Potential of solid-state fermentation for laccase production

J. L. Toca-Herrera[1], J. F. Osma[2], and **S. Rodríguez Couto[*,2]**

[1]Biosurfaces Unit, CICBiomaGUNE, Paseo Miramón 182, 20009 San Sebastián, Spain
[2]Department of Chemical Engineering, Rovira i Virgili University, Av. Països Catalans 26, 43007 Tarragona, Spain

Solid-state fermentation (SSF) processes involve the growth of microorganisms (typically fungi) on a solid material in the absence or near absence of free-flowing water. The wide range of solid materials used in SSF can be classified into two great categories: inert (synthetic materials) and non-inert (organic materials). The former only acts as an attachment place for the fungus, whereas the latter also functions as a source of nutrients, due to which it is called support-substrate. Utilisation of agro-industrial residues as support-substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilised residues. SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi, since they reproduce the natural living conditions of such fungi. In the present chapter the production of laccase enzyme by white-rot fungi under SSF is described.

Keywords: solid-state fermentation; laccase; agro-wastes; filamentous fungi; bioprocesses

1. Introduction

Enzyme production is an increasing field of Biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the use of the solid-state fermentation (SSF) technique to produce several enzymes. SSF is known from ancient times in Asian countries thus, SSF is used, for example, in the production of koji and sake. However, in western countries SSF was nearly ignored after 1940. This was due to fact that SmF had become a model technology for production of any compound by fermentation as a result of the development of penicillin. Table 1 gives a brief summary of the historical evolution of SSF [1]. This technique reproduces the natural microbiological processes like composting and ensiling. This natural process can be utilised in industrial applications in a controlled way to produce a desired product. In addition, it presents several advantages over the traditionally employed SmF (Table 2) [2].

A direct comparison between SSF and SmF cultivation techniques is difficult to make because the two processes are quite different. Studies on fungal enzyme production in SSF have shown that SSF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes with a higher temperature or pH stability. Also, the fermentation time is shorter and the degradation of the produced enzymes by undesirable proteases is minimised [3]. Castilho et al (2000) [4] performed a comparative economic analysis of SSF and SmF processes for the production of lipases by the ascomycete *Penicillium restrictum*. They found that for a plant producing 100 m^3 lipase concentrate per year, the process based on SmF needed a total capital investment 78% higher than the one based on SSF and its product had a unitary cost 68% higher than the product market price. In addition, Viniegra-González et al. (2003) [5] compared the productivity of three fungal enzymes, invertase, pectinase and tannase, using SSF and SmF techniques. They reported that the higher titres found in SSF were due to SSF cultivation works as a fed-batch culture with fast oxygenation but slow sugar supply and, in addition, SSF has the added advantage of being a static process without mechanical energy expenditures. Moreover, recently Roy et al. (2006) [6] reported that SSF was a better treatment method for rubber biodegradation than SmF.

[*] corresponding author: e-mail: susana.rodriguez@urv.cat, Phone: +34 977559617

Despite the numerous both processing and biological advantages that SSF offers over SmF [3], there are few designs available in the literature for bioreactors operating in SSF conditions. This is principally due to several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer, moisture and agitation [7]. SSF lacks the robust control mechanisms that are usually associated with SmF. Control of the environment within the bioreactors is also difficult to achieve, particularly temperature and moisture. Several authors have reported remarkable results in terms of yield or quality when cultivating microorganisms on solid substrates at laboratory-scale bioreactors. However, when these processes are reproduced at larger scales results are mostly disappointing. Scaling-up of SSF processes is difficult and unreliable [8-12]. This can be attributed to the radically different growing conditions microorganisms find in a large-scale reactor. At lab-scale, conditions such as temperature, water activity and pH can easily be maintained homogeneous and constant at optimum levels throughout the fermentation processes [10–12]. In contrast, bed heterogeneity in large-scale SSF bioreactors is inevitable as simulations with a distributed parameter model [13] and experiments with a pilot-scale SSF bioreactor [14] have shown. This is due to the low heat and mass transfer rates characteristic of the solid porous bed and that stirring is limited to avoid damaging the microorganisms [8, 10–12, 15]. Effective control strategies have shown to reduce time and space variability in growing conditions in large-scale SSF bioreactors [16, 17]. However, process complexity and the lack of reliable and affordable instrumentation make designing control strategies difficult. Computer simulation, on the other hand, is an alternative way to test new automatic control designs, since even hundreds of simulation runs are fast and relatively inexpensive. Nevertheless, only simulations with complex models provide credible results. Recently, Fernández-Fernández and Pérez-Correa (2007) [18] developed a complex model for a packed-bed solid-state bioreactor, which will be useful in the design of effective control systems for intermittently mixed SSF bioreactors. Also, Sahir et al. (2007) [19] developed a mathematical model for a packed-bed solid-state bioreactor utilising the *N*-tanks in series approach which reduced computational complexities, thus, facilitating the design of packed-bed SSF bioreactors.

Table 1. History and development of SSF [1]

Period	Development
2,600 BC*	Bread making by Egyptians
BC in Asia (recorded history 1,000 BP**)	Cheese making by *Penicillium roqueforti*
2,500 BP	Fish fermentation/preservation with sugar, starch, salts, etc Koji process
7th Century	Koji process from China to Japan by Buddhist priests
18th Century	Vinegar from pomace Gallic acid used in tanning, printing, etc
1860-1900	Sewage treatment
1900-1920	Fungal enzymes (mainly amylases), kojic acid
1920-1940	Fungal enzymes, gluconic acid, rotary drum fermenter, citric acid
1940-1950	Fantastic development in fermentation industry. Penicillin production by SSF and SmF
1950-1960	Steroid transformation by fungal cultures
1960-1980	Production of mycotoxins, protein enriched feed
1980-present	Various other products like alcohol, gibberellic acid

*BC, before Christ
**BP, before present

Table 2. Advantages and disadvantages of SSF over SmF (extracted from Pérez-Guerra et al., 2003 [2])

Advantages	Disadvantages
Similar or higher yields than those obtained in the corresponding submerged cultures	Only microorganisms that can grow at low moisture levels can be used
The low availability of water reduces the possibilities of contamination by bacteria and yeast. This allows working in aseptic conditions in some cases	Usually the substrates require pre-treatment (size reduction by grinding, rasping or chopping, homogenisation, physical, chemical or enzymatic hydrolysis, cooking or vapour treatment)
Similar environment conditions to those of the natural habitats for fungi which constitute the main group of microorganisms used in SSF	Biomass determination is very difficult
Higher levels of aeration, especially adequate in those processes demanding an intensive oxidative metabolism	The solid nature of the substrate causes problems in the monitoring of the process parameters (pH, moisture content, and substrate, oxygen and biomass concentration)
The inoculation with spores (in those processes that involve fungi) facilitates their uniform dispersion through the medium	Agitation may be very difficult. For this reason static conditions are preferred
Culture media are often quite simple. The substrate usually provides all the nutrients necessary for growth	Frequent need of high inoculum volumes
Simple design reactors with few spatial requirements can be used due to the concentrated nature of the substrates	Many important basic scientific and engineering aspects are yet poor characterised. Information about the design and operation of reactors on a large scale is scarce
Low energetic requirements (in some cases autoclaving or vapour treatment, mechanical agitation and aeration are not necessary)	Possibility of contamination by undesirable fungi
Small volumes of polluting effluents. Fewer requirements of dissolvents are necessary for product extraction due to its high concentration	The removal of metabolic heat generated during growth may be very difficult
The low moisture availability may favour the production of specific compounds that may not be produced or may be poorly produced in SmF	Extracts containing products obtained by leaching of fermented solids are often of viscous nature
In some cases, the products obtained have slightly different properties (e.g. more thermotolerance) when produced in SSF in comparison to SmF	Mass transfer limited to diffusion
Due to the concentrated nature of the substrate, smaller reactors in SSF with respect to SmF can be used to hold the same amounts of substrate	In some SSF, aeration can be difficult due to the high solid concentration
	Spores have longer lag times due to the need for germination
	Cultivation times are longer than in SmF

2. General aspects of SSF

SSF is a microbial process occurring mostly on the surface of solid materials, which can absorb or contain water, in the presence or absence of soluble nutrients [20]. SSF comprises two very different modes [21]. In the first one, a divided and humidified solid (organic material) acts as both support and nutrient source and the process essentially occurs in the absence of free water [22-24] (Fig. 1). In the second mode, a nutritionally inert solid (synthetic material), which exclusively acts as a support, is

soaked in a nutrient solution (Fig. 2). This model is less used, but it reports some advantages. The use of a defined liquid medium and an inert support with a homogenous physical structure improves controlling and monitoring the process and the reproducibility of fermentations. However, the use of inert supports presents economical disadvantages [25].

Fig. 1. Solid-state cultures of the white-rot fungus *T. pubescens* grown on different natural supports: mandarin peelings (left), banana skins (center) and wheat bran flakes (right)

Fig. 2. The white-rot fungus*T. hirsuta* grown on cubes of nylon sponge (inert support) under SSF conditions

In both cases, the success of the process is directly related to the physical characteristics of the support (particle size, shape, porosity, consistency), which favour both gas and nutrient diffusion and the attachment of the microorganisms [26]. Generally, smaller substrate particles provide a larger surface area for microbial colonisation but if they are too small may result in substrate agglomeration as well as poor growth. In contrast, larger particles provide better aeration but a limited surface for microbial colonisation. Therefore, a compromised particle size must be selected for each particular process [27]. Availability and cost are also criteria of great importance. In the case of the organic materials the chemical composition also plays an important role [28]. Thus, Rodríguez Couto et al. (2003) [29] studied the lignin peroxidase (LiP) production by the white-rot fungus *Phanerochaete chrysosporium* under solid-state conditions utilising organic materials with different lignin content. They found that the materials with higher lignin content led to the higher LiP activities. This stimulating effect of lignin was also observed for laccase production by the white-rot fungus *Trametes hirsuta* [30]. More recently, Osma et al. (2007) [31] reported that the white-rot fungus *Trametes pubescens* grown on banana skin produced laccase with high ability to decolourise synthetic dyes. Also, Rodríguez Couto (2007) [32] found that laccase produced by *T. hirsuta* grown on paper cuttings was able to decolourise synthetic dyes at alkaline pHs. This illustrates the enormous importance of selecting a suitable support-substrate for each particular purpose.

SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi [33, 34], since they reproduce the natural living conditions of such fungi [27] due to which they may be more capable of producing certain enzymes with high productivity in comparison to SmF. Fig. 3

shows a photograph of the white-rot fungi *T. pubescens* and *T. hirsuta* as grow in nature. In addition, the morphology of filamentous fungi allow them colonising and penetrating the solid support-substrates in search for nutrients. Fig. 4 shows scanning electron microscopy (SEM) microphotographs of banana skin with and without fungus. It can be observed that the fungus grew well attached to the skin. The application of agro-industrial residues in SSF bio-processes not only provides an alternative substrate but reduces the pollution problems caused by their accumulation.

Fig. 3. The white-rot fungi *T. pubescens* (A) and *T. hirsuta* (B) as grow in nature

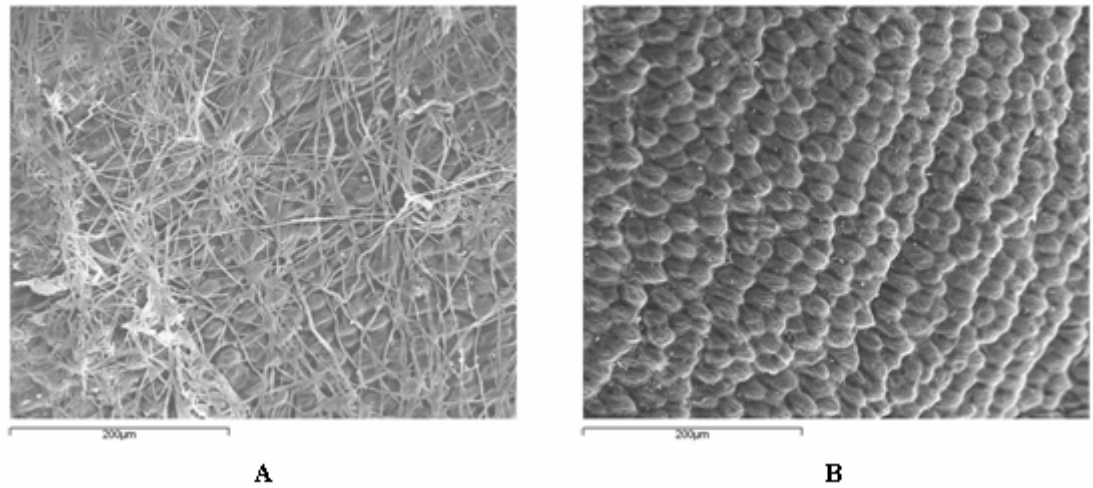

Fig 4. SEM micropotographs of banana skin: A (with fungus); B (without fungus) (extracted from Osma et al., (2007) [31])

3. Example of SSF application: laccase production

White-rot fungi are the only organisms able to degrade the whole wood components [35] due to the secretion of an extracellular ligninolytic complex during their secondary metabolism in response to nutrient limitation. The main components of this ligninolytic complex consist of a family of peroxidases named lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs) and a family of multicopper oxidases named laccases.

To study the ligninolytic ability of white-rot fungi the oxidation of model compounds such as 2,2'-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) and the polymeric dye Poly R-478 on low-nutrient agar plates is performed. Positive ligninolytic fungi turned ABTS from light green to dark green and Poly R-478 from purple to yellow. Fig. 5 and Fig. 6 show the ligninolytic ability of *T. pubescens* grown on ABTS and PolyR-478 agar plates, respectively.

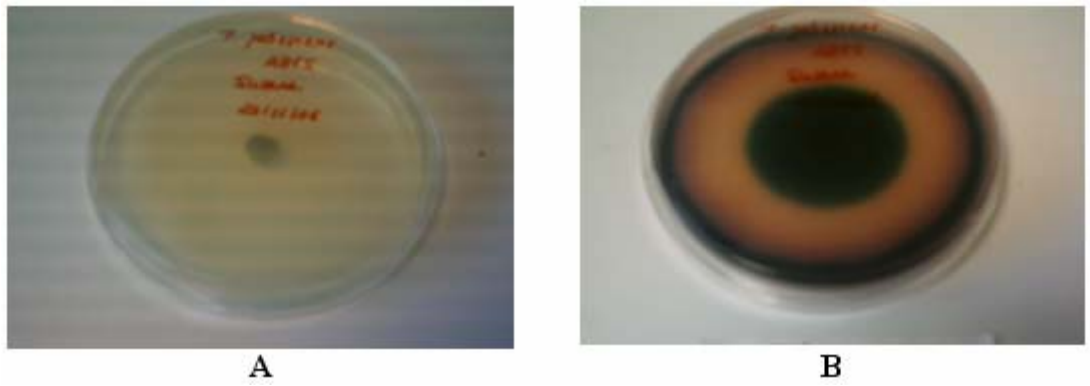

Fig. 5. ABTS oxidation by *T. pubescens* on agar plates, A: day 0, B: day 8

Fig. 6. Poly R-478 oxidation by *T. pubescens* on agar plates, A: day 0, B: day 11

Laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are particularly abundant in white-rot fungi. Laccases catalyse the oxidation of both phenolic and non-phenolic compounds [36] and are able to mineralise a wide range of synthetic dyes [37-40]. Fig. 7 shows a typical laccase-catalysed reaction where a diphenol is oxidised to form a free radical, which can further undergo a second enzymatic catalysis to form a quinone,

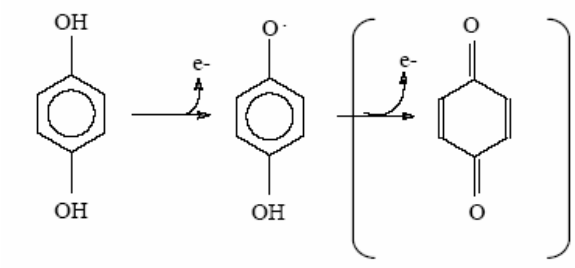

Fig. 7. Typical laccase-catalysed reaction for a diphenol (extracted from Tavares (2006) [41])

Laccases have been subject of intensive research in the last decades because they have the following properties: broad substrate specificity [42], do not need the addition or synthesis of a low molecular weight cofactor, as their cosubstrate – oxygen – is usually present in their environment, most laccases are extracellular enzymes, making the purification procedures very easy and they generally exhibit a considerable level of stability in the extracellular environment. Such characteristics make laccases very suitable for their application to several bioprocesses such as biopulping, biobleaching and the treatment of industrial wastewater.

The application of laccases to the above-mentioned processes requires the production of large amounts of enzyme at low cost. Therefore, research in this area is oriented towards the search for efficient production systems. A good strategy for this purpose is the production of laccase by SSF using agro-industrial wastes as a support-substrate. The food, agricultural and forestry industries produce large volumes of wastes annually world-wide which cause a serious disposal problem. In addition, the reutilisation of biological wastes shows a great interest, since due to legislation and environmental reasons the industry is more and more forced to find an alternative use(s) for its residual matter. Most of such wastes are rich in soluble carbohydrates and also contain inducers of laccase synthesis, ensuring an efficient production of laccase [43-46]. Furthermore, agro-wastes have shown to produce higher laccase activities than inert supports for the same fungal strain and culture conditions [47-50]. Table 3 reports the laccase production by several white-rot fungi grown on different natural supports under solid-state conditions.

Table 3. - Laccase production by different white-rot fungi grown on different natural supports under SSF conditions

Support	Microorganism	Reference
Sugarbeet bagasse	*Trametes versicolor*	[51]
Wheat straw	*Phlebia radiata*	[52]
Ballico seed	*Botryosphaeria sp.*	[53]
Corn stalks	*Lentinus edodes* strain CS-495	[54]
Straw	*Pleurotus sp.*	[55]
Cotton wastes	*Pleurotus ostreatus, Pleurotus cystidiosus, Pleurotus pulmonarius, Pholiota nameko*	[56]
Barley bran	*P. chrysoporium*	[57]
Cotton stalks	*P. chrysosporium, Funalia trogii*	[58]
Corncob	*P. chrysoporium*	[59]
Sawdust, grapewine cuttings	*Coriolus hirsutus, Daedaleopsis confragosa, Marasmius allaceus, P. chrysosporium*	[60]
Wheat straw	*P. ostreatus*	[61]
Corncob	*P. chrysosporium, P. radiata*	[62]
Wheat bran, wheat straw	*P. pulmonarius*	[63]
Neem hull, wheat bran, sugarcane bagasse	*P. ostreatus, P. chrysosporium*	[64]
Wheat straw, barley straw, wood shavings, barley bran	*T. versicolor*	[47]
Barley bran, apple peelings, orange peelings, potato peelings	*T. hirsuta*	[48]
Canola roots	*Cyathus olla*	[65]

Eucalyptus grandis	*Ceriporiopsis subvermispora*	[66]
Grape seeds, barley bran	*T. hirsuta*	[30]
Wheat straw	*Fomes sclerodermeus*	[67]
Banana waste	*P. ostreatus, Pleurotus sajor-caju*	[43]
Barley bran	*T. versicolor*	[68]
Corncob	*P. pulmonaris*	[69]
Barley bran	*T. hirsuta, T. versicolor*	[49]
Chestnut shell, barley bran	*Coriolopsis rigida*	[70]
Coconut flesh	*T. hirsuta*	[61]
Kiwi fruit	*T. hirsuta*	[45]
Wheat bran flakes	*T. pubescens*	[72]
Groundnut seeds Groundnut shells	*T. hirsuta*	[73]
Grape seeds	*T. hirsuta*	[50]
Rubberwood sawdust, oil palm frond parenchyma tissue, sago hampas	*Pycnoporus sanguineus*	[74]
Banana skin	*T. pubescens*	[31]
Paper cuttings	*T. hirsuta*	[32]
Orange peelings	*T. hirsuta*	[75]

Given the potential applications of laccases and the need for the development of economical methods for improving laccase production from fungi with an overall aim of reducing the cost of the industrial processes, the use of SSF, especially using agro-wastes as a support-substrate, is an appalling alternative. However, there are few designs available in the literature for bioreactors operating in solid-state conditions, especially at a large-scale due to the reasons stated in the introduction section. Moreover, the use of agro-wastes as a support presents additional problems, which are not found operating with inert supports, such as support degradation and/or support accretion may occur during the fermentation process. This would cause mass and oxygen restrictions into the reactor bed hampering its proper performance. Therefore, advances in the design of SSF bioreactors are needed for the industrial explotation of SSF.

4. Conclusions

SSF is a very promising cultivation technique for the production of industrially-relevant enzymes such as laccases, especially utilising agro-wastes as support-substrates. The scarcity of bioreactor designs to perform solid-state processes together with the advantages offered by such processes promote the necessity of developing new bioreactor configurations or modifying the designs that already exist. These bioreactor designs should be able to operate in continuous mode with high enzyme productivity for prolonged periods of time as well as permit the scale-up of the process.

Acknowledgements SRC and JLTH are *Ramón y Cajal Senior Research Fellows*. The authors thank the Spanish Ministry of Education and Science for promoting the *Ramón y Cajal Programme*. JFO is supported by a predoctoral fellowship from the Rovira i Virgili University (Tarragona, Spain).

References

[1] A. Pandey, Process Biochemistry **27**, 109 (1992).
[2] N. Pérez-Guerra A. Torrado-Agrasar, C. López-Macias and L. Pastrana, Electronic Journal of Environmental, Agricultural and Food Chemistry **2**, 343 (2003).
[3] U. Hölker, M. Hofer and J. Lenz, Applied Microbiology and Biotechnology **64**, 175 (2004).
[4] L.R. Castilho, C.M.S. Polato, E.A. Baruque, G.L. Sant'Anna Jr. and D.M.G. Freire, Biochemical Engineering Journal **4**, 239 (2000).
[5] G. Viniegra-González, E. Favela-Torres, C. Noe Aguilar, J. de Jesús Romero-Gómez, G. Díaz-Godínez and C. Augur, Biochemical Engineering Journal **13**, 157 (2003).
[6] R.V. Roy, M. Das, R. Banerjee and A.K. Bhowmick, Process Biochemistry **41**, 181 (2006).
[7] B.K. Lonsane, N.P. Ghildyal, S. Budiatman and S.V. Ramakrishna, Enzyme and Microbial Technology **7**, 258 (1985).
[8] J.R. Pérez-Correa and E. Agosin in: Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, M. Flickinger and D. Drew (eds.), Wiley, New York, 1999, pp. 2429-2446.
[9] M. Raimbault, Electronic Journal of Biotechnology **1**, 26 (1998). http://www.ejbiotechnology.info.
[10] F. Nagel, J. Tramper, M. Bakker and A. Rinzema, Biotechnology and Bioengineering **72**, 219 (2000).
[11] F. Nagel, J. Tramper, M. Bakker and A. Rinzema, Biotechnology and Bioengineering **72**, 231 (2000).
[12] M. Peña y Lillo, R. Pérez-Correa, E. Agosin and E. Latrille, Biotechnology and Bioengineering **76**, 44 (2001).
[13] O.F. von Meien and D.A. Mitchell, Biotechnology and Bioengineering **79**, 41 (2002).
[14] J.R. Pérez-Correa, M. Fernández, O.F. von Meien, L.F.L. Luz Jr and D.A. Mitchell, in: Solid-state fermentation bioreactors: fundamentals of design and operation, D.A. Mitchell, N. Krieger and M. Berovic (eds.). Springer, Berlin, 2006, pp. 387-403.
[15] M. Raimbault, Fermentation en milieu solide, corissance de champignons filamenteuxsur substrat amylaceé, Toulouse, France, University Paul Sabatier, 1981, pp. 130-133.
[16] M. Fernández and R. Pérez-Correa, Proceedings of the international conference on integrated modelling and analysis in applied control and automation. Bergeggi, Italy, 2004. pp. 141-149.
[17] O.F. von Meien, L.F.L. Luz, D.A. Mitchell, J.R. Pérez-Correa, E. Agosin, M. Fernández-Fernández and J.A. Arcas, Chemical Engineering Science **59**, 4493 (2004).
[18] M. Fernández-Fernández and J.R. Pérez-Correa, Process Biochemistry **42**, 224 (2007).
[19] A.H. Sahir, S. Kumar and S. Kumar, Biochemical Engineering Journal **35**, 20 (2007).
[20] G. Viniegra-Gonzalez, C.P. Sarralde-Corona and F. López Isunza, in: Advances in bioprocess engineering, E. Galindo and O.T. Ramírez (eds.) Kluwer Academic, Amsterdam, 1994, p. 295
[21] M.A. Murado, M.P. González and L. Pastrana, in: Production of microfungal metabolites on inert solid supports, S.G. Pandalai (ed.), Research Signpost, India,1, 1998, pp. 405-432.
[22] P.K.R. Kumar and B.K. Lonsane, Biotechnology and Bioengineering **30**, 267 (1987).
[23] M.V. Ramesh and B.K. Lonsane, Applied Microbiology and Biotechnology **33**, 501 (1990).
[24] L.M. Pastrana, M.P. Gonzalez, J. Pintado and M.A. Murado, Enzyme and Microbial Technology 17, **784** (1995).
[25] T. Crommentuijn, J.A. Stab, A. Doornekamp, O. Espoppey and C.A.M. Van Gestel. British Ecotoxicological Society, Functional Ecology **9**, 734 (1995)
[26] D.A. Mitchell, in: Solid State Cultivation, H.W. Doelle, D.A. Mitchell and C.E. Rolz (eds.), Elsevier Applied Science, London and New York, 1992, pp. 17-28.
[27] A. Pandey, P. Selvakumar, C.R. Soccol and P. Nigam, Current Science **77**, 149 (1999).
[28] S. Rodríguez Couto and A. Sanromán, Biochemical Engineering Journal **22**, 219 (2005)
[29] S. Rodríguez Couto, D. Moldes, A. Liébanas and A. Sanromán, Biochemical Engineering Journal **15**, 21 (2003).
[30] D. Moldes, P.P. Gallego, S. Rodríguez Couto and A. Sanromán. Biotechnology Letters **25**, 491 (2003).
[31] J.F. Osma, J.L. Toca Herrera and S. Rodríguez Couto, Dyes and Pigments **75**, 32 (2007).
[32] S. Rodríguez Couto. Engineering in Life Sciences **7**, 229 (2007).
[33] A. Pandey, W. Azmi, J. Singh and U.C. Banerjee, in: Biotechnology: Food Fermentation, V.K. Joshi and A. Pandey (eds.), Educational Publishers, New Delhi, 1999, pp. 383-426.
[34] M. Moo-Young, A.R. Moreira and R.P. Tengerdy, in: The Filamentous Fungi, J.E. Smith, D.R. Berry and B. Kristiansen (eds.), Edward Arnold Publishers, London, 1983, pp. 117-144.
[35] T.K. Kirk and P. Fenn, in: Decomposer Basidiomycetes British Mycological Society Simposium 4, Franland, Hedges and Swift (eds.), Cambridge University Press, 1982, pp. 67-90.
[36] R. Bourbonnais and M.G. Paice, FEBS Letters 267, **99** (1990).
[37] E. Rodríguez, M.A. Pickard and R. Vázquez-Duhalt, Current Microbiology **38**, 27 (1999).

[38] J. Swamy and J.A. Ramsay, Enzyme and Microbial Technology **24**, 130 (1999).
[39] Y. Wong and J. Yu, Water Research **33**, 3512 (1999).
[40] E. Abadulla, T. Tzanov, S. Costa, K.H. Robra, A. Cavaco-Paulo and G. Gübitz, Applied and Environmental Microbiology **66**, 3357 (2000).
[41] A.P.M. Tavares, Produção de lacase para potencial aplicação como oxidante na indústria papeleira, doctoral thesis. University of Aveiro, Portugal, 2006.
[42] T. Saito, P. Hong, K. Kato, M. Okazaki, H. Inagaki, S. Maeda and Y. Yokogawa, Enzyme and Microbial Technology, **33**, 520 (2003).
[43] V. Elisashvili, H. Parlar,E. Kachlishvili, D. Chihua, M. Bakrazde, N. Kokhreidze and G. Kvesitadze, Advances in Food Science **23**, 117 (2001).
[44] G.V. Reddy, B.P. Ravindra P. Komaraiah, K.R.R.M. Roy and I.L. Kothari, Process Biochemistry **38**, 1457 (2003).
[45] A.N. Kapich, B.A. Prior, A. Botha, S. Galkin, T. Lundell and A. Hatakka, Enzyme and Microbial Technology **34**, 187 (2004).
[46] E. Rosales, S. Rodríguez Couto and Mª Á. Sanromán, Journal of Food Engineering **66**, 419 (2005).
[47] S. Rodríguez Couto, M. Gundín, M. Lorenzo and A. Sanromán, Process Biochemistry **38**, 249 (2002).
[48] E. Rosales, S. Rodríguez Couto and A. Sanromán, Biotechnology Letters **24**, 701 (2002).
[49] S. Rodríguez Couto, E. Rosales, M. Gundín and Mª Á. Sanromán, Journal of Food Engineering **64**, 423 (2004).
[50] S. Rodríguez Couto, E. López and Mª Á. Sanromán, Journal of Food Engineering **74**, 263 (2006)
[51] M. Pal, A. Calvo, M.C. Terron and A.E. Gonzalez, World Journal of Microbiology and Biotechnology **11**, 541 (1995).
[52] T. Vares, M. Kalsi and A. Hatakka, Applied and Environmental Microbiology **61**, 3515 (1995).
[53] T.C.P. Barbosa, A.P. Luz, M.L. Bedin and N.H. Gabilan, International Biodeterioration and Biodegradation **37**, 122 (1996).
[54] A. D´Annibale, D. Celleti, M. Felici, E. Di Mattia and G. Giovannozzi-Sermani, Acta Biotechnologica **16**, 257 (1996).
[55] E. Lang, F. Nerud, E. Novotna and R. Martens, Folia Microbiologica **41**, 489 (1996).
[56] M. Jaszek, E. Malarczyk and A. Leonowicz, Investigation of ligninolytic enzymes during solid state fermentation of cotton wastes by selected strains of basidiomycetes, 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, 16-18 June 1998.
[57] S. Rodríguez Couto and M. Rättö, Biodegradation **9**, 143 (1998).
[58] S. Sik and A. Ünyayar, Turkish Journal of Biology **22**, 287 (1998).
[59] S. Rodríguez Couto, M. A. Longo, C. Cameselle and A. Sanromán, Acta Biotechnologica **19**, 17 (1999).
[60] V. Elisashvili, H. Parlar, E. Kachlishvili, D. Chichua and G. Kvesitadze, G., Advances in Food Science **23**, 117 (2001).
[61] P. Baldrian and J. Gabriel, Folia Microbiologica **47**, 385 (2002).
[62] D.R. Cabaleiro, S. Rodríguez, A. Sanromán and M. A Longo, Process Biochemistry **37**, 1017 (2002).
[63] C.G. Marques de Souza, A. Zilly and R.M. Peralta, Journal of Basic Microbiology **42**, 83 (2002).
[64] V. Pradeep and M. Datta, Applied Biochemistry and Biotechnology **102-103**, 109 (2002).
[65] T.C. Shinners-Carnelley, A. Szpacenko, J.P. Tewari and M.M. Palcic, Phytoprotection **83**, 31 (2002).
[66] M. Fenice, S.G. Giovannozzi, F. Federici and A. D'Annibale, Journal of Biotechnology **100**, 77 (2003).
[67] V.L. Papinutti, L.A. Diorio and F. Forchiassin, Journal of Industrial Microbiology and Biotechnology **30**, 157 (2003).
[68] S. Rodríguez Couto, D. Moldes, A. Liébanas and Á. Sanromán, Biochemical Engineering Journal **15**, 21 (2003).
[69] G.K. Tychanowicz, A. Zilly, C.G, Marques de Souza and R.M. Peralta, Process Biochemistry **39**, 855 (2004)
[70] J. Gómez, M. Pazos, S. Rodríguez Couto and Sanromán**,** Journal of Food Engineering **68**, 315 (2005).
[71] S. Rodríguez Couto and Mª Á. Sanromán, Journal of Food Engineering **71**, 208 (2005).
[72] J.F. Osma, J.L. Toca Herrera and S. Rodríguez Couto, Laccase production by *Trametes pubescens* grown on wheat bran under solid-state conditions, 6th European Symposium on Biochemical Engineering Science, Salzburgo (Austria), 27-30 Agosto, 2006
[73] S. Rodríguez Couto and Mª Á. Sanromán, Journal of Food Engineering **73**, 388 (2006).
[74] S. Vikineswary, N. Abdullah, M. Renuvathani, M. Sekaran, A. Pandey and E.B.G. Jones, Bioresource Technology **97**, 171 (2006).
[75] E. Rosales, S. Rodríguez Couto and Mª Á. Sanromán, Enzyme and Microbial Technology **40**, 1287 (2007).

