_2O + \text{Heat.} \qquad (2.1)$$

This model is in direct contrast to the model used in most liquid fermentation cultivation systems, in that it requires oxygen as an input. A typical (Berry, 1982) fermentative metabolic model used for glucose ($C_6H_{12}O_6$) is described by Equation (2.2).

$$C_6H_{12}O_6 \text{ (+ yeast)} \rightarrow 2C_2H_5OH + 2CO_2. \qquad (2.2)$$

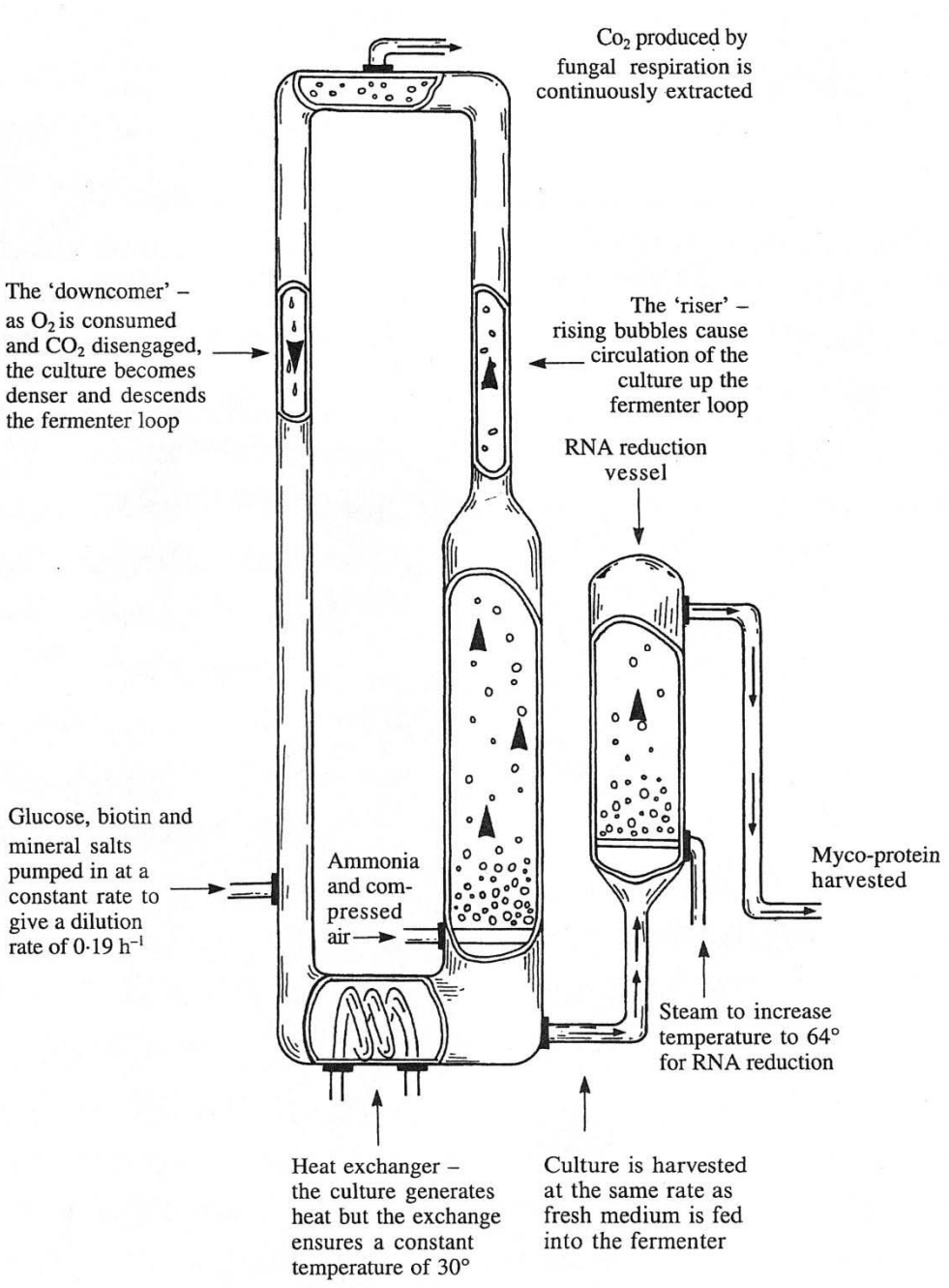

Figure 2.1: Example of a commercial (pressure driven) air-lift stirred culture system, "*Diagrammatic representation of the air-lift fermenter used by Marlow Foods at Billingham for the production of myco-protein in continuous flow culture.*" Reproduced from Trinci (1992) with editor's permission.

The capability of some fungi to degrade lignin in a liquid culture is indirectly referred to by some researchers, *e.g.* the reference to laccase production by Lorenzo *et al*. (2002) or Singh *et al*. (1987), and also can be inferred *e.g.* from the extracellular enzyme production results of Grappelli *et al*., (1991) where both laccase and peroxidase were found to be produced by *Lentinus* in a submerged culture.

Three different types of submerged cultivation systems are normally distinguished by the motion or agitation, within or without, of the vessel, or flask, that contains the liquid. These three systems are normally referred to as being either: static, shaken, or, stirred.

For both static and shaken systems, the cultivation container is ordinarily a non-porous vessel. Such a vessel generally facilitates heat exchange with the ambient environment, *i.e.* the surface supporting the vessel and the room air around it, and is usually fitted with either a plug that seals the vessel to obstruct any gas exchange with the room air, or conversely, fitted with a cotton plug to facilitate gas exchange with the room air.

Normally, for stirred systems the cultivation container is a flask. Due to its widespread application in the baking and brewing industries, this system is often referred to as a stirred-tank fermenter. Recall that fermentation is a biological process where the micro-organism used is yeast, which is a unicellular fungus. This observation is made explicit to emphasise the commonly perceived suitability of this cultivation system for fungal growth to the exclusion of alternative systems.

The principal benefit normally credited to a flask or double jacket vessel, compared to a single-walled vessel, is the ability to control the thermal state of the cultivation system independently of the state of the surrounding surface and room air conditions. Consequently, this feature is normally associated with stirred systems. However, no rational limitations are apparent to limit the application of flasks to either static or shaken systems. Hence, it is important to note that independent thermal controllability is a vessel characteristic as opposed to a motion-associated feature.

An alternative principal comparative feature that is sometimes beneficially credited to the stirred system compared to the shaken system is good mixing. However, to the author's knowledge, no study of stirred systems and, baffled and/or unbaffled shaken systems has been undertaken to establish their relative performance in this regard. Hence, there appears to be no great scientific evidence to support a contention that a stirred system will provide improved spatial homogeneity, the implicit objective of good mixing, compared to a shaken system.

A third comparative feature used in system selection is agitation, which in the context of mixing is discussed in the previous paragraph. Agitation, whether internal in the case of a stirred system, or external in the case of a shaken system, affects both mixing and gas-liquid mass transfer area. Again, to the author's knowledge, no study of stirred systems and, baffled and/or unbaffled shaken systems has been undertaken to establish their relative performance in this regard. However, Büchs's (2001) observation that it is "easy to determine" the gas-liquid mass transfer area for an unbaffled shaken bioreactor and "difficult to determine" for a stirred tank fermenter provides insight as to why such a study may not have been undertaken.

Although it is generally not associated explicitly, improved aeration or ventilation is a characteristic frequently associated with stirred systems compared to shaken systems. Again, to the author's knowledge, no study of comparative stirred systems and, baffled and/or unbaffled shaken systems has been undertaken to establish their relative performance in this regard. No rational limitations are apparent to limit the application of an aeration sub-system to stirred systems. Hence, it is important to note that independent ventilation controllability is a sub-system characteristic as opposed to a motion-associated feature and has been applied to a shaken system (Akgüen *et al.*, 2004?).

Grappelli *et al*.'s, (1991) results showed that cultures grown in a shaker produced less enzymes than those grown in a "bioreactor" (fermenter ?). This difference was explained as "enzymatic activity appears considerably higher with a peak level when the cultures are better aereated in a bioreactor", thus emphasising the need for a readily available oxygen supply in the liquid culture. Note from this comment that there is an implication that a "bioreactor" provides better aeration. It is suggested that a system independent critical comparison between the effect of aeration/ventilation rate and enzyme activity would be more appropriate in a decontamination context.

A final but important comparative feature is a system's susceptibility to the ingress of pathogens and their subsequent removal or vessel decontamination. Frequently, an external motor drives the mixing shaft in a stirred tank fermenter. The seal on this shaft adds one additional point of ingress for pathogens compared to a static or shaken vessel. Furthermore, the mixing shaft and mixing paddles add additional retentative surfaces for pathogenic reservoirs compared to a shaken system. These additional surfaces pose extra problems from a decontamination perspective. Consequently, it is asserted that a static or shaken system is inherently superior to a stirred system in a decontamination context.

Commercially, liquid fungal cultivation chiefly occur in the food, *e.g.* baking, brewing, protein supply (Trinci, 1992), pharmaceutical, *e.g.* anti-biotics (*e.g.* Penicillin), bio-insecticides (Helyer *et al*., 2003), bio-remediation (Boyle *et al*., 1998), and science laboratory sectors. Within these sectors, to the knowledge of two mycologists (Downes; Kavanagh, 2003) no liquid fungal cultivation system could not be classified as either static, shaken, or, stirred. Therefore, it would appear from this overview of these three systems, that the selection criteria for a liquid fungal cultivation system should ideally include at least the following items.

1. thermal controllability – independent or ambient dependent ?
2. mixing quality – spatially clustered/dispersed or spatially homogenous or (directional) gradients ?
3. gas-liquid mass transfer area – limitations may induce anerobic and/or spatially clustered/dispersed conditions ?
4. ventilation rate – limitations may induce anerobic and/or spatially clustered/dispersed conditions?
5. decontamination facilitation – after infection, is it easy to remove pathogenic reservoirs or bugs ?

In a multi-stage fungal growth context, liquid cultivation systems are sometimes used as a source of mycelial innoculum for further growth in a solid-state reactor. Consequently, the study objectives and results analysis of such multi-stage studies have to be approached with caution, *e.g.* injectability is not an obvious system associated candidate criterion to evaluate a fungus for its lignin degradation capabilities. Nonetheless, some of the caveats developed are directly transferable, *e.g.* "the fermenter used to produce spawn of edible fungi is distinct from the traditional fermenter fitted with stirring gear" (Kewu & Jinhua, 1998), because of the requirement for "less cutting power … harmless to mycelia".

3. Static Culture Technology

By way of introduction to static liquid cultivation, agitated liquid systems are first distinguished. Agitated liquid cultivation systems are categorised in this report depending on whether the container's agitation mechanism is located within or without the container. Whilst systems where the agitation mechanism is external to the container can almost universally be described as shake flask cultivation, the same is not true for systems where the agitation mechanism is internal. Hence, although all systems where the agitation mechanism is internal are classified as stirred culture technology, different stirred culture technologies can be distinguished based on the stirring mechanism(s) used.

The objective of agitation is to mix the fluid in a container. As agitation mechanisms vary, it is suggested that the mixing quality is dependent on the agitation mechanism used, *e.g.* the mixing of liquid and (over-liquid) gas, is arguably better in a shaken flask system than in a stirred-tank system. For static systems, agitation is absent and hence it is to be expected that mixing quality in a static system is poorer than an equivalent agitated system. Recalling that diffusion is a slow process, the main effect of the absence of agitation is that spatial homogeneity inside the vessel or flask is extremely unlikely. This is generally regarded as a negative characteristic. Therefore, due to the observation that static culture technology is a subset of agitated culture technology, it is only considered here to the extent that the motion parameter is set to zero, *i.e.*, there is no shaking or stirring.

Consequently, the reading intent here is that the following two sections on agitated technology should be read, with static technology perceived as a subset of agitated technology, under the constraint of poor spatial

homogeneity. It is recognised that this is a non-trivial constraint that has several important ramifications, of which the following are examples:

- a particular enzyme was only produced by *Pleurotus ostreatus* under static culture conditions (Okamoto *et al*., 2002),
- under static culture conditions, mycelial harvesting of filamentous fungi is often facilitated using a (membrane) filter plate in the submerged culture (Kavanagh, 2003), which could act as an undesirable baffle in an agitated design, and,
- fragmentation of filamentous fungi can occur in agitated cultivation (*e.g*. Shamlou *et al*., 1994) and this can affect population dynamics (*e.g*. Krabben *et al*., 1997).

A secondary rationale for including static culture as a subset of agitated technology, is that sometimes cultivation is undertaken as a two-stage process. For example, Fang and Zhong (2002) found that ganoderic acid "was considerably enhanced" by a "4-day shake-flask fermentation followed by a 12-day static culture". In this context, it is suggested that changing the value of the agitation parameter is analogous to changing the value of the temperature parameter, as is done in commercial mushroom growth, *i.e.* the temperature reduction used to initiate mushroom primordia formation. Such a change in parameter value is not normally used to distinguish one technology from another, and this is the convention followed here.

As a final note on static cultivation systems, it has been observed that mycelial pellet formation occurs in static or poorly agitated flasks (Martin & Demain, 1978). Increasing the shearing action of an agitation system, *e.g*. a higher shaking or stirring rate, or using a baffled flask, can help avoid pellet formation. This is important commercially for several fungal derived products, *e.g*. penicillin, citric acid, *etc*. Therefore, one of the agitation selection criteria is dependent on the cultivation objective, *i.e.* whether pellet formation is desired or not. In this respect, the observation of Smith and Berry (1974) is appropriate; "Since it is impossible to maintain homogenous growth conditions during pellet formation, conditions in which pellet formation does not occur are normally sought."

4. Shaken Culture Technology

Shaken cultivation is frequently associated with batch growth. However, it is possible to use shaken cultivation in a continuous mode and this has been achieved (Akgüen *et al*., 2004?). Figure 4.1 illustrates the system layout used by Akgüen *et al.* for continuous cultivation. Therefore, this technology should not be disregarded on the basis of its assumed temporal operation.

Shaken, regardless of the shaking motion, *e.g*. rotary, orbital, linear oscillation, *etc*., technologies are not a fail-safe space-friendly technology. This is because, failure of the vessel, or flask, retaining mechanism could lead to the presence of a projectile in the local and perhaps unprotected environment, which is a most undesirable occurrence.

A sometimes recurring problem with shaken flask cultivation is the occasional failure of one or more of the various retaining mechanisms. The impact of a failure of the primary retaining mechanism has been discussed in the previous paragraph. Other retaining mechanisms can also fail, *e.g*., referring to Figure 4.1, on the ventilation supply, the feedstock supply, and/or the harvest take-off. A failure of any one of these retaining mechanisms would have a critical impact on the cultivation system.

Consequently, because a more space-friendly agitation mechanism exists, *i.e*. stirred culture technology, shaken culture technology is only briefly examined here.

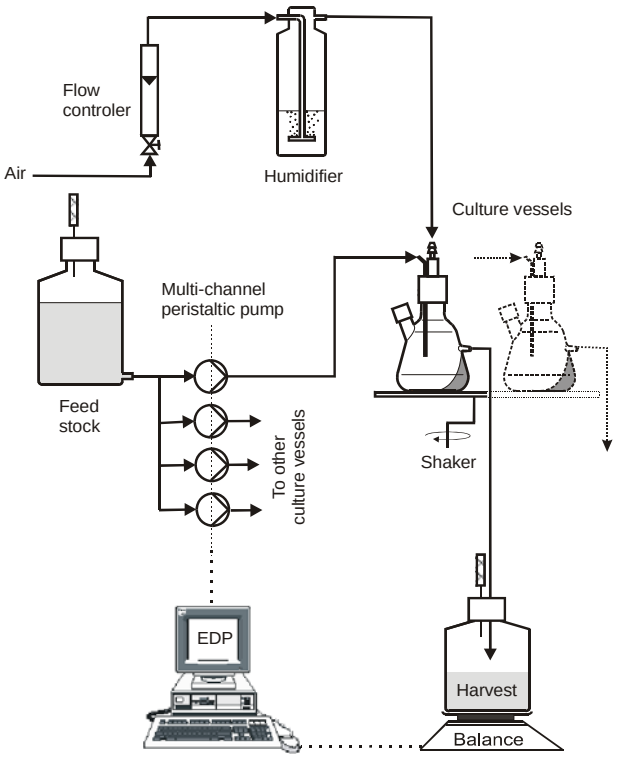

Figure 4.1: Example of a continuous shaken culture system,
reproduced from Akgüen *et al.*, (2004?) with author's permission

What are shaken cultivation systems designed to facilitate ?
Using the short list provided (Büchs, 2001), it is clear that shaken culture technology is eminently suited to:

- initial culture experiments,
- screening - recall the screening of 3,000 different organisms for Quorn® (RHM, 2003), and
- bioprocess development - establishment of optimal temperature, pH, *etc*., *e.g*., Yang *et al*. (2003).

As none of these applications match the intended final liquid cultivation use, *i.e*., a stable bioprocess using a pre-screened fungus, it is suggested that shaken culture technology is sub-optimal for the application under examination here. Consequently, shaken culture technology is not examined further. However, this technology may eminently be suited as an intermediary step, especially useful in the selection process.

5. Stirred Culture Technology

Frequently, the concepts of stirred tank and fermenter are joined together, but, it is important to note that they are not necessarily joined together. This is because fermentation is an anaerobic process (Onions *et al*., 1981) whereas most filamentous mycelial growth is undertaken as an aerobic process known as assimilation. Hence, although the term fermenter is sometimes used here, this should not be taken as an indicator that the cultivation process under discussion is fermentation. Rather, this is an acknowledgement that the source of much development work has been done for fermenters, and that, with some caveats, many results from fermenter designs are directly transferable to stirred cultivation of fungi.

What are stirred cultivation systems designed to facilitate ?
Using the short list elucidated (O'Shea, 2003), it is clear that stirred culture technology is eminently suited to:

- ventilation/aeration experiments, and
- shear stress experiments - including morphology issues, *e.g*. fragmentation.

Static, shaken, or stirred fermenter ?
A comparison by Itavaara (1987) for *Shiitake* spawn, showed that mycelial yield was lowest for the stirred fermenter and highest for the static culture, with non-homogenous growth in the shaken cultivation. However, Itavaara concluded that the stirred fermenter was best overall for automated production and subsequent inoculation by injection, subject to the condition, "If the stirring rate is set appropriately, allowing for adaptation of the hyphal cells…".

No conclusion was made by Itavaara regarding how performance could be improved, but, given the use of air-lift designs for mycelial production at commercial scale, it is suggested that an improvement over mechanical stirring is possible, *e.g.* using an air-lift design. Unfortunately, this author has been unable to find a comparative study of mechanically stirred and air-lift stirred designs.

Fermenter design considerations in respect of functional, material and sub-system issues have been understood for a number of years. Process demands form the user requirements that can be interpreted as a functional specification. This approach is used here as a mechanism to interpret the list outlined by Evans *et al*. (1970) and hence deduce an initial outline functional specification, which is itemised as follows.

1. The fungus and its supply systems should be protected from contamination. This implies the use of an enclosed system, and hence a constant volume regime under steady-state conditions, as shown at left in Figure 5.1. The cultivation vessel's volume has yet to be determined. This also implies using a sterile nutrient supply and a sterile gas supply with a sterile humidifier, scaled to match the vessel's volume.

Figure 5.1: Example of an enclosed continuous stirred culture system, a 75 litre fermenter
Outside Views, **Left**: Overall view, **Right**: Inspection window and feed pipes on cap

2. To enable steady-state conditions to be reached, the (homogenous) nutrient, or medium, supply should be regulated. Furthermore, flow rate regulation should allow a variable set-point. The set-point range has yet to be determined. For the widely used continuous overflow system of harvesting, this implies that harvest flow is indirectly regulated. Note that sometimes a load-cell is used to monitor vessel mass and its signal can be used to regulate harvest flow directly (Kristiansen & Sinclair, 1980).

3. SPATIAL HOMOGENEITY (*e.g.* a well-mixed system): presuming that the lignin to be degraded in the nutrient supply is homogenously distributed, the fungus should be well mixed, as shown at left in Figure 5.2. This is to ensure spatial homogeneity of both fungus and lignin. It needs to be emphasised that using 'standard' mixing paddles in a stirred tank bioreactor will not necessarily achieve this mixing objective. For example, some authors recommend modifying paddle motion such that the paddle impeller diameter is half the vessel diameter. However, this author has found no experimental data to show that this does indeed provide a well-mixed environment. Solomons (1980) discussed impeller type and functionality and introduced a production scale fermenter for a filamentous fungus with two impellers, one at the top and one at the bottom. The impeller at the top saves (compression) energy for aeration. Note that this well-mixed process criterion assumes that there are no biological constraints or

impediments, *e.g.* thresholding effects, to this approach. Furthermore, the range of biological conditions for lignin degradation has yet to be determined.

Figure 5.2: Inside Views, **Left**: Three sets of mixing paddles on central stirring shaft, **Right**: Anti-vortex baffles on rim and temperature sensor at right bottom

4. SPATIAL INHOMOGENEITY (*e.g.* a flow-through system): conversely, presuming that some, as yet undefined, biological condition(s), or impediment(s), exist for fungal degradation of lignin in a liquid culture, then the bioreactor needs to be equipped to satisfy such conditions. For example, a threshold effect may exist, *i.e.* degradation of lignin will not occur unless the mixing ratio of lignin is above a certain threshold. Presuming that the lignin mixing ratio is below this threshold, then it would be necessary to design the bioreactor to spatially agglomerate the lignin with the mycelium in a 'reaction' zone, such that this threshold was exceeded. This may perhaps be achieved for example, by some form of filter, *e.g.* of membrane or other construction, in a flow-through system. Note that this example is the converse of point 3 above, *i.e.* the function of a filter flow-through system is to achieve spatial inhomogeneity. However, it must also be recognised that flow-through systems can also be configured for use in a well-mixed context, by making the 'reaction' zone equal the size of the reactor. Such an approach is described in a fluidised-bed fermenter by Atkinson and Lewis (1980) where the suspension particles are used to support fungal growth.

5. The dissolved oxygen tension is regulated above the critically limiting value for assimilation and lignin degradation, wherever the lignin degradation occurs. Therefore, spatial design of the sparger (the vent in the liquid that supplies the gas bubbles), as shown at left and right in Figure 5.3, is critically dependent on selection of design path 3 or 4 above. Apart from spatial delivery aspects, sparger design is essentially a compromise between achieving small bubble size, with its attendant high surface to volume characteristic that facilitates energy and mass transfer, and the need to have sufficient bubble buoyancy, achieved via larger bubbles, to mix with the prevailing rheological environment. Note that buoyancy is a gravity related characteristic and hence a variable gravity environment impacts on the sparger's design. The critically limiting value of dissolved oxygen tension and the rheological characteristics of the culture have yet to be determined.

6. The environmental characteristics that satisfy the biological conditions for lignin degradation are regulated to the appropriate values. This may necessitate a number of controlled sub-systems, *e.g.* temperature, pH, anti-foaming, *etc*., as exemplified by the temperature probe at right in Figure 5.2. The appropriate set-point values have yet to be determined.


Figure 5.3: Inside Views, **Left**: Sparger feed pipe, **Right**: Sparger vent under bottom mixing paddles

Process demands also impact on the materials chosen for construction of the vessel. Using the same approach as above, *i.e.*, a mechanism to interpret the list outlined by King (1971), it is possible to identify the constraints on material use and hence deduce an initial outline material specification, which is itemised as follows.

7. The vessel's materials must be fit for the purpose intended, maintain their structural integrity and withstand the environmental stresses, *e.g.* temperature, pressure, pH, *etc*. regimes, for the lifespan of the bioreactor. This also applies to any probes, sensors, or actuators, immersed in or in contact with the vessel.

8. From point 1, the fungus and its supply systems should be protected from contamination. This implies the use of corrosion resistant materials and systems, to prevent trace metal or other material contamination. For example, if stainless steel is used in the construction, then, to avoid electrolytic corrosion, all the steel should be of the same grade.

9. Also from point 1, the fungus and its supply systems should be protected from toxicity. This implies the use of non-toxic materials and systems. This is to prevent toxic effects occurring at the liquid-vessel interface, in addition to the material contamination effects noted above.

10. Again from point 1, if the contamination/toxicity prevention system(s) fail, then vessel de-contamination may have to occur, *e.g.* through some form of sterilisation. This implies that the vessel materials must withstand repeated sterilisation procedures, *e.g.* through steam or chemical disinfections.

11. Visual inspection of the liquid cultivation contents and environment, *e.g.* to check whether the mycelium is initiating wall-growth, *etc.*, is a highly desirable attribute. Therefore, where feasible, transparent materials that satisfy the conditions above, should be used. If a suitable transparent material cannot be found that satisfies all the conditions, then consideration should be given to a suitably placed window, *e.g.* as shown in the mid-section of Figure 5.1 on the left, and on the cap as shown at right in Figure 5.1. The window can then be replaced as part of a maintenance program.

12. It is recommended that the design is modular to facilitate cleaning, servicing and sub-system replacement.

The stirring mechanism shown in Figure 5.2 left view, was selected as an example due to its widespread application. Note that many other techniques for stirring have been fabricated and evaluated. For example, an impellerless, cyclone column reactor that produced well mixed and aerated cultivation conditions was developed by Dawson (1963).

6. Cultivation conditions

Cultivation conditions in the bioreactor are strongly dependent on the final outcome of the fungal selection process. Preliminary results suggest the use of *Pleurotus ostreatus* or *Phanerochaete chrysosporium*, however, this selection was based on results using solid-state cultivation techniques. It is conceivable that another fungus may have improved performance in a liquid-state cultivation. For example, both *Pleurotus ostreatus* or *Trametes (Coriolus) versicolor* are known to be used in the commercial production of the enzyme laccase (Bennett, 1998; Bioscreen, 2003) and it has been claimed that both "*P. chrysosporium* and *Phlebia radiata*, are the most efficient lignin degraders" (Call and Mücke, 1997) albeit using different enzymes (lignin peroxidase / manganese peroxidase). Note that fungal selection based on the presence of a particular enzyme may be inappropriate as the question is still open as to "whether all white-rot fungi are able to produce the whole set of lignolytic enzymes" (Call and Mücke, 1997).

Therefore, the evaluation of alternative fungi for a liquid system may be of some importance given that these fungi produce some of the known principal wide-spectrum lignin-degrading enzymes. A more extensive list of fungi that produce laccase is available in Call and Mücke (1997). Once a final fungal selection has been made, then it will be easier to establish optimal condition ranges, *e.g.* for laccase in general, and *Trametes* in particular, a pH in the range 3.0-7.5 and a temperature range of 40-80°C has been established (Call and Mücke, 1997).

6.1 Hydrogen ion concentration, pH

"Louis Pasteur's theory of germs is ridiculous fiction."
Pierre Pachet, Professor of Physiology, Toulouse, 1872.

As evidenced by its role in continuous cultivation systems, a pH control system can be considered as the safety system for a bioreactor. This is because, within quite a wide range, the pH system does not limit mycelial growth. Consequently, pH control can be used to minimise bacterial contamination as shown by Fukushima *et al.* (1991). A pH control system is, therefore, an essential element in reactor design as it can limit the growth of competitive organisms. Fukushima *et al.* supported this assertion by pH control results that showed, at a set-point of about 4, in combination with the addition of acetic acid, continuous process of an agitated liquid fungal culture "for over 70 d without any microbial contamination" was possible (Fukushima *et al.*, 1993).

What is optimal pH for mycelial growth ?
The answer to this question may depend on researcher, strain, on measurement technique, *etc*. In light of the conflicting results presented, it is suggested that this subject is more fully investigated after a final selection of fungal species. However, note that for the growth of a *Pleurotus* strain, no pH optimum was found (Khan & Qadir, 1987), *i.e.*, mycelial growth in the range 4-9 pH gave a similar yield. Conversely, for a range of *Pleurotus* strains a pH optimum of a 6.0-6.5 pH was found (Bukhalo & Solomko, 1978), with a wide growth range of 3-10 pH.

6.2 Temperature

Recall from the metabolic equation, Equation (2.1), that the process being controlled is primarily a source of heat, moisture and carbon dioxide. Therefore, for a well-insulated reactor, the principal temperature control service required is the removal of heat, *i.e*. cooling.

What is optimal temperature for mycelial growth ?
In light of the various thermal spectral classifications required for fungal growth, *i.e.*, psychrophile, mesophile, or, thermophile, it is suggested that this subject is more fully investigated after a final selection of fungal species has been made. However, as an example of the range suited for one species, note that for the growth of *Pleurotus ostreatus*, a wide temperature growth range of 19.1-30.5°C was found (Singh, 1981).

In this context, it is important to note that temperature influences enzyme production and/or may also affect enzyme activity (Kavanagh, 2003).

6.3 Ventilation
Oxygen has low solubility in water and therefore can become a limiting factor unless a high ventilation/aeration rate is maintained to effect metabolic gas exchange. Conversely, carbon dioxide has high solubility in water. Hence, unlike the corresponding solid-state bioreactor, ventilation control is not easily undertaken using carbon dioxide as the controlled variable. Therefore, it is recommended that dissolved oxygen tension is used as the controlled variable for ventilation control.

6.4 Lighting
Some fungi are photo-sensitive, *e.g*. it was found in a study that mycelial growth of a *Pleurotus* strain was noticebly affected by light (Khan & Qadir, 1987), *i.e*., mycelial growth in a flask incubated in dark conditions gave a higher yield.

6.5 Foaming
Two anti-foaming strategies (Kristiansen & Sinclair, 1980) are proposed for use. Frequently an antifoam agent is added to the feedstock. Care has to be taken in selecting such an agent to ensure compatability with the fungus being used. Additionally, the agent may have to be supplied in a continuous fashion if it is metabolised, *e.g*. using the system described by Kristiansen & Sinclair (1980). The alternative to such an agent is the use of mechanical foam breakers fitted in the headspace of the fermenter.

6.6 Agitation
In the absence of pellet formation, it has been established that diffuse filamentous fungi grow exponentially in liquid media (Trinci, 1969). Therefore, the need for agitation depends on whether the formation of pellets is required or not. Note pellets can be as hard as glass and/or contain dead organic tissues in the centre (O'Shea, 2003).

6.7 Wall growth
Bull and Bushell (1976) introduce and discuss the design and operation of various fermenters and also detail the requirements for environmental control of fungal growth. They noted the common observation that wall growth was the biggest obstacle to continuous growth of mycelial organisms. Wall growth has several undesireable facets, namely: reduction in container volume, coating of sensors, blocking of supply or harvest lines, and obstruction of agitation mechanisms. Therefore, cultivation should occur in wall growth free conditions. This condition could be satisfied by at least two paths, cultivation in low pH conditions and/or use of growth resistant/repellent materials, *e.g*. glass, in the bioreactor's construction. Secondary approaches include the addition of surfactant(s) and/or the use of hydrophobic strains.

7. Reactor feedback and control

> "When you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the state of *science*." (Kelvin)

Metabolic Feedback Indicator Option Summary
Two paths present themselves for examination, metabolically indirect and morphogenically indirect. Indirect indicators of metabolism are a function of the metabolism itself. In the case of primary aerobic metabolism, as illustrated by Equation (2.1), two gases, oxygen and carbon dioxide, are respectively consumed and produced. Therefore, in the absence of other organisms, a flow rate measurement of either gas should provide feedback of the metabolic status. However, a *caveat* needs to be issued in this context, namely that oxygen has low solubility in water and carbon dioxide has high solubility in water. Hence, metabolic dynamics can be masked due to water storage effects if care is not taken in measurement system design. Also, in the case of anaerobic metabolism, as modelled by Equation (2.2), carbon dioxide can be produced without the corresponding consumption of oxygen. Therefore, it needs to be emphasised that carbon dioxide measurements indicate total metabolism, not just the aerobic part.

The performance of a fermenter is determined by its heat and mass transfer capabilities. It has been claimed (Pace, 1980) that because heat and mass transfer is some function of the fermentation broth, the broth's rheology affects the fermenter's performance. Hence, measurement of the fermenter's performance should indicate metabolic activity. Rheology is the study of the deformation and flow of matter, and one rheological indicator is a fluid's viscosity. Recall that one form of viscometer is of "paddle in infinite sea" form and is similar to many stirred tank reactor paddle forms.

It has been observed that in a liquid cultivation system the liquid's viscosity changes depending on the morphology status of the micro-organism. For example, as growth proceeds the liquid's viscosity increases. The liquid's viscosity can me measured directly or indirectly. In an agitated system, an increase in viscosity requires an increase in driving motor torque to maintain a constant agitation rate. Hence, viscosity or torque measurements can be used to indicate morphological status and infer a corresponding metabolic state. Such measurements are currently under investigation (Büchs, 2003).

Fermenter Models and Control
Due to the expertise available from other MELISSA partners in this technological field, no development or review is undertaken here. Note, however, that control design issues are addressed in the appendix to this report.

8. Overall reactor summary, size and scaling issues

The default standard configuration for a liquid-state fermenter has generally been a cylindrical stirred tank reactor, and these configurations have been applied to many specific problems. For example, construction details of a stirred tank fermenter for filamentous fungi have been available since at least 1978 (Kristiansen and Sinclair, 1980). The requirement to facilitate oxygen transfer through enhanced gas exchange has been recognised since the mid-1970's. In this regard, Kristiansen and Sinclair (1980) provide a brief review of "new fermenter configurations" to promote gas exchange, including: thin channel, tubular loop, film, circular ring, and rotating disc types. However, they do not make explicit the implicit design objective inherent in these "new" designs, namely a configuration that has a **high surface to volume ratio**.

The tank configuration shown in Figure 5.1 falls into the low surface to volume configuration category. However, the gas exchange mechanism matches the high surface to volume criterion.

The list of controlled variables, includes pH. Hence, in addition to indirect thermal control of the cultivation medium, it is foreseen that the **chemical state of the medium is directly controlled**.

For the bioreactor options proposed, the controlled variables can be separately adjusted, this enables the **use of independent climate control loops**. It is suggested that under these independent control loop conditions a simple **standard negative feedback PI control** system can be used.

The precise size of the bioreactor is a function of the degradation characteristics of the selected fungus. As a final fungal selection has not yet been confirmed, the corresponding degradation characteristics are unquantified. Consequently, it would be premature at this stage to initiate the calculations necessary to estimate the reactor's size.

9. Open questions and suggestions for future research

1. Batch and/or continuous (daily batch) process operation ?
    - Critical point that determines reactor layout and design.
    - If the reactor is not operational during flight, storage of waste must be managed.
        - This implies that there is at least one batch to be processed upon arrival.

- Fungal or bacterial pre-processing could occur during the in-flight storage phase.

2. Single or multiple stages of fungal species and/or supplementary physio-chemical reactors ?
   - Could use a sequence of species to increase overall degradation efficiency.
     - For example a soft rot and/or brown rot fungi (where lignin removal is absent/slow/partial) may be used to pre-digest the substrate, thus increasing the lignin mixing ratio, and perhaps facilitate lignin availability for increased degradation efficiency ?
   - Different species may have differing growth requirements.
     - If the selected species is/are photosensitive then a lighting system is required.
3. Full range of fungal growth phases required or limited to one phase ?
   - May require multiple reactors.
4. What are the appropriate, fungal dependent, environmental variable requirements ?
5. Energy use limitations ?
6. Pest/competitive growth control ?
   - Use of chemicals, *e.g.* acetic acid, and/or pH control approach and/or pasteurisation.
7. Treatment of air supply to remove anti-fungal volatile organic compounds that may be of internal or external origin, *e.g.*, from the higher plant growth chamber ?
8. Possibilities for enhancing performance ?
   - Ultrasonic bioreactor (*e.g.* Schlager, 1998).
9. Possibilities for using advanced control techniques ?
   - Advanced control techniques have been used for bioreactor control. For example, an adaptive/predictive control scheme has been used for simultaneous control of dissolved oxygen and dissolved carbon dioxide (Diaz *et al.*, 1996), however, it is suggested that the biological implications, *e.g.* fragmentation, of using agitation as a controlled variable may require further investigation. In this context, it is important, and is acknowledged by these authors, to note that "the poorly understood and constantly changing dynamics of the biological system" limit the validity of using model based techniques.
10. Will fungi that are lignin degraders in a solid cultivation system, degrade lignin in a liquid system ?
    - If they do, are there consequential limitations on system options, *e.g.* static only ?

10. Discussion

"The molecular steps of the fungal secretion pathway, post-translational modification of metabolites, and the release of proteins from hyphae into their environment are understood only in a descriptive sense."
(Bennett, 1998).

Until the question of whether lignin degradation is possible in a liquid cultivation system using the combination of target substrate/medium and fungus, is answered, the question of reactor configuration is of relatively secondary interest. Therefore, this question is approached first here. With this question in mind, it may be prudent to be aware of other liquid substrate delignification experiences.

In the paper pulp industry the initial research goal was that fungal enzyme degradation would remove as much lignin as possible. However, after decades of research, this goal became modified so that the objective was the modification of lignin for further downstream processing. If this experience was directly applied to the MELISSA case, then it would imply that the fungi bioreactor would be one of a series of supplementary reactors to the first compartment, *e.g.* fungi + physio-chemical.

Given the observation of Call and Mücke (1997) that although delignification was achieved via live fungal degradation, the isolated use of the three identified enzymes did not achieve such delignification. This highlights the role of auxiliary fungal secretions such as mediators/initiators and emphasises the role of multi-stage delignification. In this enzyme + mediator (+ oxygen) context, it is noteworthy that the Lignozym®-system (Call and Mücke, 1997) has achieved 33.7-61.9% lignin removal in a relatively short "retention time" of 1-4 hours. Given these outstanding results, it is suggested that future research in this area may benefit from cooperation with the developers of this system.

It can be argued that lignin degradation occurs in a solid substrate when a developing hyphal tip encounters a predominantly lingo-cellulosic region of the substrate. To exploit such a region effectively the fungus has to degrade the lignin as no other nutrient pathway is available.

Compare this with a liquid substrate where the lingo-cellulosic content consists of a small component, *x*, of the total, in the form of suspended solids. Arguably, in an agitated submerged culture, what a developing hyphal tip encounters is predominantly a (1-*x*) nutrient supply. In this situation, the condition to switch to a secondary (lignin degrading) metabolism may not be triggered.

Therefore, unless special precautions are taken, it may be inappropriate to assume that conditions that act as a switch trigger in a solid substrate will also act as a switch trigger in a liquid substrate. If this turns out to be the case, it may worthwhile trying to recreate, to some extent, in a liquid cultivation system the regional conditions that can exist in a corresponding solid substrate. This may be achieved, *e.g*., by using a membrane filter both as a growth support mechanism for the fungus and as a suspended solid aggregation mechanism. This would regionalise both the fungus and the suspended solids and hence provide some approximation of equivalent solid-state conditions.

It is this author's opinion that the configuration of the bio-reactor is critically dependent on the answer to the question posed above. Consequently, it is considered premature to draw any conclusion(s) as to what form or configuration an appropriate liquid cultivation system would be.

Another confounding issue is the subject of morphology, in particular the viscosity of the fungus/medium mix. For example, using a stirred tank as an example, agitation form is very different for high and low viscosity fluids (think of the stirring mechanism on an ice-cream and cream mixer respectively). Consequently, more than one morphological state may exist in the liquid cultivation system, *e.g*. as evidenced by the pellet formation found by Call (2003).

Similarly, it is considered that, issues such as wall growth, and one corresponding hypothesis that mutagenic morpho-genesis may induce a hydrophobic strain in which this problem does not occur, are more appropriately addressed at a later stage of development.

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