Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Science Letters:

Effects of polyurethane matrices on fungal tannase and gallic acid production under solid state culture*

TREVIÑO Lucia, CONTRERAS-ESQUIVEL Juan C.,
RODRÍGUEZ-HERRERA Raul, AGUILAR Cristóbal Noé†‡

(*Department of Food Research, Autonomous University of Coahuila, Saltillo, Coahuila 25000, Mexico*)

†E-mail: cag13761@mail.uadec.mx

Received July 23, 2007; revision accepted Aug. 17, 2007

Abstract: The influence of the physical structure of polyurethane matrix as a support in a solid state culture in tannase production and gallic acid accumulation by *Aspergillus niger* Aa-20 was evaluated. Three different polyurethane matrices were used as the support: continuous, semi-discontinuous and discontinuous. The highest tannase production at 2479.59 U/L during the first 12 h of culture was obtained using the discontinuous matrix. The gallic acid was accumulated at 7.64 g/L at the discontinuous matrix. The results show that the discontinuous matrix of polyurethane is better for tannase production and gallic acid accumulation in a solid state culture bioprocess than the continuous and semi-discontinuous matrices.

Key words: Tannase, Gallic acid, Polyurethane matrix support, Solid state culture, *Aspergillus niger* Aa-20
doi:10.1631/jzus.2007.B0771 **Document code:** A **CLC number:** TS2

INTRODUCTION

Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound, mainly used in the pharmaceutical industry for manufacturing trimethoprim, an antibacterial agent (Kar and Banerjee, 2000). Gallic acid is also used in food industries as a substrate to produce a potent antioxidant, propilgallate. Tannase or tannin-acyl-hydrolase (EC 3.1.1.20), is an extracellular and inducible enzyme, which catalyses the hydrolysis of some tannins and gallic acid esters. Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight, which differ from most other natural phenolic compounds in their ability to precipitate proteins from solutions (Lekha and Lonsane, 1997). Based on the structures and properties, they are distributed in four groups: condensed tannins, gallotannins, ellagitannins and complex tannins (Khanbabaee and van Ree, 2001). Condensed tannins, also known as polymeric proanthocyanidins, are composed of flavonoid units (Bhat *et al*., 1998). Gallotannins are esters of gallic acid linked to a sugar core, usually glucose, whereas ellagitannins are esters of ellagic acid also linked to a sugar core (Aguilar *et al*., 2007). Complex tannins are mixed compounds of flavonoid units linked to gallic acids by ester bonds.

Microorganisms are the main source for industrial enzymes due to their biochemical diversities, technical and financial advances. Tannase is usually obtained from bacteria, some yeasts (Aoki *et al*., 1976) and filamentous fungi, mainly from the following species: *Aspergillus*, *Penicillium*, *Fusarium* and *Thichoderma* (Aguilar *et al*., 2007). This enzyme is applied commercially in the industries of drinks and foods, chemical-pharmaceutics, brewing, and animal feed (Belmares *et al*., 2004). The use of tannase in large scale, however, is much limited due to a variety of factors including production cost and insufficient

‡ Corresponding author

* Project supported by the Agricultural, Livestock and Fishing Department (SAGARPA, No. 12385) and the National Forest Commission (CONAFOR, No. 2004-C01-13), and the Council of Science and Technology, Mexico

knowledge of the enzyme (Aguilar and Gutiérrez-Sánchez, 2001). Although the gallic acid production in a solid state culture has been evaluated (Kar *et al*., 1999; Kar and Banerjee, 2000; Aguilar *et al*., 2002), the influence of the physical nature of a matrix used in a solid culture to produce tannase and gallic acid remains unclear. In this study, we investigate the effects of polyurethane matrices in a solid state culture on *Aspergillus niger*-induced tannase and gallic acid production.

MATERIALS AND METHODS

Microorganism

Aspergillus niger Aa-20 spores [IRD-UAMI (Research Institute of Development, France and Metropolitan Autonomous University, Mexico) collection] used in this work were stored at –20 °C in cryoblocks® (bead storage system, Technical Service Consultants Ltd., France). Inoculum was prepared by transferring the spores to potato dextrose agar (PDA) and incubated at 30 °C for 3~5 d. The spores were then scraped into a 0.01% Tween 80 solution and counted in a Neubauer chamber.

Chemicals

Tannic and gallic acids used were analytical grade (Sigma, Mexico, Federal District). PDA agar and salts for Czapek-Dox were obtained from (Merk Reagents, Monterrey, Mexico).

Culture medium

The medium was Czapek-Dox modified with 12.5 g/L tannic acid as an inducer and a sole carbon source (Aguilar *et al*., 2002).

Support

Polyurethane foam (PUF) was used as the solid support for the solid culture. PUF matrix was structured in three patterns: continuous, semi-discontinuous and discontinuous. Continuous matrix was constructed by PUF circles of 9.0 cm in diameter and 0.5 cm in thickness; semi-discontinuous matrix was composed of PUF cubes of 0.5 cm×0.5 cm×0.5 cm; and the discontinuous matrix was pulverized PUF. Three grams of each support was placed into a 250 ml Erlenmeyer flask and 7 ml of Czapek-Dox modified media were added. Spores were inoculated at 2×10^7 per gram of PUF, and then were incubated for 48 h at 30 ºC, 70% initial humidity, 5.5 initial pH. Six samples were taken during the kinetics. Tannase and gallic acid extracts were recovered by adding 14 ml of water to each reactor. It was filtrated using 41 Whatman paper, and the filtrated liquid was used for the assay of the enzymatic activity. In addition, gallic acid accumulation and substratum consumption were determined as described in the next paragraph.

Analytical methods

Tannase production was measured by methanolic rhodanine spectrophotometric method (Sharma *et al*., 2000), using methyl gallate prepared in 0.05 mol/L citrate buffer (pH=5) as substrate. The 0.05 mol/L citrate buffer (pH=5) was used as control and also as the crude enzymatic extract. For the colour reaction, the methanolic rhodanine (0.67%) and potassium hydroxide (0.5 mol/L) were used. This reaction was monitored by measuring absorbance at 520 nm. One unit of enzyme (U) was defined as the amount of enzyme able to release 1 mol gallic acid per millilitre per minute. Gallic acid accumulation was also measured by methanolic rhodanine spectrophotometric method (Sharma *et al*., 2000). Tannic acid concentration was evaluated by spectrophotometry using the phenol-sulfuric method (λ=480 nm) reported by Aguilar (2000). Briefly the method implies a thermal reaction of 1 ml of sample with 2 ml of phenol-sulfuric reagent (1 mg/ml) during 5 min in a boiling water bath, then the sample is cooled and the absorbance is recorded at 480 nm. Biomass determination was analysed by measuring protein concentration with the biuret assay kit (Randox Laboratories Ltd., USA) following the technique reported by Córdova-López *et al*.(1996), where 0 g of fermented solid is impregnated with phosphoric acid (0.15 mol/L) and heated in a boiling water bath to hydrolyse the mycelium during 7 min; the sample is then cooled and centrifuged to obtain a mycelial protein solution; finally, 200 ml of sample is mixed with 800 ml of biuret reagent and the blue color is measured at 595 nm.

Kinetic parameters

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

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

where X (g/L) represents the biomass calculated, X_0 and $X_{\max}$ (g/L) are the initial and maximum biomass values, respectively, μ (h^{-1}) is the specific growth rate, and t (h) is the culture time. The algorithm minimizes the sum of least square errors, comparing experimental data with the theoretical values obtained.

The biomass/substrate yield, $Y_{X/S}$, is calculated by the next equation:

$$Y_{X/S}=(X_{\max}-X_0)/(S_0-S_{\text{final}}),$$

where $X_{\max}$ and X_0 (g/L) are the maximum and initial biomass values obtained, respectively, and S_0 and S_{final} (g/L) are the initial and final substrate concentration values, respectively.

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

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

where q_S is giving as grams of substrate consumed per gram of biomass per hour.

Tannase/biomass yield, $Y_{E/X}$, is estimated from the linear correlation between tannase activities, E (U/L) and biomass concentration, X (g/L). The yield coefficient is defined as $Y_{E/X}$ (units of tannase per gram X).

The specific rate of products, q_P, is defined as follows:

$$q_P=\mu/Y_{P/X},$$

where q_P is the units of tannase or grams of gallic acid produced per gram of biomass per hour, $Y_{P/X}$ is the product/biomass yield.

RESULTS AND DISCUSSION

The biomass production of *Aspergillus niger* Aa-20 reached the highest value (1.30 g/L) in the semi-discontinuous matrix, whereas discontinuous and continuous matrices gained 0.81 and 0.68 g/L, respectively (Fig.1). Table 1 presents the values of the kinetic parameters associated with the fungal growth, the specific growth rate μ and the maximum growth $X_{\max}$.

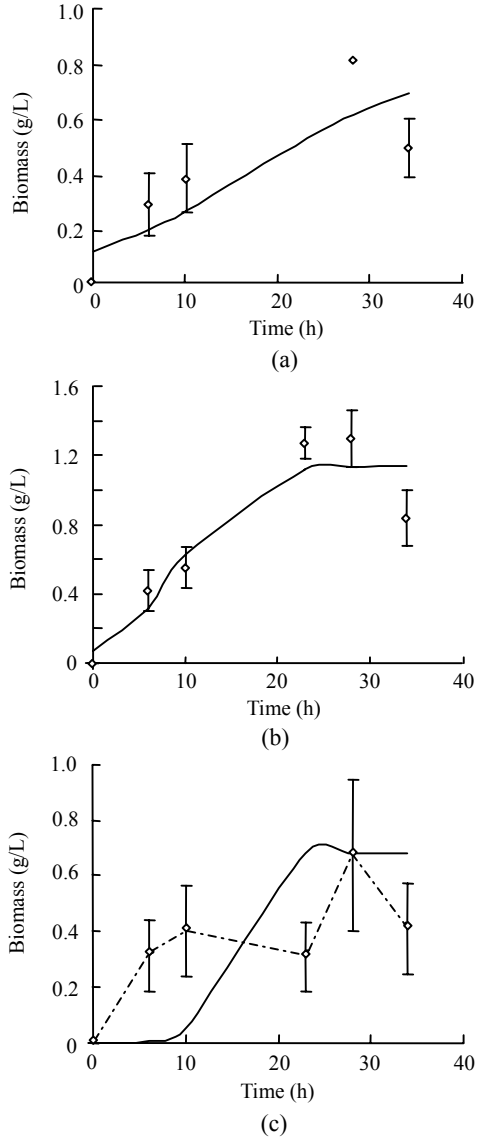

Fig.1 Growth of *Aspergillus niger* Aa-20 in the three different matrices of PUF: (a) semi-discontinuous; (b) continuous; (c) discontinuous. Line represents the calculated values and points are the experimental values (mean±*SD*)

Table 1 Kinetic parameters associated to fungal growth in the three different matrices of PUF

Parameters	μ (h^{-1})	$X_{\max}$ (g/L)
Continuous	0.10	0.81
Semi-discontinuous	0.29	1.14
Discontinuous	0.63	0.68

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

Table 2 shows the results of the substrate consumption. It was found that in the continuous matrix the tannic acid was consumed near completely, with only 0.30 g/L remained in the broth at the end of the culture. In the semi-discontinuous matrix, a concentration of 0.70 g/L was remained. However, in the discontinuous matrix, the substrate was not so efficiently consumed, since the remained was at 5.59 g/L.

Table 2 Kinetic parameters associated to substrate uptake in the three different matrices of PUF

Parameters	$Y_{X/S}$	q_S [g/(g·h)]
Continuous	0.07	0.66
Semi-discontinuous	0.11	0.39
Discontinuous	0.10	0.16

$Y_{X/S}$: Biomass/substrate yield; q_S: Substrate uptake rate (grams of substrate per gram of biomass per hour)

Fig.2 presents the results of extracellular tannase production, and Table 3 shows the corresponding kinetic parameters. The highest enzyme activity was found at the discontinuous matrix with a 2479.59 U/L value. At the continuous and semi-discontinuous matrices, maximal activities were 543.40 and 479.39 U/L, respectively. The maximal values were obtained at a really short fermentation period around the first 12 h.

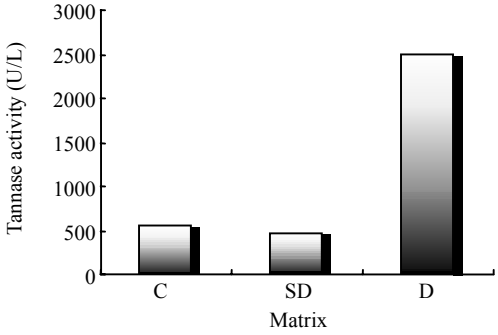

Fig.2 Tannase activity obtained in the three different matrices of PUF

The culture conditions were: incubation temperature 30 °C, humidity 70%, pH 5.5 and culture time 11.5 h. C: Continuous; SD: Semi-discontinuous; D: Discontinuous

Table 3 Kinetic parameters associated to tannase production in the three different matrices of PUF

Parameters	$Y_{P/X}$ (U/g)	q_P [U/(g·h)]
Continuous	670.86	67.09
Semi-discontinuous	359.46	104.24
Discontinuous	3646.47	2297.28

$Y_{P/X}$: Produc/biomass yield; q_P: Product formation rate

Lekha and Lonsane (1994) reported that *Aspergillus niger* PKL-104 produced 140000 U/L of tannase in the solid state culture. Kar and Banerjee (2000) found high amounts of tannase produced by *Rhizopus oryzae* using a modified-solid fermentation. Also Aguilar *et al*.(2001a; 2001b) reported an 18000 U/L of tannase as a maximum production. In these three studies, the final productions were affected by many factors including the choices of the enzyme producer microorganism and the solid support, and some culture conditions such as pH, humidity percentage, airing, etc.

Fig.3 shows that the discontinuous matrix of PUF favoured the gallic acid accumulation; 7.64 g/L was obtained during the first 12 h of the culture, whereas the continuous and semi-discontinuous matrices produced 0.74 and 0.86 g/L of gallic acid, respectively. The gallic acid accumulation was proportional to the tannase biosynthesis in the production systems (Table 4). In the discontinuous matrix supported culture system, a high accumulation of gallic acid occurred during the first 10 h, which was associated with the maximum enzymatic activity. The solid state fermentation yielded higher amounts of gallic acid during first hours of the culture, compared with a liquid fermentation (Kar and Banerjee, 2000; García-Nájera *et al*., 2002). In this study the gallic acid accumulation at 10 h is 1.2 times higher than the liquid fermentation (García-Nájera *et al*., 2002). Kar and Banerjee (2000) also reported high concentrations of gallic acid, which, however, were produced on a longer liquid fermentation.

The use of small particles of PUF favours the formation of fungal aggregates which exhibit a major

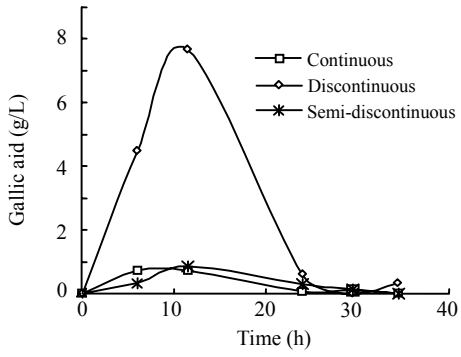

Fig.3 Gallic acid accumulation in the three different matrices of PUF

The culture conditions were: incubation temperature 30 °C, humidity 70%, pH 5.5

Table 4 Kinetic parameters associated to gallic acid accumulation in the three different matrices of PUF

Parameters	$Y_{P/X}$ (U/g)	q_P [U/(g·h)]	Accumulation (%)
Continuous	0.91	0.09	5.90
Semi-discontinuous	0.66	0.19	6.85
Discontinuous	11.23	7.08	61.13

$Y_{P/X}$: Product/biomass yield; q_P: Product formation rate

catalytic activity, while the use of bigger particles of PUF favours the growth of longer hyphaes with a lower catalytic activity. This can be supported by the results obtained by Oostra *et al*.(2001) and Rahardjo *et al*.(2002), who demonstrated that the form of fungal cells forming the mycelium in solid state culture is extremely important because it affects the respiration and, therefore, the modulation of enzymes biosynthesis. Also, Viniegra-González and Favela-Torres (2006) indicated that the diffusion of substrates on a solid support is an important physical parameter that affects the expression of enzymes and it is seriously affected by the continuity of the solid support. In this aspect, Cerda-Montalvo *et al*.(2005) demonstrated the formation of substrate gradients inside the solid support using PUF in the solid state culture for tannase production. This phenomenon was originally proposed by Georgiou and Shuler (1986) and confirmed by Mitchell *et al*.(1991; 2004) and Olsson (1994). Strong glucose gradients were observed by Nagel *et al*.(2002) in solid state culture and several models have been proposed to explain these profiles of substrates in solid state culture systems by Rajagopalan and Modak (1995a; 1995b), Mitchell *et al*.(2004), Viniegra-González *et al*.(2003) and Viniegra-González and Favela-Torres (2006).

The results of the present study show that the discontinuous matrix of polyurethane is better for tannase production and gallic acid accumulation in the solid state culture bioprocess. We conclude that the physical structure of a solid support matrix has a great influence on the physiological behavior of the fungus in the solid culture than the continuous and semi-discontinuous matrices.

References

Aguilar, C.N., 2000. Induction and Repression of Synthesis of Tannase from *Aspergillus niger* Aa/20 in Submerged and Solid State Cultures. PhD Thesis, Metropolitan Autonomous University, Mexico (in Spanish).

Aguilar, C.N., Gutiérrez-Sánchez, G., 2001. Review: sources, properties, applications and potential uses of tannin acyl hydrolase. *Food Sci. Tech. Int.*, **7**(5):373-382. [doi:10.1177/108201301772660411]

Aguilar, C.N., Augur, C., Favela-Torres, E., Viniegra-González, G., 2001a. Induction and repression patterns of fungal tannase in solid-state and submerged cultures. *Proc. Biochem.*, **36**(6):565-570. [doi:10.1016/S0032-9592(00)00251-X]

Aguilar, C.N., Augur, C., Favela-Torres, E., Viniegra-González, G., 2001b. Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid. *J. Ind. Microbiol. Biotechnol.*, **26**(5):296-302. [doi:10.1038/sj.jim.7000132]

Aguilar, C.N., Favela-Torres, E., Viniegra-González, G., Augur, C., 2002. Culture conditions dictate protease and tannase production in submerged and solid-state cultures of *Aspergillus niger* Aa-20. *Appl. Biochem. Biotechnol.*, **102-103**(1-6):407-414. [doi:10.1385/ABAB:102-103:1-6:407]

Aguilar, C.N., Rodríguez-Herrera, R., Gutiérrez-Sánchez, G., Augur, C., Favela-Torres, E., Prado-Barragán, L.A., Ramírez-Coronel, A., Contreras-Esquivel, J.C., 2007. Microbial tannases: advances and perpectives. *Appl. Microbiol. Biotechnol.*, **76**(1):47-59. [doi:10.1007/s00253-007-1000-2]

Aoki, K., Shinke, R., Nishira, H., 1976. Purification and some properties of yeast tannase. *Agric. Biol. Chem.*, **40**(1):79-85.

Belmares, R., Contreras-Esquivel, J.C., Rodríguez- Herrera, R., Ramírez-Coronel, A., Aguilar, C.N., 2004. Microbial production of tannase: an enzyme with potential use in food industry. *Lebensmit. Wiss. und-Technol.*, **37**(8):857-864. [doi:10.1016/j.lwt.2004.04.002]

Bhat, T.K., Singh, B., Sharma, O.P., 1998. Microbial degradation of tannins—a current perspective. *Biodegradation*, **9**(5):343-357. [doi:10.1023/A:1008397506963]

Cerda-Montalvo, M.L., Contreras-Esquivel, J.C., Rodríguez-Herrera, R., Aguilar, C.N., 2005. Glucose diffusion on support for solid state fermentation and its influence on tannase production profiles. *Int. J. Chem. Reactor Eng.*, **3**(5):1-10.

Córdova-López, J., Gutierrez-Rojas, M., Huerta, S., Saucedo-Castañeda, G., Favela-Torres, E., 1996. Biomass estimation of *Aspergillus niger* growing on real and model supports in solid state fermentation. *Biotechnol. Tech.*, **10**(1):1-6. [doi:10.1007/BF00161075]

García-Nájera, J.A., Medina, A., Castro, Y., Reyes-Vega, M.L., Prado-Barragán, L.A., Rodríguez-Herrera, R., Aguilar, C.N., 2002. Accumulation and Recovery of Gallic Acid in a Submerged Culture of *Aspergillus niger* Aa-20. IFT Annual Meeting, Anaheim, CA, USA, p.25.

Georgiou, G., Shuler, M.L., 1986. A computer-model for the growth and differentiation of a fungal colony on solid substrate. *Biotechnol. Bioeng.*, **28**(3):405-416. [doi:10.1002/bit.260280314]

Kar, B., Banerjee, R., 2000. Biosynthesis of tannin acyl hydrolase from tannin-rich forest residue under different fermentation conditions. *J. Ind. Microbiol. Biotechnol.*, **25**(1):29-38. [doi:10.1038/sj.jim.7000011]

Kar, B., Banerjee, R., Bhattacharyya, B.C., 1999. Microbial production of gallic acid by modified solid state fermentation. *J. Ind. Microbiol. Biotechnol.*, **23**(3):173-177. [doi:10.1038/sj.jim.2900713]

Khanbabaee, K., van Ree, T., 2001. Tannins: classification and definition. *Nat. Prod. Rep.*, **18**(6):641-649. [doi:10.1039/b101061l]

Lekha, P.K., Lonsane, B.K., 1994. Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL-104 in solid-state, liquid surface and submerged fermentations. *Proc. Biochem.*, **29**(6):497-503. [doi:10.1016/0032-9592(94)85019-4]

Lekha, P.K., Lonsane, B.K., 1997. Production and application of tannin acyl hydrolase: state of the art. *Adv. Appl. Microbiol.*, **44**(9/17):215-260.

Mitchell, D.A., Do, D.D., Greenfield, P.F., Doelle, H.W., 1991. A semimechanistic mathematical model for growth of *Rhizopus oligosporus* in a model solid-state system. *Biotechnol. Bioeng.*, **38**(4):353-362. [doi:10.1002/bit.260380405]

Mitchell, D.A., von Meien, O.F., Krieger, N., Dalsenter, F.D.H., 2004. A review of recent developments in modelling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation. *Biochem. Eng. J.*, **17**(1):15-26. [doi:10.1016/S1369-703X(03)00120-7]

Nagel, E.J., van As, H., Tramper, J., Rinzema, A., 2002. Water and glucose gradients in the substrate measured with NMR imaging during solid-state fermentation with *Aspergillus oryzae*. *Biotechnol. Bioeng.*, **79**(6):653-663. [doi:10.1002/bit.10332]

Olsson, S., 1994. Uptake of glucose and phosphorus by growing colonies of *Fusarium oxxysporum* as quantified by image analysis. *Exp. Mycol.*, **18**(1):33-47. [doi:10.1006/emyc.1994.1004]

Oostra, J., le Comte, E.P., van den Heuvel, J.C., Tramper, J., Rinzema, A., 2001. Intra-particle oxygen diffusion limitation in solid-state fermentation. *Biotechnol. Bioeng.*, **75**(1):13-24. [doi:10.1002/bit.1159]

Rahardjo, Y.S., Weber, F.J., le Comte, E.P., Tramper, J., Rinzema, A., 2002. Contribution of aerial hyphae of *Aspergillus oryzae* to respiration in a model solid-state fermentation system. *Biotechnol. Bioeng.*, **78**(5):539-544. [doi:10.1002/bit.10222.abs]

Rajagopalan, S., Modak, J.M., 1995a. Evaluation of relative growth limitation due to depletion of glucose and oxygen during fungal growth on a spherical solid particle. *Chem. Eng. Sci.*, **50**(5):803-811. [doi:10.1016/0009-2509(94)00452-W]

Rajagopalan, S., Modak, J.M., 1995b. Modelling of heat and mass transfer for solid-state fermentation process in tray bioreactor. *Bioproc. Biosyst. Eng.*, **13**(3):161-169.

Sharma, S., Bhat, T.K., Dawra, R.K., 2000. A spectrophotometric method for assay of tannase using rhodanine. *Anal. Biochem.*, **279**(1):85-89. [doi:10.1006/abio.1999.4405]

Viniegra-González, G., Favela-Torres, E., 2006. Why solid-state fermentation seems to be resistant to catabolite repression. *Food Technol. Biotechnol.*, **44**(3):397-406.

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Romero-Gomez, S.J., Diaz-Godinez, G., Augur, C., 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J.*, **13**(2-3):157-167. [doi:10.1016/S1369-703X(02)00128-6]