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Surface adhesion fermentation: a new fermentation category

Fermentación por adhesión a superficies: una nueva categoría fermentativa

Marcel Gutiérrez-Correa* y Gretty K. Villena

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Abstract

Basic knowledge on solid state fermentation and on biofilm formation is summarized and related to cell adhesion processes. These subjects are covered from the engineering and molecular biology points of view. Contrary to the common believe, the advantages of solid state fermentation are related to the adhesion of fungi to solid particles instead of being due to the low water content. Thus, solid state fermentation and biofilm fermentation (erroneously known as adsorption immobilization) are technical variants of the same biological process, and should be referred as Surface Adhesion Fermentation.

Keywords: Biofilms, solid substrate fermentation, differential gene expression, fungi.

Resumen

Se resume el conocimiento básico sobre la fermentación en estado sólido y la formación de biopelículas y se relaciona con los procesos de adhesión celular, cubriendo puntos de vista de ingeniería y de biología molecular. Contrariamente a la creencia común, la ventaja de la fermentación en estado sólido está relacionada a la adhesión de los hongos a partículas sólidas y no al bajo contenido de agua. Por lo tanto, la fermentación en estado sólido y la fermentación en biopelículas (erradamente conocida como inmovilización por adsorción) son variantes técnicas del mismo proceso biológico y deben ser referidas como Fermentación por Adhesión a Superficies.

Palabras clave: Biopelículas, fermentación en sustrato sólido, expresión diferencial de genes, hongos.

Introduction

Many of the traditional fermented food are based on the koji process that belongs to a major technical fermentation category known as «solid substrate (state) fermentation» (SSF) differing from either submerged or immobilization fermentations in the amount of free water that they contain. For a long time it has been considered that the advantages of SSF are due to water limitation of the system so that a higher product concentration and volumetric productivity are attained. However, scale up of SSF processes is the main drawback for a more ample industrial application. On the other hand, related to fungal enzyme production, submerged fermentation (SF) is the process of choice for industrial operations due to the very well known engineering aspects such fermentation modeling, bioreactor design and process control. Also, it has been considered that there would not be biochemical differences between an enzyme produced by the same fungal strain in either SSF or SF and most of the biochemical information on some important fungal enzymes comes from submerged cultures. Enzyme market in 2005 will be between 1,700 to 2,000 million dollar and thus it represents a very

*Laboratorio de Micología y Biotecnología, Universidad Nacional Agraria La Molina, Apartado 456 Lima 1, Perú.

E-mail Marcel Gutiérrez-Correa: **mgclmb@lamolina.edu.pe**

important biotechnological sector in which any technological development would be rapidly adopted (Bhat, 2000; Demain, 2000).

Nowadays, biofilm processes used mainly for waste water treatment are also being considered for metabolite and enzyme production. Although fungal biofilms are less known than bacterial biofilms, they can be used for enzyme production as it has been recently showed (Villena et al., 2001). As it will be stated in the present paper, both SSF and biofilm fermentation (BF) depend on surface adhesion and a new fermentation category was recently established. Surface adhesion fermentation (SAF) was proposed by Gutierrez-Correa (2003) as this new category and it will be sustained it further in this paper.

Solid State Fermentation Overview

As mentioned above, SSF is a process used for the production of fermented food, animal feed, fuel, enzymes, pharmaceuticals, which involves the growth of microorganisms (mainly fungi) on moist solid substrates in the absence of free-flowing water. SSF processes exhibit several advantages over SF, including improved product characteristics, higher product yields and productivities, easier product recovery and reduced energy requirements. Also, mixed culture SSF for enzyme production give higher yields than single culture SSF as it can be seen in figure 1 (Castillo et al., 1994; Dueñas *et* al., 1995; Gutierrez-Correa and Tengerdy, 1997, 1998, 1999; Gutierrez-Correa et al., 1998). Since SSF processes have been used for centuries there is a great number of references and many excellent reviews have also been published (Doelle et al., 1992; Soccol et al., 2003; Suryanarayan, 2003; Tengerdy, 1996).

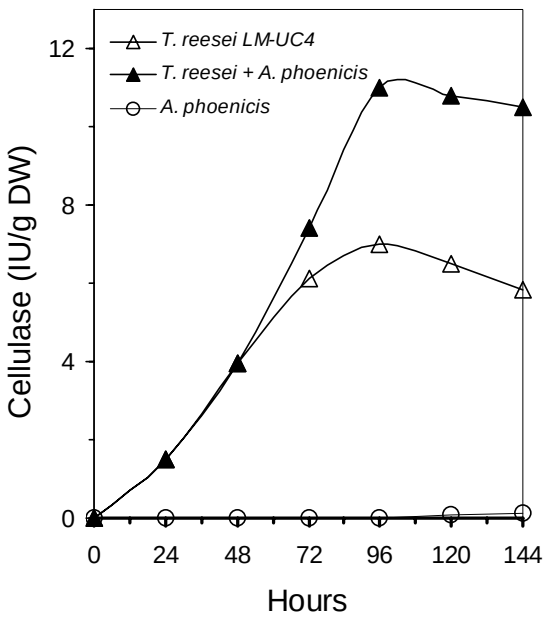

Figure 1. Cellulase production kinetics in single and mixed culture solid state fermentation on sugar cane bagasse (redrawn from Gutierrez-Correa and Tengerdy, 1999).

Regarded to the solid support used for SSF, two main types of processes are used. Firstly, SSF processes that use natural solid substrates like starch- or (lingo)cellulose residues or agro-industrial sources such as grains, and grain by-products, cassava, potato, rice, beans and sugar beet pulp. In theses cases substrate is used also as the source of carbon and nutrients for microbial growth (Ningam and Singh, 1996; Tengerdy and Szakacs, 2003). Secondly, SSF processes that use inert natural or artificial solid supports like sugarcane bagasse, perlite, amberlite, polyurethane foam and others. In these latter processes support is used only as an attachment structure for the microorganism. From the engineering point of view inert supports are better because they do not change their geometric and physical characteristics due to the microbial growth, allowing a better control of heat and mass transfer (Ooijkaas et al., 2000).

Enzyme production by SSF is not of wide use by manufacturers mainly due difficulties in bioreactor design, although the advantages of this form of microbial cultivation as compared to SF are well known. Growth patterns in SSF have been detailed in only few cases and these can be summarized in two phases: a) germination, germ tube elongation and mycelial branching to cover loosely most of the substrate; b) increase in mycelial density with aerial and penetrative hyphae development (Mitchell, 1992). However, as it

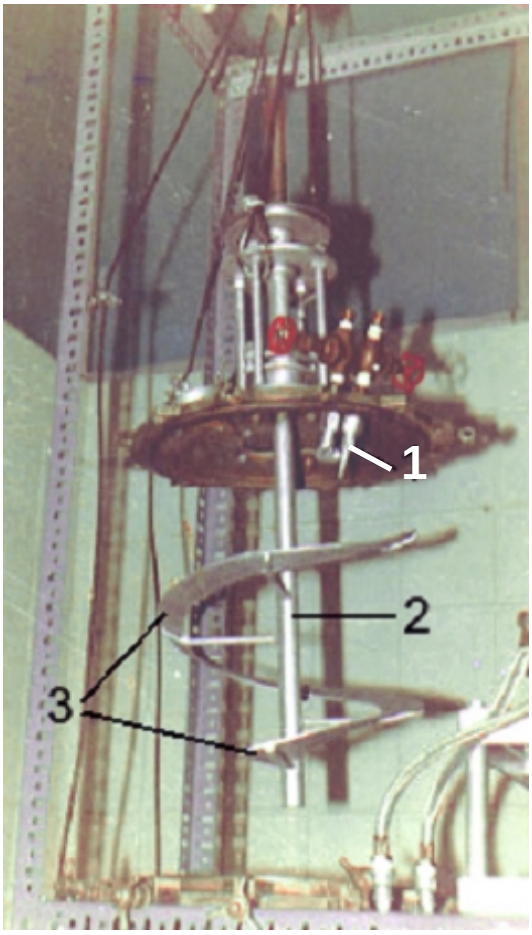

Figure 2. Cover plate of the LMB 50L semi-pilot SSF bioreactor showing stirrer details. (1) Mist nozzle for water replenishment as part of the evaporative heat control system, (2) shaft, (3) variable path helical-ribbon stirrer.

will be described below, a previous phase namely spore adhesion to surfaces has been forgotten by most researchers. Recently, a general approach to compare productivity of fungal enzymes using SSF and SF techniques was attempted by using logistic and Luedeking-Piret equations (Viniegra-González et al., 2003). Also, many attempts to develop mathematical models for SSF have been done and current models are more rational tanking into account not only mass and heat transport processes across the substrate bed (macroscale models) but also phenomena that occur on and within individual particles (microscale models) (Mitchell et al., 2003).

Several SSF bioreactor models have been designed following two general categories: laboratory-scale and pilot and industrial-scale (Durand, 2003; Fasidi et al., 1996). Many designs have been published that belong to the first category including static and agitated models but only few models are used in commercial production. We designed and built an agitated 50L semi-pilot computer-controlled SSF bioreactor with an evaporative heat control system and a variable path helical-ribbon stirrer (Figure 2; unpublished results). In general, however, many types of SSF bioreactors can be run at the bench scale level with small quantities of substrate but their scale-up is difficult due to heat generation and heterogeneity with natural supports.

Biofilm fermentation overview

The concept of a biofilm presumes either a population or a community of microorganisms living attached to a surface. Biofilms can be developed on either biotic or abiotic surfaces from a single species or as a community derived from several species (David and O'Toole, 2000; Fenchel, 2002). This way of growth is the prevailing lifestyle of microorganisms including bacteria, yeast, filamentous fungi and even micro-animals (Armstrong et al., 2001; Gilbert and Lappin-Scott, 2000; Morris et al., 1997; Watnick and Kolter, 2000). However, there is a strong tendency in microbiology and in the scientific literature to consider within the above concept of biofilms to those developed by bacteria in complex natural communities or those developed by bacteria and yeast in natural or artificial environments of medical relevance that are responsive of persistent infections (O'Toole et al., 2000. Rickard et al., 2003; Sauer, 2003; Stickler, 1999; Wimpenny, 2000). It should be noted that adhesion and subsequent differential gene expression to generate phenotypes distinct from those of free living organisms are two unifying processes of the biofilm concept (Ghigo, 2003; O'Toole et al., 2000). Also, biofilms have been used

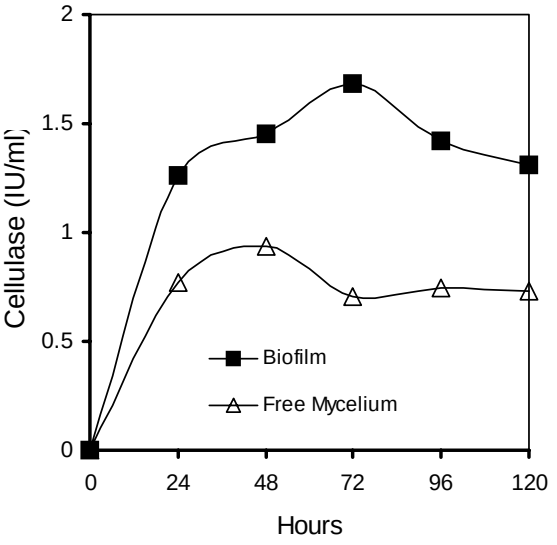

Figure 3. Biofilm fermentation for cellulase production with *Aspergillus niger* on polyester cloth (redrawn from Villena et al., 2001).

for a long time in water treatment facilities where they were called slime, mats or sludge, but not other practical used was seen until recently. This has brought that most of the available information is on bacterial and, in recent years, on yeast biofilms.

Filamentous fungi are naturally adapted to growth on surfaces and in these conditions they show a particular physiological behavior which it is different to that in submerged culture; thus, they can be considered as biofilm forming organisms according to our former concept. Differential physiological behavior of most attached fungi corresponds principally to a higher production and secretion of enzymes and also to a morphological differentiation which is absent in submerged cultures (Akao et al., 2002; Biesebeke et al., 2002). The advantages of this form of growth have been industrially exploited by two culture systems: SSF and cell immobilization although there is a lack of knowledge on the molecular basis of growth on surfaces.

Technology of cell immobilization was highly developed during the last two decades based on the operative advantages in the productive process instead of physiological issues. Natural adsorption on solid supports is an immobilization technique that it has been used with filamentous fungi thus neglecting its study as a way of biofilm formation. Actually, once spores are adsorbed to the support they grow attached to it thus forming a film. We prefer the term biofilm fermentation (BF) instead of cell immobilization because the microbe is an active and differential entity. BF has been applied for the production of enzymes, amino acids, organic acids, alcohol, aromas and in bioconversion processes (Anderson, 1983; Groboillot et al., 1994; Norton and Vuillemard, 1994) as well as in bioremediation an effluent biotreatment (Burton, 2001; Doggett, 2000; King and Shoda, 1999; Kasinath et al., 2003; Rodgers et al., 2003; Van Driessel and Christov, 2001).

We have showed that *Aspergillus* and *Trichoderma* biofilms developed on polyester cloth produce higher cellulase titers than submerged cultures. *A. niger* biofilms produce 20% more cellulolytic activity than *T.* reesei biofilms and 140% more cellulolytic activity than submerged cultures (Villena et al., 2001) (Figure 3). As in SSF, it has been possible to use mixed culture BF of *Aspergillus niger* and *Trichoderma reesei* for cellulase production (Figure 4). Mixed culture BF produced 70% more cellulase than single

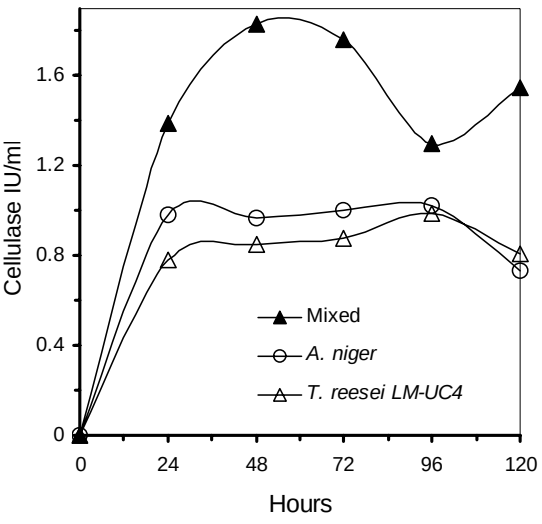

Figure 4. Cellulase production by mixed culture fungal biofilms (unpublished results).

culture BF; these results were similar to those obtained with mixed culture SSF (Gutierrez-Correa and Tengerdy, 1999).

Due to the extended used of bacterial biofilms for wastewater treatment several mathematical models of growth kinetics have been developed including estimation of biokinetic parameters, mass transfer processes, flow velocity through biofilms (Benefield and Molz, 1985; Riefler et al., 1998; Stoodley et al., 1997). Also, the engineering treatment of cell immobilization including the type that actually correspond to biofilm according to our concept together with the availability of mathematical models of biofilm kinetics have paved the way for bioreactor design and process development (Vekatasubramanian et al., 1983). Fungal biofilm kinetic models need to be developed yet since they have only recently been realized as true biofilms instead of simple cell immobilization systems. Perhaps, the easy way to treat mathematically fungal biofilms is to adapt fungal pellet growth kinetics knowledge. An advantage of biofilms is that according to the type and form of the support almost all type of submerged bioreactors can be used, including the simple stirred tank, gas-lift, packed bed columns, rotating contactors and horizontal blade-stirrer bioreactor (Keshavarz et al., 1990; Pakula and Freeman, 1996). We have tested cellulase production by fungal biofilms in both a modified internal-loop air-lift reactor in which the riser tube was replaced by a spiral-wound polyester support and in a stirred-tank reactor in which a spiral-wound polyester support was attached to the shaft instead of turbines (unpublished results; Figure 5).

Cell adhesion supports SAF processes

Cell adhesion as a biological process has been studied by cell biology particularly referred to animal cell. It has been considered it as a basic important processes for tissue and organ development that includes not only physical and chemical interactions but also vital actions such signaling and gene regulation. A number of surface protein receptor are present in animal

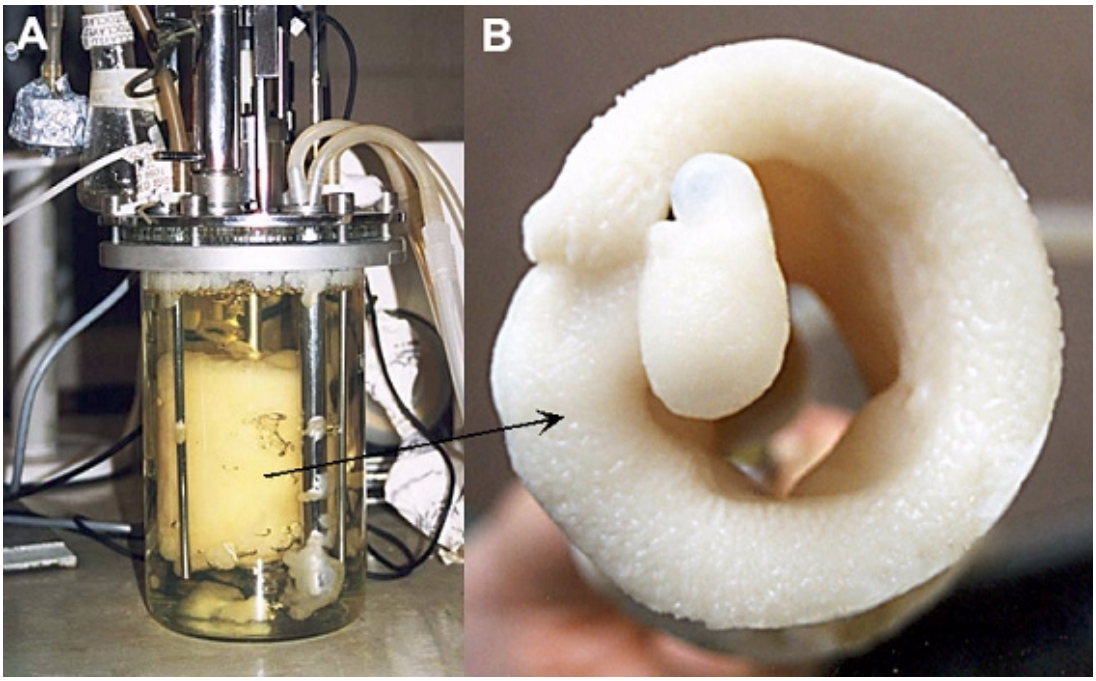

Figure 5. Modified stirred tank bioreactor (A) for fungal biofilm fermentation in which a spiral-wound polyester support has been fixed to the shaft. (B) fungal biofilm at the end of the fermentation process.

cell including E-cadherins, integrins and others (Pece and Gutkind, 2002; Schwartz and Ginsberg, 2002). Cell adhesion is also present in plants and it may play a similar role as in animal organisms. Plant cell adhesion is desirable because of its positive effects on cell physiology and biochemistry particularly on secondary metabolite production. Hence controlled cell adhesion considered as a immobilization process (as it happened with fungi) is of interest from the process engineering point of view (Panda et al., 1989; Rao and Ravishankar, 2002). For instance, enhanced production of shikonin by *Lithospermum erythrorhizon* cells immobilized on polyurethane foam matrices (thus, a plant biofilm) has been attained in packed bed columns (Park et al., 1990).

Formation and maintenance of biofilms are dynamic processes that involve complex interactions of physical and biological processes. Physical properties of support like hydrophobicity, electrostatic charge and surface roughness are important at the initial adhesion step of bacteria, yeast and filamentous fungi (Bigerelle et al., 2002; Cunliffe et al., 1999; Dufrene, 2000; Webb et al., 1999). The DLVO (for Dejarguin, Landau, Verwey, and Overbeek) theory of adhesion of colloidal particles considers the van der Waals attractive interactions and the electrostatic interactions between the surfaces (Gerin et al., 1995; Rouxhet and Mozes, 1990). For the adhesion of microbial cells, the DLVO theory is usefully completed by considering the influence of cell wall macromolecules which can either bridge the cell to the surface or prevent adhesion by steric hindrance.

In bacteria cell structures like fimbriae, pili and flagella participate in the process of

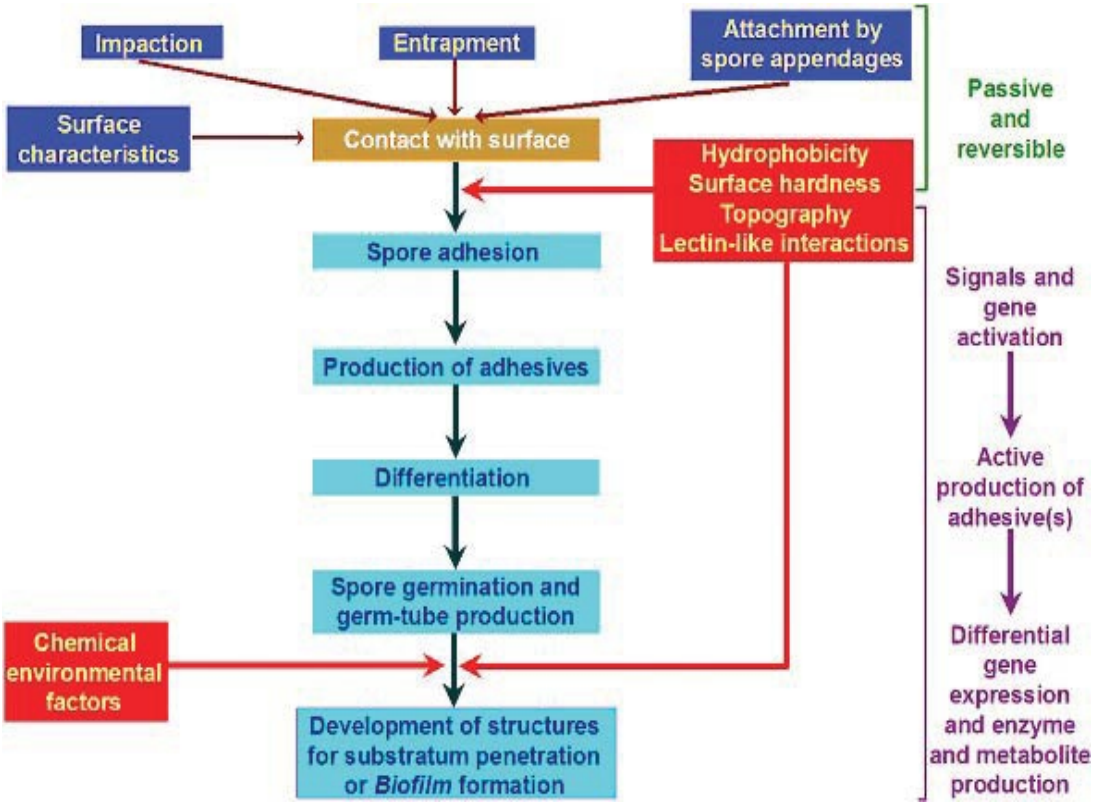

Figure 6. Stages in fungal adhesion applied to both solid state and biofilm fermentations (modified from Jones, 1994).

recognition and colonization of surfaces and extracellular polysaccharide production stabilizes adhesion (Davey and O'Toole, 2000; Ghigo, 2003; Lejeune, 2003; Purevdorj et al., 2002; Stickler, 1999; Watnick and Kolter, 2000). In yeasts, adhesion depends on the expression of surface glycoproteins, floculins or adhesins, that modify the properties of cell walls and they are differentially expressed during haploid and diploid stages, or in dimorphic yeast-like or hyphal-like stages, and also the production of extracellular polysaccharides is a quite common process (O'Toole et al., 2000; Reynolds y Fink, 2001; Sundstrom, 1999).

Adhesion in filamentous fungi is mediated principally by a class of small amphipathic proteins called hydrophobins that are produced by ascomycetes and basidiomycetes, and may also be produced by zygomycetes. Hydrophobins stabilize the adhesion of spores and mycelium to both natural and artificial hydrophobic surfaces resulting in morphogenetic signals (Mankel et al., 2002; Scholmeijer et al., 2001, 2002; Wosten y Willey, 2000). Other molecules like glycoproteins participate in adhesion as found in *Colletotrichum lindemuthianum* (Hughes et al., 1999). There is also evidence on the production of an extracellular matrix that enhance adhesion (Doss, 1999).

We have described the growth pattern of *A. niger* BF by using scanning electron microscopy. Biofilm formation can be divided into three phases: 1) adhesion, which is strongly increased by *Aspergillus* spore hydrophobicity; 2) initial growth and development phase from spore germination to surface colonization; 3) maturation phase in which biomass density is highly increased and an internal channel organization that assures medium flow through biofilm is clearly evident (Villena and Gutierrez-Correa, 2003). Based on early studies of Jones (1994) on fungal adhesion, we have summarized the stages of this process by including recent findings on cell signaling and differential gene expression, since the latter processes are most important in SSF and BF (Figure 6).

Biochemical and Molecular characteristics of SAF

The ample analytical study of bacterial biofilms has demonstrated that adhesion launches the expression of a set of genes that ends with the typical biofilm phenotype, particularly with an enhanced resistance to antimicrobial agents (Davey and O'Toole, 2000; Goldberg, 2002; Stickler, 1999; Watnick and Kolter, 2000). Differential expression implies the activation or repression of genes that comprise between 1% to 38% of the bacterial genome. In gram-negative bacteria like *E. coli*, differential expression comprises 230 genes while in *Pseudomonas* 1% of its genome is differentially expressed when growing as biofilm. In gram-positive bacteria like *Bacillus* it has been found that 519 genes are expressed during biofilm formation (Ghigo, 2003; Oosthuizen et al., 2002; Sauer, 2003; Schoolnik and Yildiz, 2000).

In yeast like *Candida* genes of the *ALS* (agglutinin-like) family that code for proteins related to adhesion to surfaces are differentially expressed. Likewise, it is possible that biofilm resistance to antimicrobial agents may be due to a higher expression of drug resistance gene families (Chandra et al., 2001). Moreover, *Candida albicans* biofilms are structured communities composed of a mixture of yeast cells and hyphal elements, suggesting a pivotal role for the dimorphic switch in the development of biofilms and the Efg1 regulator protein being responsible for the filamentous growth (Ramage et al., 2002). In *Saccharomyces cerevisiae FLO11* gene has been identified as responsible for cell adhesion and the formation of aggregates and biofilm (Reynolds and Fink, 2001).

As mentioned above, biofilms of filamentous fungi have not been realized until recently thus biochemical and molecular information is lacking. However, as SSF processes have been traditionally related to water limitation and not to adhesion, it is considered that there are not biochemical

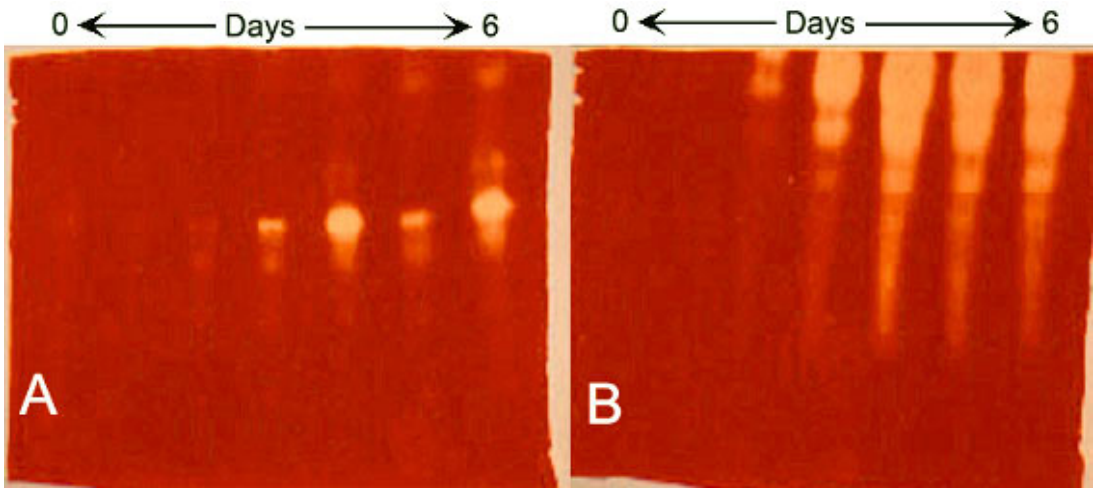

Figure 7. Electrophoretic zymograms of *Trichoderma reesei* endoglucanase produced by submerged fermentation (A) and solid state fermentation (B) of delignified sugar cane bagasse. 100 μg protein/well concentrated enzyme samples were electrophoresed in a 3 – 15 % density gradient PAGE and zymograms were then developed by the agar replica technique and stained with Congo red.

differences between enzymes produced by SSF and those produced by SF; thus, work in this subject is scarce. Molecular comparison of cellulases produced by *Trichoderma reesei* in SF and SSF showed that those produced in SSF were strongly different from those produced in SF (Figure 7; unpublished work), the latter being of low molecular weight. *Aspergillus niger* produced higher amounts of two different feruloyl esterases in SSF of sugar beet pulp as compared to SF (Asther et al., 2002). Likewise, it was found that *Phanerochaete chrysosporium* (Vallim et al., 1998) and *Aspergillus oryzae* (Hata et al., 1998) show a differential gene expression for cellobiohydrolase and glucoamylase, respectively, when they are cultured in SSF conditions. These findings have been recently confirmed for *A. oryzae* in which a group of SSF-specific genes were found (Akao et al., 2002). Also, it has been found that the transcription factor encoded by the gene *brlA* that regulates sporulation in *A. oryzae* is expressed only in SSF together with two unidentified proteases (Biesebeke et al., 2002). These examples of differential gene expression have been regarded as a result of the low water activity present in SSF systems. However, a different explanation was given by Gutierrez-Correa (2003). Many filamentous fungi live on natural lignocellulosic solid substrates (wood, grasses, etc.) by adhering themselves to the surface of such substrates and degrading them with enzymes produced by their mycelia. This mode of living is imitated in SSF processes in which adhesion is the main biological factor that is involved in and it should be considered as the principal responsible for the induction of SSF-specific genes through surface adhesion signaling instead of the water limitation. Thus both SSF and biofilm fermentation depend on the adhesion process and represent technical variants of the same biological process.

Concluding remarks

Due to the lack of knowledge on the molecular biology of fungal biofilms we can only speculate that as in SSF a similar differential gene expression will be found in biofilm fermentation. Techniques of functional genomics will help in testing this hypothesis and if so biofilm fermentation would be more suited for the scale up to industrial operations

than SSF since many of the engineering concepts of SF can be easily adapted to this type of fermentation. We should now consider a new major technical fermentation category, named «surface adhesion fermentation» (SSF and biofilm fermentation).

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