

Involvement of Physicochemical Parameters on Pectinase Production by *Aspergillus niger* HFD5A-1

I. Darah, W. Haritharan and S.H. Lim*

Industrial Biotechnology Research Laboratory, School of Biological Sciences,
University Sains Malaysia, 11800, Penang, Malaysia.

(Received: 11 January 2013; accepted: 24 February 2013)

This study was aimed to study the effect of physicochemical parameters on *Aspergillus niger* HFD5A-1 growth and pectinase production. The newly isolated fungal strain, *Aspergillus niger* HFD5A-1 was cultivated in 250 ml Erlenmeyer flask under submerged fermentation and physicochemical characteristics were studied in order to enhance the pectinase production. Fermentation conditions improved were pH 4.5, cultivation temperature of 30°C, inoculum size of 2% (v/v) 1×10^6 cell/mL and agitation speeds of 150 rpm, together with citrus pectin of 1.80% (w/v) and 0.40% (w/v) of peptone as carbon and nitrogen sources, respectively had produced the highest pectinase production of about 2.51 U/mL and 6.58 g/L fungal growth after 6 days of incubation time. The results show that after improvement of physicochemical parameter the pectinase production was increased about 97.6%. The enzyme production by this isolate was found that not growth dependent. Improvement of physicochemical parameters is an important factor in pectinase production in submerged fermentation system.

Key words: Pectinase, *Aspergillus niger*, submerged fermentation, physicochemical parameter.

White biotechnology is an economical beneficial technology has drawn a great interest in food processing industries. Nevertheless, enzymes are becoming increasingly important in sustainable technology and green chemistry rather than traditional chemical process¹. The utilization of microbial pectinases comprises a vast spectrum in industrial application processes. Pectinases are heterogenous group of enzyme hydrolyzes pectic substances mainly found in plants and fruits. Pectinases are divided into three groups: hydrolases which consisting of polygalacturonase (EC 3.2.1.15); lyase/trans-eliminases which comprising pectin lyase (EC 4.2.2.10), and pectate lyase (EC 4.2.2.2); pectin esterase (EC 3.1.1.11)^{2,3}.

Pectins are polysaccharides in the plant kingdom and constitute the major component of middle lamella and plant cell walls that covers one third of plant tissues⁴. Pectic substances represent between 0.5 - 4.0% of fresh weight plant material⁵. Pectinases commonly used in fruit juice industry for juice extraction and clarification of fruit juices⁶. Besides that, it has been also applied in coffee and tea fermentation^{7, 8}, vegetable oil extraction⁹, improvement of chromaticity and stability of red wines¹⁰, textile¹¹, paper and pulp industries and in waste treatment⁵. Microbial pectinases can be produced by both submerged fermentation and solid state fermentations. Submerged fermentation system has been extensively employed in production of highly priced materials and physiology aspects of synthesis enzymes.

Pectinase is produced by a large number of microorganisms¹² including bacteria¹³, actinomycetes¹⁴, yeasts¹⁵, and filamentous fungi. Among the microbial pectinases, filamentous fungi

* To whom all correspondence should be addressed.
Tel.: +6012-4629487; Fax: +604-6565125;
E-mail: limshehong77@gmail.com

such as *Aspergillus* spp.^{7, 16}, *Penicillium* spp.^{17, 18}, *Trichoderma viride*, *Mucor piriformis* and *Yarrowia lipolytica* were studied extensively^{19, 20}. However, industrial production of pectinases makes use almost exclusively of *A. niger* strains²¹. Moreover, *A. niger* is classified as GRAS (Generally Regarded As Safe) claimed that it is acceptable for used in food industry¹⁵.

In the present study, we are reporting the production of pectinase by a strain of *Aspergillus niger* HFD5A-1 which we had successfully isolated from a rotten Malaysia pomelo (*Citrus grandis*). This fungal strain has an ability to produce extracellular acidic pectinase and therefore improvements through physicochemical parameters were carried out.

MATERIALS AND METHODS

Microorganisms, culture maintenance and inoculum preparation

Aspergillus niger HFD5A-1 which was isolated from rotten oranges was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal culture was maintained on potato dextrose agar slant supplemented with 1.0% citrus pectin (w/v) at 30°C for 72 hours aerobically until sporulate before storing them at 4°C until further used. The subculturing was performed every month to ensure its survival.

Inoculum preparation

The inoculum preparation was performed by adding 5.0 mL of sterile distilled water containing 0.1% Tween 80 to a sporulated culture. The spores were dislodged using sterile inoculation loop and shaking vigorously. The spore suspension was adjusted to 1×10^7 spores/mL using haemocytometer chamber (Neubauer Germany) and 1.0 mL was taken as the inoculum.

Cultivation medium

Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL of modified medium proposed by Maldonado and Strasser de Saad²². The medium consisted of (w/v): KH_2PO_4 , 0.4%; Na_2HPO_4 , 0.2%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02%; CaCl_2 , 0.001%; $(\text{NH}_4)_2\text{SO}_4$, 0.2%; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.007%; H_3BO_3 , 0.001% and citrus pectin, 1.5%. The initial pH of the medium was adjusted to 5.0. Fermentation

was carried out after inoculation with 1×10^7 spores/mL suspension and incubated at 30°C and 150 rpm of agitation for 10 days. The samples were withdrawn at every 48 hourly intervals and were assayed for pectinase activity and fungal growth determination.

Enzyme recovery and fungal biomass determination

The culture broth was filtered through Whatman No. 1 filter paper to separate the fungal mycelium. The cell-free culture filtrate containing the crude enzyme was then assayed for pectinase activity.

The filter paper containing biomass were dried at 80°C until constant weight and the fungal cell dry weight was obtained by deducting the weight of filter paper. The fungal cell dry weight was then expressed as g/L. All the experiments were performed in triplicate and the values were reported as standard deviations.

Improvement of cultural conditions

Various physical parameters for maximal pectinase production were studied. These included initial pH medium (2.0, 4.0, 4.5, 5.0, 6.0, and 8.0), temperature (20, 30, 37, 40 and 50°C), agitation speed (0, 50, 100, 150, 200 and 250 rpm), and inoculum sizes (1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , and 1×10^8 spores/mL). All the experiments were performed in triplicate and the values were reported as standard deviations.

Improvement of chemical compositions

Pectinase production was enhanced by adding various nutritional factors. Carbon sources including pectin, glucose, fructose, lactose, sucrose, starch, carboxy methyl cellulose (CMC) with final concentration of 1.0% (w/v) were tested. The selected carbon source with maximal pectinase activity was then investigated with different concentration (0.9%, 1.2%, 1.5%, 1.8% and 2.1%; w/v). Different organic and inorganic nitrogen sources such as ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, peptone, yeast extract and urea were supplemented separately to a final concentration of 0.2% (w/v) were also studied. Nitrogen source that exhibited maximal pectinase activity was tested with different concentration (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%; w/v). All the experiments were performed in triplicate and the values were reported as standard deviations.

Assay for pectinase activity

Pectinase assay was carried out by measuring reducing sugars release from pectin hydrolyzation. One aliquot of sample was added to a solution containing one aliquot of 1% pectin in 0.1 M citrate buffer, pH 4.5. After incubation at 45°C for 30 min, reducing sugars were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959) using galacturonic acid as a reference. The enzymatic activity was expressed in terms of Unit (U). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of galacturonic acid per mL of culture filtrate per minute under assay conditions.

RESULTS AND DISCUSSION

Environmental and nutritional factors (physicochemical) are known to have marked effects on enzyme production by microorganisms. There are variations in optimum conditions for pectinase enzyme production that needed to be investigated in order to have maximal yield.

Cultivation time for maximal pectinase activity

Study on the cultivation time was investigated prior to the improvement of physicochemical parameters. *Aspergillus niger* HFD5A-1 was cultured in submerged fermentation to determine the fungal growth and pectinase activity at an interval of 48 hours for 10 days. It is important to determine the optimal day of cultivation for maximal pectinase production in order to carry out other followed up parameters involved in this study. The results obtained is shown in Figure 1 which indicated the production of pectinase was increased with cultivation time and achieved its maximal production on the 6th day of cultivation time of 1.27 U/mL. The enzyme production dropped gradually after achieving its maximal production. This may be due to the depletion of nutrients and accumulation of toxic metabolites in the cultivation medium²³. Maximum production of different fungi varies from 1 to 6 days of cultivation time²⁴. Moreover, Sarvamangala and Dayanand²⁵ observed a gradual increased in the production of pectinase from deseeded sunflower head by *Aspergillus niger* after 72 hours of cultivation in submerged fermentation. The time of incubation depends on nature of the medium, concentration of the nutrients, growth rate of the

microorganism, and its enzyme production pattern. In addition, the fungus was also observed to attain its dramatically growth on the 4th day of cultivation time with about 2.92 g/L. This indicated that pectinase production by this fungus was not directly growth related as the higher fungal biomass was achieved approximately 2 days earlier than the maximal enzyme production.

Effect of temperature

Temperature is one of the prime factors contributing in affecting various types of cell metabolic process. These include enzymatic inhibition, promotion or inhibition on the production of a particular metabolite, protein denaturation and cell death²⁶. Figure 2 shows that maximal pectinase production of 1.40 U/mL was obtained at the incubation temperature of 30°C, where the maximal fungal growth (2.77 g/L) was also achieved. Results obtained in this study were similar to those observed by Kutateladze *et al.*,²⁷ and Mathew *et al.*,²⁸ whom also found that their strain grew well at 30°C. However, Fawole and Odunfa²⁹ reported that 40°C as optimal for pectinase production by their strain of *Aspergillus niger*. The enzyme activity and fungal growth predominantly decreased due to inactivation of enzymes at temperature higher than that of 30°C, indicating mesophilic nature of the fungus. Besides that, temperature less than 30°C may have resulted in freezing of protoplasmic membrane which leads to stress condition in solute transport systems in the cells³⁰. Another possible reason for initial increase in pectinase activity and then decreased was because atoms in the enzyme molecule received more energy with increased temperature which have greater tendency to overcome the weak interactions forces holding the globular protein structure together that finally deactivate enzymes.

Effect of pH

The optimal pH for the highest pectinase production by *A. niger* HFD5A-1 was 4.5 with about 1.58 U/mL (Fig. 3). However the highest fungal growth was achieved at pH 4 which was 2.32 g/L. The acidic pH where the fungus revealed its maximal pectinase activity was due to the nature of this fungus, which was isolated from rotten pomelo that was in acidic base. Besides, the fungal growth was highest (2.32 g/L) at pH 4.0, this condition again showed the nature of the fungus. Therefore, the pectinase produced by this *A. niger*

HFD5A-1 was an acidic pectinase. Bacteria produce mainly alkaline and thermostable pectinases, whilst fungi are the major producers of acidic ones³¹. They also reported that *A. aculeatus* produced pectinase (polygalacturonase) at pH 3.0. These results were in agreement with pectinase production by *Penicillium griseoroseum*³² and

*Penicillium viridicatum*¹⁷ which were acidic pectinases. Optimum production of pectinase enzyme from many filamentous fungi has been reported to be within the acidic range of 3.0 to 6.5^{31,33}. The drop of pectinase production could be due to the instability of enzymes at very low or very high pH values with the fact that they are

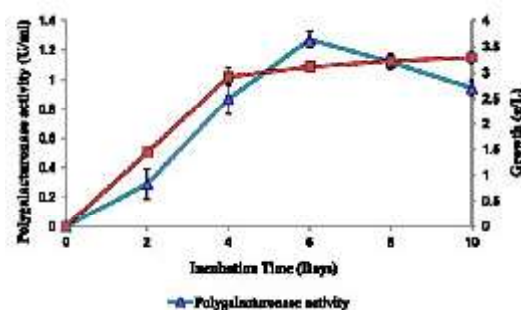


Fig. 1. Time course profile of polygalacturonase production in shake flask system by *Aspergillus niger* HFD5A-1 before enhancement of physicochemical characteristics

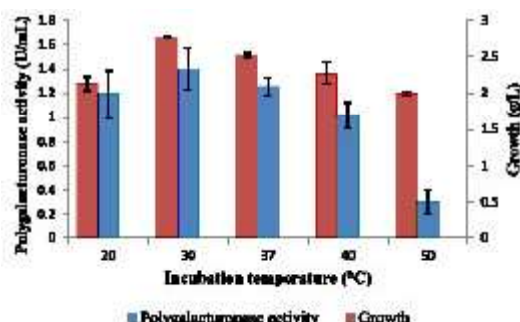


Fig. 2. Effect of incubation temperature on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1

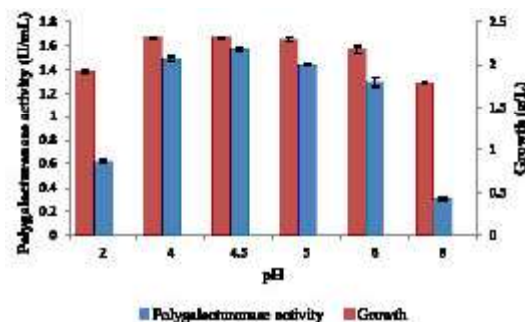


Fig. 3. Effect of pH on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1

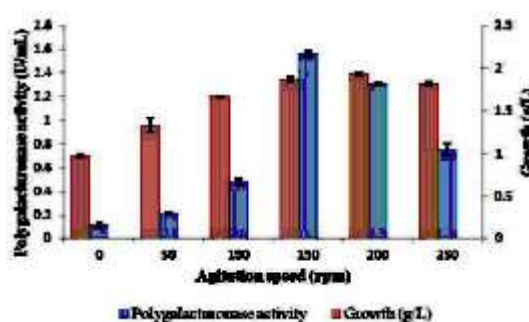


Fig. 4. Effect of agitation speed on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1.

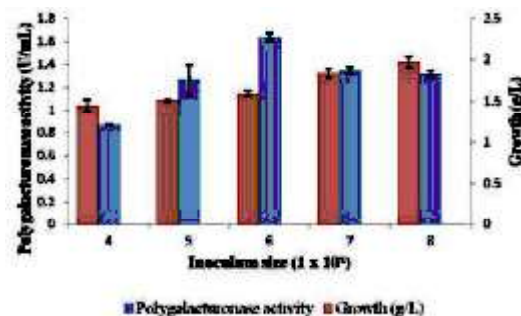


Fig. 5. Effect of inoculum size on biomass & polygalacturonase production by *Aspergillus niger* HFD5A-1

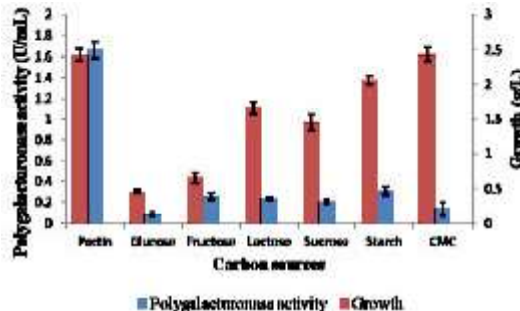


Fig. 6. Effect of carbon sources on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1

proteins which are generally denatured at extreme pH values. Moreover, it is well established that production of enzymes at different pH values also depends on the nature of strains especially in the variation of metabolic activities, architecture of the microbial cell and specific genetic diversity.

Effect of agitation speed

Enzyme activity increased with the increasing of agitation speed up to 150 rpm (Fig. 4), where it achieved its maximal pectinase activity of 1.56 U/mL. However, its highest fungal growth was achieved at 200 rpm with 1.94 g/L. The agitation speed higher than 150 rpm resulted in low pectinase production due to fungal cell disturbances caused by shear stress or shear force. On the other hand, decrease in enzyme activity at lower than 150 rpm could be due to lower dissolved oxygen³⁴. Friedrich *et al.*,³⁵ reported that the range of agitation speed could be from 100-300 rpm

depending on the types of isolates and its growth. Palaniyappan *et al.*,³⁶ reported that pectinase produced by *Aspergillus fumigatus* MTCC 870 was optimal at 160 rpm. Therefore, sufficient dissolved oxygen supply is needed by the fungus to grow and produced its secondary metabolites, and this condition can be obtained from optimal agitation speed.

Effect of inoculum size

Different inoculum sizes were studied and the results showed that there was an increased in pectinase production until it achieved its maximal production at the inoculum size of 1×10^6 spores/mL with 1.64 U/mL (Fig. 5). This result was almost similar with the results obtained by Mosjov³⁷ who achieved the optimal pectinase production at 6×10^6 spores/mL. Pectinase production was found to be decreasing before optimal inoculum size because lower levels of inoculum size may not have

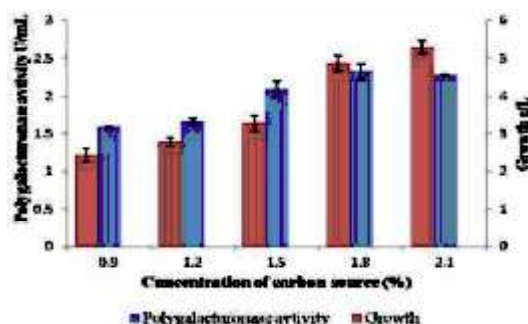


Fig. 7. Effect of concentration of carbon source on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1

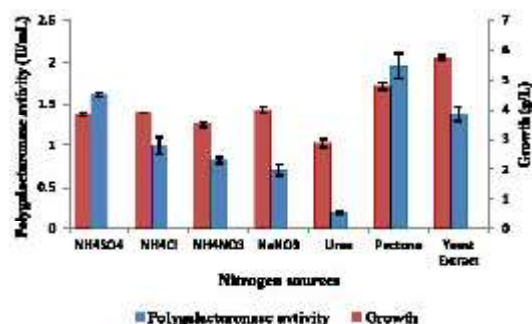


Fig. 8. Effect of nitrogen sources on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1

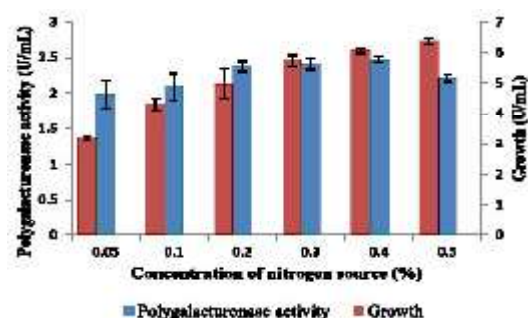


Fig. 9. Effect of concentration of nitrogen source on biomass and polygalacturonase production by *Aspergillus niger* HFD5A-1

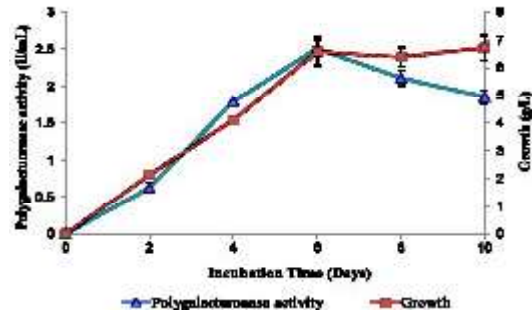


Fig. 10. Time course profile of polygalacturonase production in shake flask system by *Aspergillus niger* HFD5A-1 after enhancement of physicochemical characteristics

sufficient viable spores for initiating growth and enzyme synthesis. An increased in the number of spores however, ensures a rapid proliferation of biomass and enzyme synthesis³⁸. On the other hand, a decreased in pectinase production after optimal inoculums size may be due to depletion of nutrients. Roheena *et al.*,³⁹ reported that decreasing in enzyme production by *A. oryzae* after the optimal inoculums size could be due to the over growth of the fungus that may produced anaerobic conditions during the fermentation, and it consumed majority of substrate for growth and metabolic processes.

Effect of carbon sources

An adequate supply of carbon as energy source is critical for optimum growth which later affecting the fungal growth and its secondary metabolite production. Among the various sole carbon sources studied for pectinase production, maximal enzyme production was observed in the medium supplemented with citrus pectin (Fig. 6) with about 1.66 U/mL and 2.42 g/L of fungal growth. Similar results were reported by Phutela *et al.*⁴⁰ stated that pure pectin gave maximum yield of pectinase production by a thermophilic *Aspergillus fumigatus*. Aguilar and Huitron⁴¹ reported that the pectinase production from many filamentous fungi induced by the presence of pectic substrates in the cultivation medium with four times higher. The results obtained from this study revealed that pectinase production by *A. niger* HFD5A-1 was substrate inducible. In addition, Fawole and Odunfa²⁹ observed that pectin and polygalacturonic acid promoted the production of pectic enzyme by *A. niger* but unfortunately decreased the pectinolytic activity in cultures supplemented with glucose. Low pectinase activity with other carbon sources was probably due to catabolite repression^{11,19}. Pectinase production was further increased by enhancement of pectin concentration. It is found that 1.8% of pectin yield the highest activity of pectinase production with 2.33 U/mL (Fig. 7), beyond that pectinase activity was decreasing probably because of excessive accumulation of galacturonic acid after pectin degradation.

Effect of nitrogen sources

Both, organic and inorganic nitrogen sources were tested in this study (Fig. 8). *A. niger* HFD5A-1 produced the highest pectinase yield in

the pectin medium supplemented with peptone which was about 1.95 U/mL and fungal growth of 4.82 g/L. Moreover, maximal pectinase production was found to be with the addition of 0.4% peptone with 2.47 U/mL and fungal growth of 6.06 g/L (Fig. 9). Vivek *et al.*⁴² found that the fermentation medium supplemented with organic nitrogen produced higher yield of enzyme compared to inorganic nitrogen. Kashyap *et al.*,⁴³ reported that yeast extract, peptone and ammonium chloride were found to enhance pectinase production but the addition of glycine, urea and ammonium nitrate suppressed pectinase production.

The results obtained were in agreement with studies done by Mathew *et al.*²⁸ and Anguilar *et al.*,⁴⁴ who observed that organic nitrogen sources were the best inducer in pectinase production by *A. niger*. Reda *et al.*,⁴⁵ also found that maximal value of pectinase production by *Bacillus firmus*-1-10104 in the presence of peptone as a nitrogen source. Moreover, Vivek *et al.*,⁴² (2010) reported that organic nitrogen sources showed higher endo-, exo- pectinase activities than inorganic nitrogen sources.

Enzyme production using improved physicochemical parameters

After the improvement of physicochemical parameters, a time profile study was conducted to view the cumulative effect of various parameters. The experiment was conducted by incorporating all the improved physical and chemical parameters, and the samples were observed at 2 days interval. Figure 10 shows the time profile of the pectinase production after improvements. The maximal pectin production of 2.51 U/mL and 6.58 g/L fungal growth were obtained at the cultivation period of 6 days. There was about 97.6% of increment in pectinase production after improvement compared to before improvement of physicochemical parameters (1.27 U/mL).

Since acidic pectinase play an important role in bringing down the cloudiness and bitterness of fruit juices, pectinase produced by this fungal strain has good demand in food and beverage industries. Thus, we are now trying to further enhance the enzyme production by immobilizing the fungal cells in scouring sponge based on entrapment method as described previously^{34,46,47}. The results will be published later.

CONCLUSION

This study revealed that a locally isolated fungal strain *Aspergillus niger* HFD5A-1 was a potential pectinase producer. It produced maximal pectinase production of 2.51 U/mL and fungal growth of 6.58 g/L on the 6th day of cultivation after physicochemical parameters improvement which consisted of initial medium pH of 4.5, cultivation temperature of 30°C, agitation speed of 150 rpm with inoculum size of 1×10^6 spores/mL and supplemented with 1.8% citrus pectin and 0.4% peptone in submerged fermentation using shake flask system. There were many factors contributing to the maximal production of enzymes.

ACKNOWLEDGMENTS

The authors are sincerely grateful to Universiti Sains Malaysia for awarding Research Assistant fellowship to Haritharan to complete his Master of Science study.

REFERENCES

1. Bruhlmann, F., Leupin, M., Erismann, K.H., Fiechter, A. Enzymatic degumming of ramie bast fibers. *J. Biotechnol.*, 2000; **76**: 43-50.
2. Visser, J., Bussink, H.J., Witteveen, C. (2004) In: Smith A (ed) Gene expression in recombinant microorganisms. New York: Marcel Dekker, Inc., 2004; pp 241-306.
3. Yadav, S., Yadav, P.K., Yadav, D., Singh, Y.K.D. Purification and characterization of pectin lyase produced by *Aspergillus terricola* and its application in retting of natural fibers. *Appl. Biochem. Biotechnol.*, 2009; **159**: 270-283.
4. Gupta, S.M., Srivastava, S., Gupta, S., Ahmed, Z. Genetic manipulation of fruit ripening: using antisense mRNA strategies. *J. Appl. Biosci.*, 2008; **34**: 115-123.
5. Jayani, R.S., Saxena, S., Gupta, R. Microbial pectinolytic enzymes: a review. *Process Biochem.*, 2005; **40**(9): 2931-2944.
6. Gupta, R., Singh, S. Apple juice clarification using fungal pectinolytic enzymes and gelatin. *Indian J. Biotechnol.* 2004; **3**: 573-576.
7. Angayarkanni, J., Palaniswamy, M., Murugesan, S., Swaminathan, K. Improvement of tea leaves fermentation with *Aspergillus* spp. pectinase. *J. Biosci. Bioeng.*, 2002; **94**: 299-303.
8. Quattara, H.G., Koffi, B.L., Karou, G.T., Sangare, A., Niamke, S.L., Diopoh, J.K. Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation. *World J. Microb. Biotechnol.* 2008; **24**: 1753-1760.
9. Demir, H., Gogus, N., Tari, C., Heerd, D., Lahore, M.F. Optimization process parameters for the utilization of orange peel to produce polygalacturonase by solid-state fermentation from an *Aspergillus sojae* mutant strain. *Turkish J. Biol.*, 2012; **36**: 394-404.
10. Roldán, A., Palacios, V., Peñatez, X., Benitez, T., Pérez, L. Use of *Trichoderma* enzymatic extracts on vinification of Palomino fino grapes in the Sherry region. *J. Food Eng.* 2006; **75**: 375-382.
11. Ahlawat, S., Dhiman, S.S., Battan, B., Mandhan, R.P., Sharma, J. Pectinase production by *Bacillus subtilis* and its potential application in biopreparation of cotton and microcopy fabric. *Process Biochem.*, 2009; **44**(5): 521-526.
12. Murad, H.A., Azzaz, H.H. (2011) Microbial pectinases and ruminant nutrition. *Res. J. Microbiol.*, 2011; **6**(3): 246-269.
13. Kumar, A., Sharma, R. Production of alkaline pectinase by bacteria (Cocci sps.) isolated from decomposing fruit materials. *J. Phytol.*, 2012; **4**(1): 01-05.
14. Bruhlman, F., Kim, K.S., Zimmerman, W., Fiecher, A. Pectinolytic enzymes from actinomycetes for the degumming of ramie bast fibers. *Appl. Env. Microbiol.*, 1994; **60**: 2107-2112.
15. Arevalo-Villena, M., Fernandez, M., Lopez, J., Briones, A. Pectinases yeast production using grape skin as carbon source. *Adv. Biosci. Biotechnol.*, 2011; **2**: 89-96.
16. Mellon, J.E., Cotty, P.J. Expression of pectinase activity among *Aspergillus flavus* isolates from southwestern and southeastern United States. *Mycopathologia*, 2004; **157**: 333-338.
17. Silva, D., Martins, E.S., Silva, R., Gomes, E. Pectinase production from *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural residues and agro-industrial by-product. *Brazilian J. Microbiol.*, 2002; **35**: 318-324.
18. Pericin, D.M., Madarev, S.Z., Radulovic, L.M., Skrinjar, M. Production of exo-pectinase by *Penicillium roqueforti* using pumpkin oil cake. *Natural Sci.*, 2007; **113**: 313-320.
19. Solís-Pereira, S., Favela-Torres, E., Viniegra-González, G., Gutiérrez-Rojas, M. Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentations. *Appl. Microbiol. Biotechnol.*, 1993; **39**(1): 36-41.

20. Patil, N.P., Chaudari, B.L. Production and purification of pectinase by soil isolate *Penicillium* sp. and search for better agro-residue for it SSF. *Recent Res. Sci. Technol.*, 2010; **2**(7): 36-42.
21. Dinu, D., Nechifor, T.M., Stoian, G., Costache, M., Dinischiotu, A. Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16. *J. Biotechnol.*, 2007; **131**:128-137.
22. Maldonado, M.C., Strasser de Saad, A.M. Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. *J. Ind. Microbiol. Biotechnol.*, 1998; **20**:34-38.
23. Shruti, S., Sudev, K.M. Enhanced production of pectinolytic enzymes from immobilized cells of mixed *Aspergillus* species. *Int. J. Appl. Biol. Pharma. Technol.*, 2012; **3**: 20-27.
24. Ghildyal, N.P., Ramakrishna, S.V., Nirmala, P., Devi, B.K., Lowsane, A.H.A. Large scale production of pectolytic enzyme by solid state fermentation. *J. Food Sci. Technol.*, 1981; **18**: 243-251.
25. Sarvamangala, R.P., Dayanand, A. Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid state conditions. *J. Bioresour. Technol.*, 2006; **97**: 2054-2058.
26. Darah, I., Sumathi, G., Jain, K., Lim, S.H. Involvement of physical parameters in medium improvement for tannase production by *Aspergillus niger* FETL FT3 in submerged fermentation. *J. Biotechnol. Res. Int.*, 2011; doi:10.4061/2011/897931.
27. Kutateladze, L., Zakariashvili, N., Jobava, M., Urushadze, T., Khvedelidze, R., Khokhashvili, I. Selection of microscopic fungi - pectinase producers. *Bulletin Georgian Nat. Academy. Sci.*, 2009; **3**(1): 136-141.
28. Mathew, A., Eldo, A.N., Molly, A.G. Optimization of culture conditions for the production of thermostable polygalacturonase by *Penicillium* SPC F 20. *J. Ind. Microbiol. Biotechnol.*, 2008; **35**: 1001-1005.
29. Fawole, O.B., Odunfa, S.A. Some factors affecting production of pectic enzymes by *Aspergillus niger*. *J. Int. Biodeterioration Biodeg.*, 2003; **52**: 223-227.
30. Papagianni, M. Fungal morphology and metabolite production in submerged mycelia process. *J. Biotechnol. Adv.*, 2004; **22**(3): 189-259.
31. Favela-Torres, E., Volke-sepúlveda, T., Viniegra-gonzález, G. Production of hydrolytic depolymerising pectinases. *Food Technol. Biotechnol.*, 2006; **44**: 221-227.
32. Piccoli-Valle, R.H., Silva, D.O., Passos, V.J.F., Passos, L.M.F. Production of pectin lyase by *Penicillium griseoroseum* in bioreactors in the absence of inducer. *Brazilian J. Microbiol.*, 2001; **32**(2): 135-140.
33. Rasheedha, A.B., Kalpana, M.D., Gnanaprabhal, B.V., Pradeep, B.V., Palaniswamy, M. Production and characterization of pectinase enzyme from *Penicillium chrysogenum*. *Indian J. Sci. Technol.*, 2010; **3**: 377-381.
34. Darah, I., Sumathi, G., Jain, K., Lim, S.H. Tannase enzyme production by entrapped cells of *Aspergillus niger* FETL FT3 in submerged culture system. *Bioprocess Biosyst. Eng.*, 2011; **34**(7): 795-80.
35. Friedrich, J., Cimerman, A., Steiner, W. Submerged production of pectolytic enzymes by *Aspergillus niger*: Effect of different aeration /agitation regimes. *Appl. Microbiol. Biotechnol.*, 1989; **31**: 490-494.
36. Palaniyappan, M., Vijayagopal, V., Viswanathan, R., Viruthagiri, T. Screening of natural substrates and optimization of operating variables on the production of pectinase by submerged fermentation using *Aspergillus niger* MTCC 281. *African J. Biotechnol.*, 2009; **8**(4): 682-686.
37. Mosjov, K. Experimental investigations of submerged fermentation and synthesis of pectinolytic enzymes by *Aspergillus niger*: effect of inoculums size and age of spores. *Appl. Technol. Innov.*, 2010; **2**(2): 40-46.
38. Kashyap, P., Sabu, A., Pandey, A., Szakacs, G. Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. *Process Biochem.*, 2002; **38**: 307-312.
39. Roheena, A., Ikram-ul-haq, Mohsin, J. Optimization of cultural conditions for the production of alpha amylase by wild and mutant strain of *Aspergillus oryzae* in stirred fermenter. *Pakistan J. Bot.*, 2011; **43**(1): 715-723.
40. Phutela, U., Dhuna, V., Sandhu, S., Chandha, B.S. Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposing orange peels. *Brazilian J. Microbiol.*, 2005; **36**: 63-69.
41. Aguilar, G., Huitron, C. Stimulation of the production of extracellular pectinolytic activities of *Aspergillus* sp. by galacturonic acid and glucose addition. *J. Enzym. Microb. Technol.*, 1987; **9**: 690-696.
42. Vivek, R., Rajasekharan, M., Ravichandran, R., Sriganesh, K., Vaitheeswaran, V. Pectinase production from orange peel extract and dried orange peel solid as substrate using *Aspergillus*

- niger*. *Int. J. Biotechnol. Biochem.*, 2010; **6**: 445-453.
43. Kashyap, R., Soni, K.S., Tewari, R. Enhanced production of pectinase by *Bacillus* sp. DT7 using solid state fermentation. *J. Bioresour. Technol.*, 2003; **77**: 215-227.
 44. Anguilar, G.B., Trejo, B., Garcia, J., Huitron, G. Influence of pH an endo and exo-pectinase production by *Aspergillus* species CH-Y-1043. *Can. J. Microbiol.*, 1991; **37**: 912-917.
 45. Reda, A.B., Hesham, M.Y., Mahmoud, A.S., Ebtsam, Z.A. (2008) Production of bacterial pectinase(s) from agro-industrial wastes under solid state fermentation conditions. *J. Appl. Sci. Res.*, 2008; **4**: 1708-1721.
 46. Darah, I., Ibrahim, C.O. Laboratory-scale production of lignin-degrading enzymes by free and entrapped cells of *Phanerochoete chrysosporium* in a tubular air-lift bioreactor. *Folia Microbiologica*, 1998; **43**(2): 161-168.
 47. Sandai, D., Ibrahim, D., Kassim, J. Calcium alginate entrapped cells of *Penicillium digitatum* FETL DS1 for the improvement of tannase production. *Indian J. Biotechnol.*, 2012; **6**(2): 27-34.