



Journal of Applied Sciences

ISSN 1812-5654

science
alert

ANSI*net*
an open access publisher
<http://ansinet.com>

Bioprocess Development for Large Scale Production of Anticancer Exo-Polysaccharide by *Pleurotus ostreatus* in Submerged Culture

^{1,2}H. El-Enshasy, ³A. Daba, ⁴M. El-Demellawy, ⁴A. Ibrahim, ⁵S. El Sayed and ^{2,5}I. El-Badry

¹Chemical Engineering Pilot Plant, Faculty of Chemical and Natural Resources Engineering, University Technology Malaysia (UTM), 81310 UTM, Skudai, Johor, Malaysia

²Department of Bioprocess Development,

Mubarak City for Scientific Research (MuCSAT), New Burg Al Arab, Alexandria, Egypt

³Department of Pharmaceutical Products, MuCSAT, Alexandria, Egypt

⁴Department of Medical Biotechnology, MuCSAT, Alexandria, Egypt

⁵Biotechnology Pilot Plant, MuCSAT, Alexandria, Egypt

Abstract: In the recent years, mushroom derived active metabolites have emerged as an important class of bioactive compounds with several therapeutic applications. Most of the production methods were based on cultivation of mushroom on solid substrate in controlled temperature green house. For the production of bioactive compounds for therapeutic application, production should be carried out under sterile and well controlled condition. Thus, the interest of cultivation of mushroom in bioreactors for bioactive compound production was increased during the last few years. In the present study, mushroom cells were cultivated in submerged culture for the overproduction of anticancer exo-polysaccharides (EPS). Cell cultivation was optimized in fully controlled stirred tank bioreactor in batch and fed-batch culture to improve the process and to increase the anticancer EPS production. In the first part of this study, significant improvement in EPS production was achieved upon transferring the process from shake flask to 15 L bioreactor. Further development in the process was conducted through optimization of some process parameters in bioreactor batch culture. In fed-batch culture, among different feeding strategies, optimized glucose feeding based on using the in-line data for oxygen and carbon dioxide obtained from out-gas analyzer was the best. The maximal yield of EPS obtained was 2.1 g L⁻¹ in optimized fed-batch culture. The obtained EPS was almost two fold higher than those obtained in batch culture.

Key words: *Pleurotus ostreatus*, exo-polysaccharide, submerged fermentation, fed-batch

INTRODUCTION

Since, centuries, mushrooms have been eaten and appreciated for their flavor, economical, ecological and medicinal values. Beside the known nutritional value of mushrooms, as one of the rich sources of protein, carbohydrates and lipids (Morais *et al.*, 2000; Sanchez, 2004), they have been used in traditional medicine in many cultures all over the world. Many mushrooms contain different types of antimicrobial active compounds with potential applications against different types of bacteria (Smania *et al.*, 1999; Mothana *et al.*, 2000), fungi (Smania *et al.*, 2003) and viruses (Brandt and Piraino, 2000; Awadh *et al.*, 2003). These beside the traditional use of different types of mushroom extracts for the treatment of many other non-microbial related diseases based on their activities as hypoglycemic (Mizuno, 1999;

Wasser *et al.*, 2002), antioxidant (Lin *et al.*, 2004), anti-inflammatory (Zhang *et al.*, 2002), hepato-protectant (Chen and Yu, 1993) and immune-modulator (Lin, 2004). Moreover, many attractive polysaccharides of anti-tumor activity have been also isolated from different types of mushrooms (Mizuno, 1999; Lindequist *et al.*, 2005; Wang *et al.*, 2005).

Traditionally, mushrooms are usually cultivated on solid substrate under controlled temperature and humidity in green house. The main disadvantages of this method are: long cultivation period up to several months, the difficulties to cultivate many wild mushrooms, low yield of active metabolite, long process of bioactive compound purification and ease of contamination by other microorganism in this open cultivation system. Thus, submerged cultivation of mushrooms is viewed as a promising alternative system for efficient production of

mushroom medicinal product since it can also fulfill the cGMP requirements for bioactive compounds production. Of different medicinal mushrooms, *Pleurotus ostreatus*, an edible mushroom belonging to the family Basidiomycetes, is potential source for anticancer polysaccharide when cultivated in either solid or submerged cultures. This polysaccharide can be produced extracellularly in submerged culture. In our previous work the structure of this type of polysaccharide was identified by IR and ¹³C NMR. The spectral pattern showed a typical highly branched chain of peptidoglycan with 1→3 and 1→6 linkage (Daba *et al.*, 2005). The isolated polysaccharide possesses immune-stimulatory effect and anticancer activity against different types of cancer cells. In this context, the current study was focused on the improvement of the production process of this type of polysaccharide and bioprocess optimization in 15 L bioreactor using different production processes.

MATERIALS AND METHODS

Microorganism and cultivation conditions:

Basidiomycetes fungus *Pleurotus ostreatus* NRRL 366 was kindly provided by the agriculture research service Peoria, USA. The strain was maintained and reactivated monthly in Petri dishes containing a sterile solid potato dextrose agar medium (PDA, Oxoid, UK). Cells were incubated at 26°C for 14 days and stored in a refrigerator at 5°C.

Medium for cell growth and EPS production in bioreactor: The medium used in bioreactor experiments was composed of (g L⁻¹): glucose, 20.0; KH₂PO₄, 0.46; K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.5; peptone, 2.0 and yeast extract, 2.0. Glucose was sterilized separately and added to the cultivation medium before inoculation. The pH was adjusted to 5.5 after sterilization.

Inoculum preparation and cell cultivation: *Pleurotus ostreatus* was initially grown on PDA medium in a petri dish for 14 days at 28°C. The surface mycelia with spores were harvested in sterile saline solution and used as inoculum. Cells were gently homogenized to prevent the formation of large aggregates in submerged culture. Inoculum for bioreactor was in form of 50 mL mycelium/spore suspension with optical density of 1 OD. Shake flask cultivations were performed using 250 mL Erlenmeyer flask with working volume of 50 mL. The inoculated flasks were shaken at 200 rpm on a rotary shaker (Model, 4230 Innova, New Brunswick, NJ, USA) with eccentricity of 2.0 cm and cultivation temperature

26°C. The bioreactor cultivations were carried out in 15 L stirred tank bioreactor (Biostat-C, Sartorius BBI Systems, Melsungen, Germany) with working volume of 9 L. The stirrer was equipped with two 6-bladed rushton turbine impellers (d_1 (impeller diameter) = 85 mm; d_2 (tank diameter) = 214 mm, $d_1/d_2=0.397$). Agitation was adjusted at 200 rpm and temperature was controlled at 30°C throughout the cultivation time. Aeration was performed using compressed air, sterilized using hydrophobic microbiological air filter and supplied continuously to the bioreactor with rate of 1 v v⁻¹ m⁻¹. Air flow was adjusted and controlled using mass flow controller (F102D, Bronkhorst High-Tech B.V., Nijverheidstraat, The Netherlands) coupled with the control console CDU of the bioreactor. Foam was suppressed by the addition of the antifoam agent Struktol (Schill+Seilacher Gruppe, Hamburg, Germany). During the cultivation process, the dissolved oxygen concentrations were analyzed using a polarographic electrode (Ingold, Mittler-Toledo, Switzerland).

During cell cultivation, the out-gas of the bioreactor was analyzed continuously using O₂ and CO₂ out-gas analyzer (Sartorius BBI, Melsungen, Germany). The in-line data of the out-gas analyzer was continuously recorded using MFCS supervisory control system. In fed-batch experiment (with continuous glucose feeding using CO₂ out-gas data), peristaltic pump (Watson Marlow, Wilmington, MA, USA) was connected to the control system.

Sample preparation and cell dry weight determination:

Samples in form of two flasks or 20 mL of broth in case of bioreactor culture were taken at different time intervals and collected in pre-weighed centrifugation tube of 50 mL (Falcon, USA), centrifuged at 5°C with 5000 rpm for 20 min. Supernatant was frozen at -20°C for sugar and EPS determination. The cell pellets were washed twice by distilled water, centrifuged again and dried in an oven at 60°C for determination of cell dry weight.

Determination of glucose: Glucose was determined in the fermentation media by enzymatic method using a glucose determination kit (Glucose kit Cat. No. 4611, Biocon Diagnostic GmbH, Burbach, Germany).

Extraction of polysaccharide from mycelial culture: After sample centrifugation, the resulting culture filtrate was mixed with equal volume of absolute ethanol, stirred vigorously and kept overnight at 4°C. The precipitate exo-biopolymer was centrifuged at 10.000 g for 20 min. After discarding the supernatant, the precipitate of pure

EPS was washed separately with ethanol, acetone and ethyl ether then lyophilized (Bae *et al.*, 2000). After complete lyophilisation cycle of about 4 days, the obtained precipitate was weighed.

RESULTS

Cultivations in shake flask culture: Cells were cultivated in shake flask cultures to evaluate the potency of *P. ostreatus* for EPS production in submerged culture. As shown in Fig. 1, cells grew reaching the maximal cell mass of about 4.5 g L^{-1} after about 225 h cultivation. The production of EPS was firstly observed in culture after 50 h cultivation and increased gradually in parallel to cell growth. The maximal polysaccharide production of 0.69 g L^{-1} was obtained after 240 h. As shown also in Fig. 1, glucose concentration was gradually decreased in culture throughout the cultivation time. However, during the growth phase (the first 225 h of cultivation) the glucose consumption rate was about 0.053 g/L/h . The glucose consumption rate was decreased thereafter to only 0.021 g/L/h for the rest of production time. It is also noteworthy to mention that the pH value of culture was dropped gradually from 5.5 at the beginning of cultivation to 3.6 after 144 h and increased again gradually reaching 3.9 at the end of cultivation time. The growth

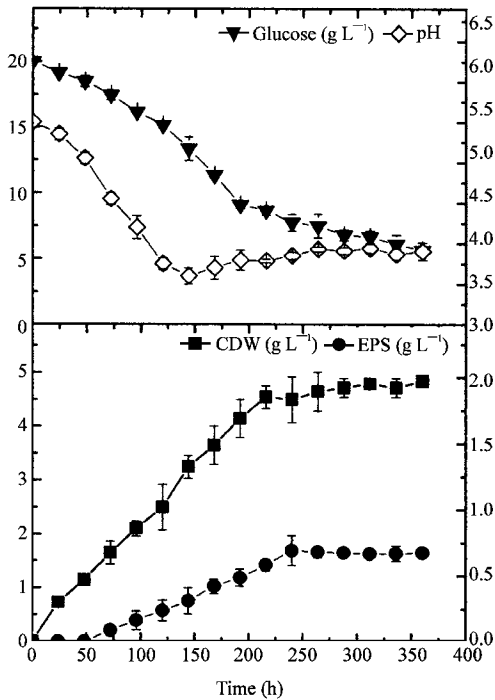


Fig. 1: Cell growth and EPS production by *P. ostreatus* in shake flask culture

morphology of mushroom in shake flaks culture was mainly in pellet form. The pellet size was obvious in culture after only 48 h cultivation and increased gradually with time.

EPS production in batch culture in stirred tank bioreactor:

The production of EPS was conducted in pilot scale 15-L stirred tank bioreactor under fully aerobic condition as described in materials and methods part. Fig. 2, summarizes the cell growth, EPS production and other changes in culture during this cultivation process. As shown, cells grew exponentially with rate of 0.036 g/Lh reaching maximal cell mass of about 6 g L^{-1} after 168 h. During that time glucose was consumed by cells with rate of 0.08 g/L/h and totally consumed after 240 h. In parallel, EPS was produced in culture after 48 h and accumulated in culture with production rate of 5.3 mg/L/h reaching maximal volumetric production of about 1.12 g L^{-1} after 216 h and kept more or less constant for the rest of production time. On the other hand, the DO in culture was dropped

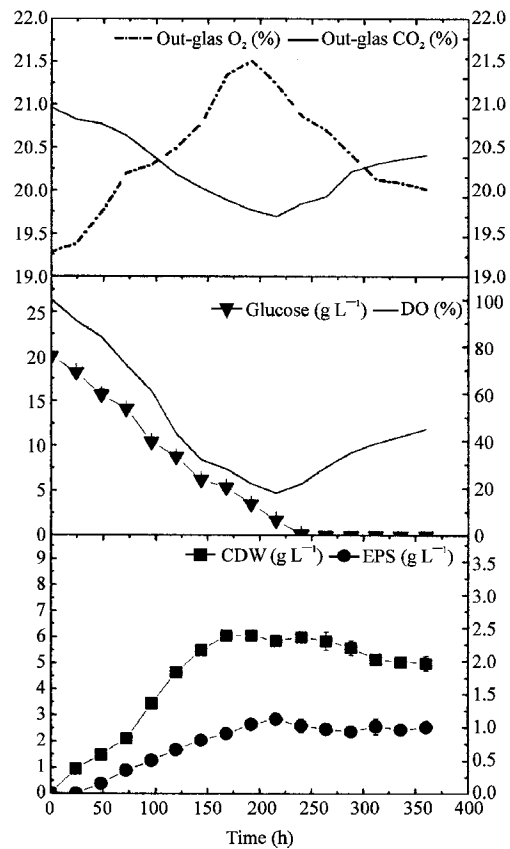


Fig. 2: Cell growth and EPS production by *P. ostreatus* in batch culture in stirred tank bioreactor under controlled pH conditions

significantly during the growth phase reaching about 20% saturation and increased again gradually as cell reached stationary phase. The out-gas analysis shows also that significant decreased in oxygen percent in out gas concomitant with an increased in carbon dioxide content during the growth phase. This also indicates the active cell metabolism during this phase. As cells entered the stationary phase, oxygen ratio in outgas decreased gradually with significant reduction of carbon dioxide concentration in out-gas. However, cell morphology of culture was different from those obtained in shake flask. In bioreactor culture, cells were aggregated during the early phase and form small and lose pellet. As cultivation time increase, the pellet size increased gradually. In general, the pellets in bioreactor culture were smaller with more hairy surface compared to those obtained in shake flask cultures.

Fed-batch cultivation with intermittent glucose addition:

As shown in the previous experiment, glucose was limited in culture after 240 h cultivation. Thus, the termination of EPS production may be attributed to the glucose limitation. Therefore, fed-batch cultivation was designed with addition of glucose of 90 g at 216 h (time at which glucose concentration reached less than 2 g L⁻¹ in batch culture). Figure 3 shows the time course of cell growth and EPS production in fed-batch culture. As shown, cells grew exponentially during the first 168 h during the batch phase. As glucose added to culture no further growth was observed. On the other hand, the production of EPS was increased gradually after glucose feeding with same production rate and reached 1.74 g L⁻¹ at the end of cultivation time. The DO in culture reached 20% saturation at the end of growth phase and kept more or less constant for the rest of cultivation time. On the other hand, the percent of oxygen in out gas increased gradually after cell entering the stationary phase. This indicates the higher cell physiological activity compared to those in batch culture after entering the stationary phase. Meanwhile, the data of carbon dioxide concentration in out-gas analyzer showed also that the decrease of the percent of carbon dioxide in out-gas after entering the stationary phase was less than in batch culture. These all together support the idea that the cell activity in fed-batch culture was higher than batch culture in stationary phase.

Fed-batch cultivation with on-line glucose feeding strategy using out-gas analysis data: In the present experiment, EPS was produced in fed-batch culture under

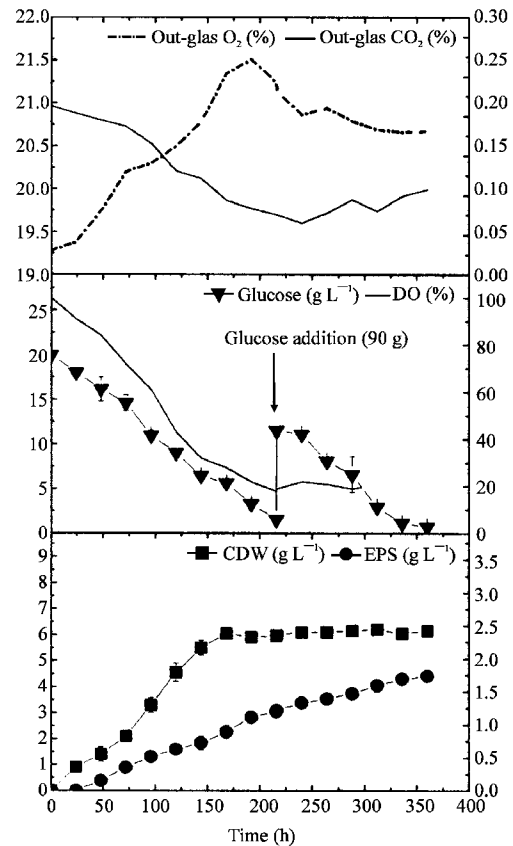


Fig. 3: Cell growth and EPS production in fed-batch culture. Arrow show the time at which glucose was fed to the bioreactor in single shot addition

controlled feeding conditions based on the value of carbon dioxide of out-gas. As shown in the previous experiments, oxygen consumption is decreased (indicated by increased fraction of oxygen in out-gas) and carbon dioxide production decreased (indicated by the decrease of CO₂ fraction in out-gas) under glucose limitation. However, cultivation of mushroom is fully aerobic process and the high CO₂ in out-gas indicates high metabolic activity. Thus, it was taken as key parameter for glucose feeding. The glucose feeding pump was cascaded to the value of outgas to keep the carbon dioxide concentration in out-gas at the maximal value achieved before glucose limitation. As shown in Fig. 4. The value glucose feeding started after 192 h as cells entered the stationary phase. Feeding rate was varied between 0 and 0.08 g/L/h (the value of glucose consumption rate in batch culture during the growth phase). Based on this feeding strategy, EPS production increased gradually and reached about 2.1 g L⁻¹ at the end of cultivation time. However, neither glucose nor oxygen was limited throughout the cultivation time. Thus, we can conclude that glucose was

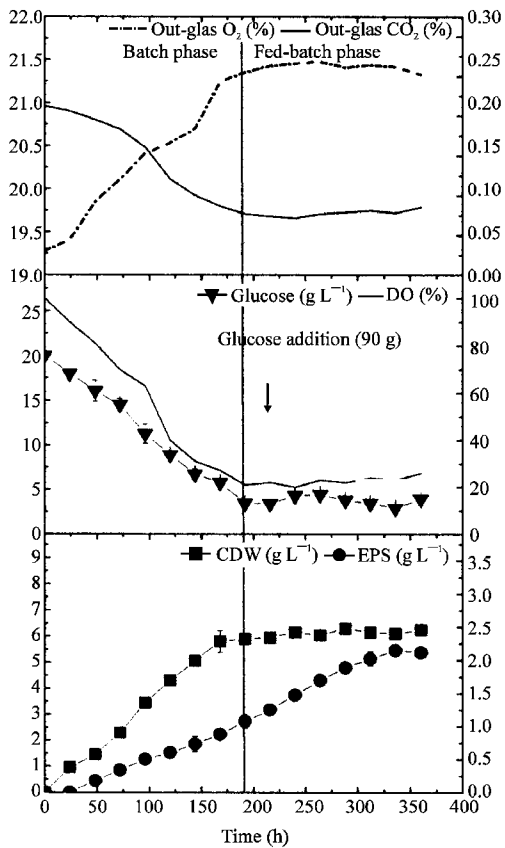


Fig. 4: Cell growth and EPS production in fed-batch culture in CO₂ stat culture. Glucose was fed to maintain constant concentration of carbon dioxide in out-gas of the bioreactor

the limiting nutrient for EPS production but not for growth since growth was terminated after 168 h (as in case of batch culture).

DISCUSSION

Water extract of many mushrooms used in traditional Chinese medicine and other folk medicines have long history in various diseases treatment. Nowadays, based on the discovery of many novel molecules in mushrooms, increased interest has been observed on using the medicinal mushroom extracts for cancer treatment. Long cultivation period, extensive purification steps and low yield are the main drawbacks of using mushroom fruit bodies for the production of anticancer bioactive compounds. Thus, during the last few years the cultivations of mushroom cells in submerged culture for exo-polysaccharide (EPS) production was very attractive topic for many researchers (Yang and Liao, 1998; Cho *et al.*, 2006). In this study, we developed

new production process of EPS by oyster mushroom *P. ostreatus* in submerged culture. As shown from the results of the early experiment in shake flask, the production of EPS was associated with cell growth and terminated as cell entered the stationary phase. However, glucose was not limited in culture in that time. This incomplete utilization of glucose in culture may due to the decrease of glucose consumption as growth was mainly in form of large pellet. During the growth phase the pH dropped significantly in culture. This decrease of pH was due to acid formation as also reported by other authors (Rajarathnam *et al.*, 1992). This may also due to insufficient oxygenation in shake flask and the growth of cells in form of large pellet. In general, the growth of fungal cells in large pellet increases the tendency for acid formation and large fraction of cells is growing under anaerobic conditions (El Enshasy *et al.*, 1999). As the production process was transferred to stirred tank bioreactor under controlled pH of 5.5, the cell mass increased by about 15% this was concomitant with an increased in EPS production by about 25%. In this culture, glucose was fully consumed after 250 h. However, the better cell growth and EPS production may attributed to the better mixing characteristics and oxygenation in stirred tank bioreactor compared to shake flask (El Enshasy, 2007; Maier and Büchs, 2001). As glucose was limited after 220 h, both of growth and EPS production were terminated accordingly.

Generally, mushroom use glucose as their primary carbon source for both growth and also contributed significantly in EPS production, but when glucose concentration is high in the medium, mycelia growth is inhibited (Boyle, 1998). It have been also reported that to minimize the glucose repression effect on different types of mushroom growth, glucose should not exceed the range of 20-30 g L⁻¹ (Azuma and Kitamoto, 1994). When cultivation was conducted in fed-batch culture by different modes, significant increase in EPS production was observed. In first fed-batch experiment, glucose was added in one shot of 90 g to increase the glucose concentration in culture up to 10 g L⁻¹. Glucose addition resulted in the continuous production of EPS in culture and reached maximal concentration of 1.74 at the end of cultivation time without any significant increase cell mass production compared to batch culture. Thus, we can conclude that glucose was the limiting substrate for EPS production but not for growth.

On using this alternative new method of glucose feeding based on the out-gas analyzer data for CO₂ concentration of the out-gas from the bioreactor to keep it at the same value when cell entered the stationary phase, EPS production was increased significantly. This increase in EPS production was not due to further cell

growth. Thus, we can conclude that fed-batch cultivation increased the volumetric EPS production through the increase of cell productivity.

CONCLUSION

In the present study, the optimized submerged culture conditions for mycelia growth and EPS production by *P. ostreatus* in large scale was addressed. From the series of experiments, we determined that glucose was not the limiting substrate for cell growth but was limiting substrate for EPS production. By scaling up the process from shake flask to pilot scale stirred tank bioreactor significant increase in EPS was observed. Further improvement in EPS production process was achieved by cultivating the cells in fed-batch culture. Among different feeding strategies applied, cascading the glucose feeding rate to the value of carbon dioxide in out-gas was the best feeding strategy and yielded 2.1 g L⁻¹ EPS at the end of cultivation time (this value was almost double of those obtained in batch culture). Thus we can conclude that fed-batch culture for EPS production using mono-glucose feeding controlled by out-gas analysis data is the most suitable system for large scale production of antitumor EPS by *P. ostreatus*.

ACKNOWLEDGMENTS

The Authors wish to thank Mr. Ahmed Abdel Fattah, Mr. Ramadan Mohamed and Mr. Aboul Soud for their technical assistance.

REFERENCES

- Awadh, A.A.N., R.A.A. Mothana, A. Lesnau, H. Pilgrim and U. Lindequist, 2003. Antiviral activity of *Inonotus hispidus*. *Fitoterapia*, 74: 483-485.
- Azuma, S. and Y. Kitamoto, 1994. Nutritional environment for mycelial growth and fruit body formation in *Lentinus edodes*. *Mushroom Sci. Biotechnol.*, 1: 7-13.
- Bae, J.T., J. Sinha, J.P. Park, C.H. Song and J.W. Yun, 2000. Optimization of submerged culture conditions for exo-biopolymer production by *Paecilomyces japonica*. *J. Microbiol. Biotechnol.*, 10: 482-487.
- Boyle, D., 1998. Nutritional factors limiting the growth of *Lentinus edodes* and other white-rot fungi in wood. *Soil Biol. Biochem.*, 30: 817-823.
- Brandt, C.R. and F. Piraino, 2000. Mushroom antivirals. *Recent Res. Dev. Antimicrob. Agents Chemother.*, 4: 11-26.
- Chen, R. Y. and D.Q. Yu, 1993. Studies on the triterpenoid constituents of the spores from *Ganoderma lucidum* karst. *J. Chinese Pharm. Sci.*, 2: 91-96.
- Cho, E.J., J.Y. Oh, H.Y. Chang and J.W. Yun, 2006. Production of exopolysaccharides by submerged mycelia culture of a mushroom *Tremella fuciformis*. *J. Biotechnol.*, 127: 129-140.
- Daba, A., M. El-Demellawy and H. El-Enshasy, 2005. Anticancer Activity of Polysaccharides Produced by *Pleurotus ostreatus* in Submerged Culture. In: *Genetic and Cellular Biology of Basidiomycetes*, Pisabarro, A.G. and L. Ramirez (Eds.). Pamplona University Press, Spain, pp: 43-55.
- El-Enshasy, H., 2007. Filamentous Fungal Cultures: Process Characteristics, Products and Applications. In: *Bioprocessing for Value-Added Products from Renewable Resources*, Yang, S.T. (Ed.). Elsevier Press, New York, pp: 225-261.
- El-Enshasy, H., K. Hellmuth and U. Rinas, 1999. Fungal morphology in submerged cultures and its relation to glucose oxidase excretion by recombinant *Aspergillus niger*. *Applied Biochem. Biotechnol.*, 81: 1-11.
- Lin, Y., P. Lai, Y. Huang and H. Xie, 2004. Immune-competent polysaccharides from the submerged cultured mycelium of culinary-medicinal mushroom *Lentinus strigellus* (Agaricomycetidae). *Int. J. Med. Mushrooms*, 6: 49-55.
- Lin, Z.B., 2004. Focus on anti-oxidative and free radical scavenging activity of *Ganoderma lucidum*. *J. Applied Pharmacol.*, 12: 33-137.
- Lindequist, U., T.H.J. Niedermeyer and W.D. Julich, 2005. The pharmacological potential of mushrooms. *Evid. based Complement. Aleternat. Med.*, 2: 285-299.
- Maier, U. and J. Büchs, 2001. Characterisation of the gas-liquidmass transfer in shaking bioreactors. *J. Biochem. Eng.*, 7: 99-106.
- Mizuno, T., 1999. The extraction and development of antitumor-active poly-saccharides from medicinal mushrooms in Japan. *Int. J. Med. Mushrooms*, 1: 9-30.
- Morais, M.H., A.C. Ramos, N. Matos and E.J. Santos-Oliveira, 2000. Note: Production of shiitake mushroom (*Lentinus edodes*) on ligninocellulosic residues. *Food Sci. Technol. Int.*, 6: 123-128.
- Mothana, R.A.A., R. Jansen, W.D. Jülich and U. Lindequist, 2000. Ganomycin A and B, new antimicrobial farnesyl hydroquinones from the basidiomycete *Ganoderma pfeifferi*. *J. Nat. Prod.*, 63: 416-418.
- Rajarathnam, S., M.N. Shashireka and Z. Bano, 1992. Biopotentialities of basidiomycetes. *Adv. Applied Microbiol.*, 37: 233-361.
- Sanchez, C., 2004. Modern aspects of mushroom culture technology. *Applied Microbiol. Biotechnol.*, 64: 756-762.

- Smania, A., F.D. Monache, E.F.A. Smania and R.S. Cuneo, 1999. Antibacterial activity of steroidal compounds isolated from *Ganoderma applanatum* Pat. (*Aphyllphoromycetidea*) fruit body. *Int. J. Med. Mushrooms*, 5: 31-35.
- Smania, E.F.A., F.D. Monache, A. Smania and Y.R.A. Cuneo, 2003. Antifungal activity of sterols and triperpenes isolated from *Ganoderma annulare*. *Fitoterapia*, 74: 375-377.
- Wang, J.C., S.H. Ju, Z.C. Liang and C.J. Yeh, 2005. Optimization of the production of water-soluble polysaccharide from *Pleurotus citrinopileatus* in submerged culture and its antitumor effect. *Applied Microbiol. Biotechnol.*, 67: 759-766.
- Wasser, S.P., K.K. Tan and V.I. Elisashvili, 2002. Hypoglycemic, interferonogenic and immunomodulatory activity of *Tremellastin* from the submerged culture of *Tremella mesenterica* etz. Fr. (*Heterobasidiomycetes*). *Int. J. Med. Mushrooms*, 4: 215-227.
- Yang, F.C. and C.B. Liao, 1998. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochem.*, 33: 547-553.
- Zhang, Y., G. Millis and M.G. Nair, 2002. Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom *Grifola frondosa*. *J. Agric. Food Chem.*, 50: 7581-7585.