Optimization of Cellulase Production by *Aspergillus ornatus* by the Solid State Fermentation of *Cicer arietinum*

Yasmin Toor[1] and Umbrin Ilyas[1*]

[1]Department of Botany, University of Gujrat, Gujrat (50700), Pakistan
***Corresponding** Email: umbrin.ilyas@gmail.com

Abstract

Agrowastes are quite suitable substrates for enzyme producing microbes. Agrowastes are good carbon source and provide rich growth media for a range of microorganisms which produce industrially important enzymes. In the present studies, the process of cellulase production by *Aspergillus ornatus* on *Cicer arietinum* was optimized by adjusting various process parameters in solid state fermentation at Department of Botany, University of Gujrat, Pakistan, 2011. Chickpea agrowaste supplied with 0.2% ammonium sulphate as nitrogen source at 28°C at pH of 4, initial moisture of approximately 75% incubated upto the 72th hour of fermentation produced maximum cellulase enzyme. The results indicated that chickpea agrowaste might be a very good potential source of carbon and might be used at larger scales for cellulase production due to its cost effectiveness and ease of availability.

Keywords: *Aspergillus ornatus,* Cellulase, Chickpea waste, Solid State Fermentation

{**Citation:** Yasmin Toor, Umbrin Ilyas. Optimization of cellulase production by *Aspergillus ornatus* by the solid state fermentation of *Cicer arietinum*. American Journal of Research Communication, 2014, 2(1): 125-141} www.usa-journals.com, ISSN: 2325-4076.

Introduction

Cellulose is considered as a huge reservoir of renewable energy source (Coral *et al.*, 2002). Plant wastes are chiefly composed of cellulosic and lignocellulosic materials, which are biodegradable polymers and can be converted into a vast range of valuable products (Howard *et al.*, 2003). Cellulase is a complex enzyme whose basic function is the conversion of complex carbohydrates which are present in lignocellulosic biomass efficiently into glucose monomers (Hoelker *et al.*, 2004). It also breaks down cellulose into other oligosaccharide compounds beside glucose monomers (Chellapandi and Jani, 2008), and the enzymatic hydrolysis of celluloses is a very effective alternative method for the generation of sugars (Carere *et al.*, 2008; Dashtban *et al.*, 2009), which claims cellulases extremely important from the commercial as well as industrial point of view. Cellulases are employed in a variety of industrial processes, such as starch processing, fodder industry, cereal alcohol fermentation, malting, breweries, vegetable and fruit juice extraction, pulp and paper industry as well as in the textile industry. Replacing traditional fossil fuels with bioethanol generated from biomass has been a major goal in this millennium. Loads of research has been done to revolutionize cellulase enzyme production processes (Li *et al.*, 2009).

The complete degradation of cellulose into glucose is brought about by the combined action of three kinds of cellulases, namely endo 1, 4-β glucanases, exo 1, 4-β-D glucanases and β-D glucosidases (Pothiraj *et al.*, 2006). The first kind of cellulose arbitrarily degrades soluble and insoluble β-1, 4 glucan substrates. The other two act on the yield of the first kind to give D-glucose, cellobiose and related monomer subunits. Solid state fermentation, broadly defined as the process of fermentation that takes place in the absence or near absence of free water, with the substrate mostly in a physically solid form, has become a potential technology over the last

decade. It has proved to have potential for the economically feasible production of many microbial products that relate to the food industry, fuel industry, and industrial chemicals as well as in the pharmaceutical industry (Panday, 2003). Solid state fermentation (SSF) holds great potential for the manufacture of enzymes. SSF has many returns over other fermentation processes in a sense that the culture media are simpler and many solid media upon being supplied with appropriate nutrients can be utilized directly as growth media (Perez-Guerra *et al.,* 2003). Moreover the product of interest is obtained in a much higher concentration, which makes the purification process easier and cost effective. Contamination risks are also greatly decreased due to the minimal moisture content in the scheme. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source (Tengerdy and Szakacs, 1998). A vast range of microbes, for instance bacteria, yeast and fungi make diverse types of enzymes, among which hydrolytic enzymes (cellulases, xylanases, pectinases, etc.) are secreted by fungal cultures because these enzymes are a part of their growth and metabolic reactions (Panday *et al*., 1999).

Solid substrates make a perfect supporting and nourishing environment to the microbial flora including bacteria, yeast and fungi. Filamentous fungi in particular are the best studied in relation to SSF because of their hyphal growth. These have the ability to grow on the surface as well as into the substrate particles (Bisen & Sharma, 2012). *Aspergillus* species have been experimentally proved to be very efficient in the production of cellulases, and many scientists have described cellulase synthesis by various species of *Aspergillus* (Lockington *et al.,* 2002; Wang *et al.,* 2006; Gao *et al.,* 2008).

Several agrowastes and agro-industrial residues like wheat bran, rice bran, sugarcane pulp, cassava pulp, numerous oil cakes (e.g. copra oil cake, nut cake, soybean cake, ground nut

provender, etc), fruit pulps (e.g. apple), corn cobs, saw dust, seeds (e.g. tamarind, jack fruit), low husk and occasional pulp, tea waste, spent production grains, etc square measure the foremost usually and usually used substrates for SSF processes. Throughout the expansion on such substrates hydrolytic exo-enzymes square measure synthesised by the micro-organisms and excreted outside the cells that produce and facilitate in accessing straightforward merchandise (carbon supply and nutrients) by the cells. This successively promotes biogenesis and microbial activities. There are a number of publications that report recent work done on the solid state fermentation of agro-industrial residues like rice bran, rice husk, potato wastes, cassava husk, wheat bran, sugarcane pulp, sugarbeet pulp, nut cake, rice straw, cocoa pod, fruit wastes etc. SSF done on such substrates ends up in the assembly of a good variety of industrially vital merchandise like alcohol, enzymes, antibiotics, biofuel, biogas, mushrooms, organic acids as well as valuable secondary metabolites (Panday and Soccol, 1998; Panday *et al.*, 1999; Soccol and Vandenberghe, 2003; Prasertans, 1996; Villas-Boas *et al.*, 2002).

In the present studies, an effort has been made to utilize agrowaste of green Chickpea (*Cicer arietinum*) for the production of cellulase. Different physio-chemical parameters will be optimized to assess their effect on cellulase production. The main objective of the study was to investigate the effect of these different process parameters on the yield of cellulase and the optimization of initial moisture, initial pH, temperature and nitrogen sources.

Materials & Methods

Cellulosic Substrate

Fresh Chickpea agrowaste was collected from Sabzi Mandi, Gujrat, Pakistan, in its mature stage. The plant was sundried, chopped into pieces and ground to a very fine powder.

Microorganism

Aspergillus ornatus, the experimental microorganism was isolated directly from infested wood bark sample of *Ficus benghalensis* from local area of Hafiz Hayat, Gujrat, Pakistan, and screened for its cellulolytic potential on CMC agar. Sterile PDA slants were maintained at 4^0C and regularly subcultured.

Spore Suspension

Spore suspensions were made from the PDA slants by the method given by Noomrio & Dahot (1992) for inoculation of the substrate. Spore count of the suspension was approximately 1x 10^5 spores/ml by Haemocytometer.

Solid State Fermentation

Liquid media salt (LMS) was used for fermentation process whose composition per litre of distilled water was as follows: $NH_4(SO_4)_2$; 3.5g, KH_2PO_4; 3g, $MnSO_4$; 0.5g, $CaCl_2$; 0.5 g (Juhasz *et al.*, 2005). The solid state fermentation was carried out in 250ml Erlenmeyer flasks. Eight gram of chickpea agrowaste was taken in the individual flasks and 5ml of LSM & 2.5 ml of distilled water were added into the individual flasks and autoclaved at standard conditions (121°C, 20 min). 1ml of the spore suspension prepared was added aseptically into each flask via Micropipette. The flasks were then stored for 6 days within static conditions at 28°C to allow the fermentation process to occur.

Crude Enzyme Extraction

The crude enzyme was extracted by the method given by Gao *et al.* (2008) with slight modifications. 25ml of distilled water was added to fermented flasks. The flasks were then transferred into a shaker incubator at 120 rpm and 28°C for 30 min. The liquid extract was

filtered with a muslin cloth into Eppendorff tubes and centrifuged at 10,000 rpm for 10 minutes to obtain clear supernatant which serves as crude enzyme extract.

Enzyme Assay

Cellulase activity was determined by following the method given by Acharya *et al.,* (2008) at 40°C by using carboxymethylcellulose as a substrate. 1 ml of crude enzyme extract was added into a test tube and 0.5 ml CMC was added to it. The mixture was incubated at 40°C for 15 min. The reducing sugar released as a result of breakdown of carboxymethylcellulose by cellulase enzyme was measured using 3, 5-dinitrosalicyclic acid (DNS) (Miller, 1959). 3 ml of DNS solution was added to the reaction mixture. The mixture was boiled at 100°C for 10 min in order to terminate the reaction. One unit of cellulase activity was taken equal to the quantity of enzyme needed to release 1mM of reducing sugar per ml per minute under the above assay condition.

Optimization of Moisture Level

Optimization of moisture was carried out by adding different quantities of distilled water ranging from 2.5 ml to 15 ml to fermenting substrate. Three replicates were maintained for each moisture level. The flasks were kept at static condition for 6 days at 28°C.

Optimization of Temperature

The temperature was optimized by incubating the chickpea waste containing fermentation media at different temperatures. The incubation was carried out at 24°C, 28°C, 32°C and 36°C respectively in incubator.

Optimization of Nitrogen Source

Different Ammonium salts were used in order to determine the salt which served as the best nitrogen source. 0.2 g of inorganic nitrogen source per 8 g of carbon source was added to the

substrate. The original LMS was replaced by another liquid media salt which had the same composition as the former but without the nitrogen source. The flasks were kept at static conditions at 28°C for 6 days.

Optimization of pH

The pH was optimized by adjusting the pH of LMS over a range of pH values from 4-7. The pH of the medium was adjusted by treatment of 1N NaOH and 1N HCl respectively. Three replicates were maintained for each pH value. After inoculation with 1ml of spore suspension, the flasks were kept at static conditions for 6 days.

Optimization of Incubation Period

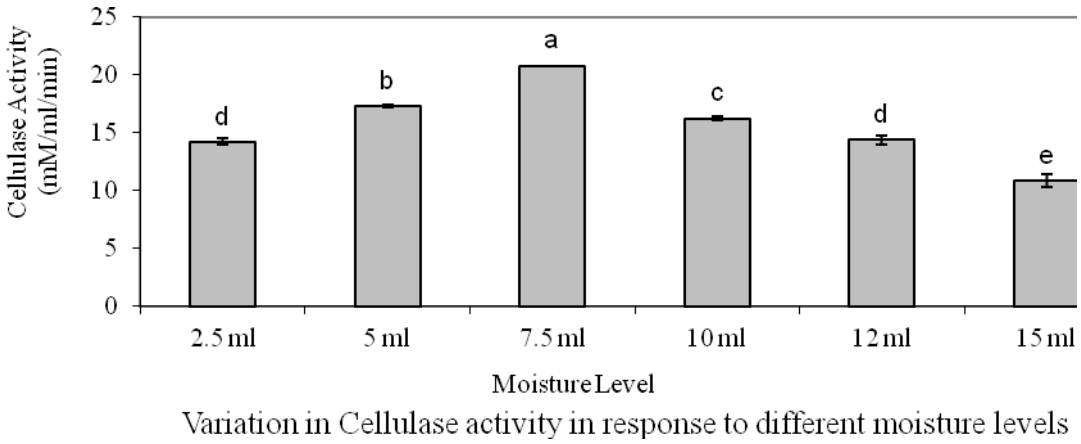
Enzyme in crude form was harvested for various periods of incubation ranging from 24 hours to 168 hours of fermentation and enzyme assay was performed to determine the amount of cellulase activity at a regular 24-hour interval to determine the hours of maximum activity.

Results & Discussions

Optimization of Moisture Level

Cellulase activities observed at a water content of 2.5 ml, 5 ml, 7.5 ml, 10 ml, 12 ml and 15 ml were 14.21, 17.285, 20.7785, 16.246, 14.3675 and 10.815 mM/ml/min respectively, indicating a maximum degradation of the substrate at a water content of 7.5 ml added to the substrate. Cellulase production was considerably slowed down both at lower and higher levels of moisture. This corresponds to the highest level of cellulase activity for the different water contents provided to the fermentation media. Results indicated that moderate moisture level supports

highest rate of cellulase production whereas lower as well as higher moisture contents decrease the amount of cellulase activity (Fig. 1).



Variation in Cellulase activity in response to different moisture levels

Fig 1. Variation in Cellulase Activity (mM/ml/min) in response to different moisture levels for the fermentation of chickpea agrowaste by *Aspergillus ornatus*.

Optimization of Temperature

The cellulase activities at 24°C, 28°C, 32°C and 36°C were 17.369, 20.502, 18.431 and 13.216 mM/ml/min respectively, indicating highest enzyme activity at 28^0C. . Similarly, Acharya *et al*. (2008) reported maximum cellulase activity of 0.0925 IU/ml for fermentation of sawdust with *Aspergillus niger* at 28°C. Enhanced cellulase activity at the same temperature was also observed by Narasimha *et al.* (2006) for fermentation of sawdust with the same *Aspergillus* species which Acharya *et al.* (2008) used in their experiments. Experiments performed for cellulase production by Singhania *et al*. in 2006 using fungal cultures of *Trichoderma reesei* also revealed maximum cellulase production at 28°C, showing that most of the *Aspergillus* species have an optimum temperature of 28°C for cellulase production on various substrates (Fig. 2).

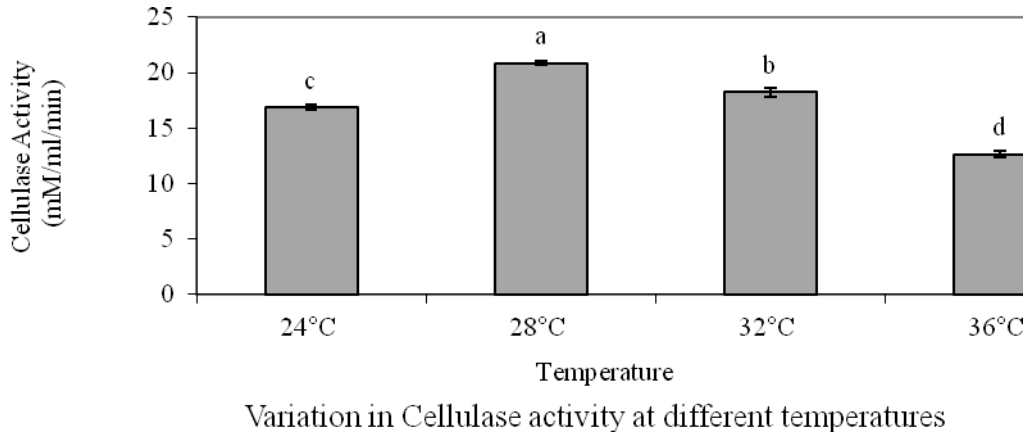


Fig 2. Variation in Cellulase Activity (mM/ml/min) in response to different temperatures for the fermentation of chickpea agrowaste by *Aspergillus ornatus*.

Optimization of Nitrogen Source

Enzyme activity of *Aspergillus ornatus* was observed for each salt given to the substrate i.e. 20.51, 30.11, 34.07, 27.37, 21.64 and 18.32 mM/ml/min respectively. Maximum cellulase activity was observed with ammonium sulphate. Singhania *et al.* (2006) reported maximum activity with ammonium nitrate as nitrogen source in their studies on solid state fermentation using *Trichoderma reesei*. The present results also revealed that ammonium nitrate gave second highest yield of cellulase enzyme (see Fig. 3), which is in accordance with the study of Singhania *et al*. (2006). The observations of Ilyas *et al*. (2011) reported maximum cellulase activity with ammonium sulphate as nitrogen source which supports the result of the present study conducted. The efficiency of ammonium sulphate as nitrogen providing source may be due to its direct availability as nitrogen source for protein production (Mandels, 1975).

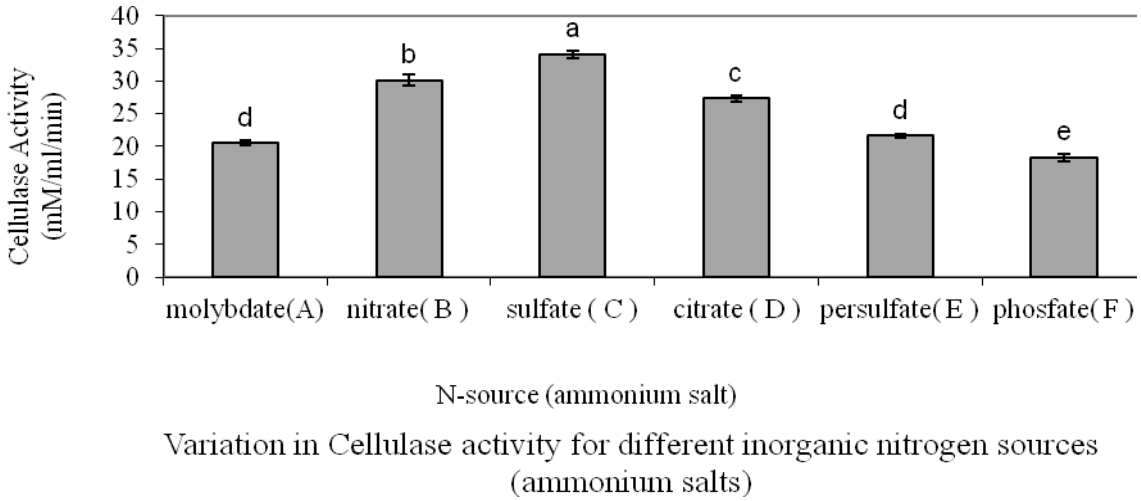


Fig 3. Variation in Cellulase Activity (mM/ml/min) in response to different inorganic nitrogen sources (ammonium salts) for the fermentation of chickpea agrowaste by *Aspergillus ornatus*.

Optimization of pH

Different cellulase activity was found at pH 3, pH 4, pH5, pH 6 and pH 7 which were 32.20, 37.71, 29.99, 30.03 and 31.51 mM/ml/min respectively, indicating the highest activity occurred at acidic conditions at a pH of 4, corresponding to the maximum degradation of cellulosic chickpea agrowaste substrate (see Fig. 4). Maximum cellulase activity of 0.925 IU/ml at a pH of 4 was also observed by Acharya *et al.* (2008) when they used *Aspergillus niger* for the solid state fermentation of sawdust. Sohail *et al.* (2009) reported maximum cellulase activity at a pH of 4 for *Aspergillus niger*. Sugarcane waste was fermented for cellulase production via SSF by Juwaied *et al.* (2011) using *Aspergillus niger* and *Trichoderma viride*, findings reported optimum pH of 4.5. Singhania *et al.* (2006) observed optimum cellulase activity at pH 7 for *Trichoderma reesei*. Ilyas et al. (2011) reported optimum pH of 4.5 in their studies for cellulase production optimization.

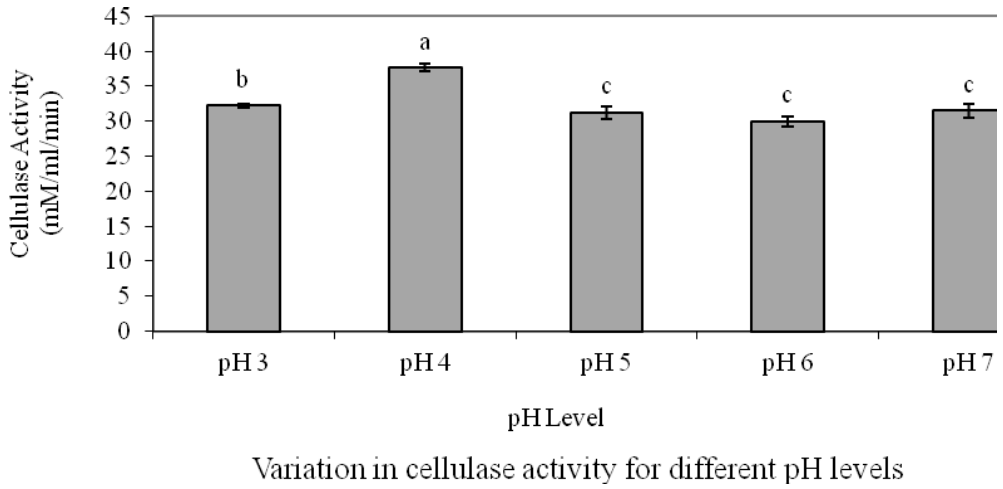


Fig 4. Variation in Cellulase Activity (mM/ml/min) in response to different pH levels for the fermentation of chickpea agrowaste by *Aspergillus ornatus*.

Optimization of Incubation Period

Enzyme in crude form was harvested after 24, 48, 72, 96, 120, 144 and 168 hours of incubation giving a cellulase activity of 36.4, 39.55, 43.54, 39.28, 35.56, 36.43 and 34.77 mM/ml/min respectively. Maximum cellulase production was recorded at the 72th hour of fermentation (see Fig. 5). Cellulase activity observed was 43.54 mM/ml/min. In another experiment performed by Sherief and others in 2010 using *Aspergillus fumigatus* for the solid state fermentation of mixed substrate of rice straw and wheat bran, the highest readings for cellulase activity were recorded on the 96th hour of fermentation. Maximum cellulase activity in solid state fermentation using *Aspergillus flavus* was recorded after 12 hours of fermentation by Ojumu *et al.*, (2003). Acharya *et al*., (2008) used sawdust as a substrate for cellulase production via solid state fermentation by *Aspergillus niger*. Maximum enzyme activity was recorded at the 96th hour of fermentation. Alam *et al*., (2005) conducted experiments on the solid state fermentation of oil palm biomass

for cellulase production by *Trichoderma harzianum* and observed maximum cellulase activity of 0.0413 units at 72 hours of fermentation. Likewise 72 hours of fermentation were observed to be the optimum incubation period for cellulase production by fungal cultures of *Trichoderma reesei* (Singhania *et al.*, 2006). Pothiraj *et al.* (2006) observed maximum cellulase activity at 240th hour of fermentation of cassava waste using *Rhizopus stolonifer*. Ilyas *et al.* (2011) reported a maximum cellulase activity at the 96th hour of fermentation in their studies with *Aspergillus niger*.

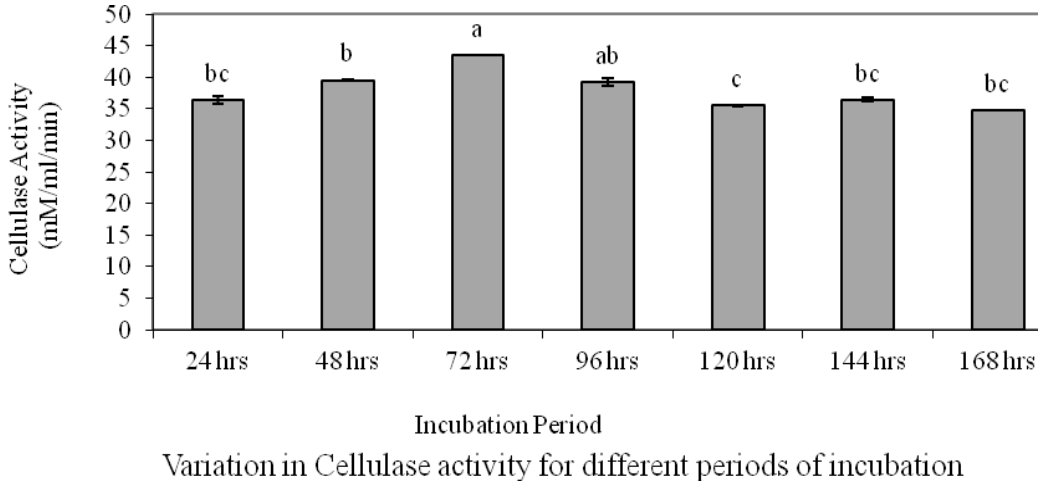


Fig 5. Variation in Cellulase Activity (mM/ml/min) in response to different incubation periods for the fermentation of chickpea agrowaste by *Aspergillus ornatus*.

Conclusion

In accordance with the results of these experiments performed, it is shown that *Aspergillus ornatus* is arelatively good producer of cellulose under a range of conditions. The present studies have shown valueable results which imply that chickpea agrowaste can be a very good source for

the production of cellulose enzyme in the laboratory. However, for optimizing the culture conditions to make it applicable to industrial scale will require thoughtful planning and further research to make it fruitful for various commercial processes at a large scale.

References

Acharya PB, Acharya DK and Modi HA. (2008): "Optimization for cellulose production by *Aspergillus niger* using saw dust as substrate." *African Journal of Biotechnology*. 22: 4147-4152.

Alam MZ, Muhammad N and Mahmat ME. (2005): "Production of cellulase from oil palm biomass as substrate by solid state bioconversion.: *American Journal of Applied Science*. 2: 569-572.

Bisen PS and Sharma A. (2012): "Introduction to Instrumentation in Life Sciences." *CRC Press*.

Carere CR, Sparling R, Cicek N, and Levin DB. (2008): "Third generation biofuels via direct cellulose fermentation." *International Journal of Molecular Sciences*. 9: 1342-1360.

Chellapandi P and Jani HM. (2008): "Production of endoglucanase by the native strains of *Strptomyces* isolates in submerged fermentation." *Brazilian Journal of Microbiology*. 39: 122-127.

Coral G, Arikan B, Unaldi MN, and Guvenmes H. (2002): "Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain." *Turkish Journal of Biology*. 26: 209-213.

Dashtban M, Schraft H and Qin W. (2009): 'Fungal bioconversion of lignocellulosic residues: Opportunities and perspectives." *International Journal of Biological Sciences*. 5: 578-595.

Gao J, WengH , Zhu D,Yuan M, Guan F and Xi Y. (2008): "Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid state cultivation of corn stover." *Bioresource* Technology. 99: 7623-7629.

Hölker U, Höfer M, and Lenz J. (2004): "Biotechnological advantages of laboratory-scale solid-state fermentation with fungi." *Applied Microbiology and Biotechnology*. 64(2):175-186.

Howard RL, Abotsi E, JansenVREL, Howard S. (2003): "Lignocellulose Biotechnology: Issue of Bioconversion and Enzyme production." *African Journal of Biotechnology*. 2: 602-619.

Ilyas U, Majeed A, Hussain K, Nawaz K, Ahmad S, and Nadeem M. (2010): "Soild State fermentation of *Vigna mungo* for cellulase production by *Aspergillus niger*." *World Applied Sciences Journal*. 12:1172-1178.

Juhasz T, Egyhazi A and Reczey K. (2005): "β galactosidase production by *Trichoderma reesei*." *Applied Biochemistry Biotechnology*. 121: 243-254.

Juwaied AA, Al-amiery AAH, Abdumuniem Z, and Anaam U. (2011): "Optimization of cellulase production by *Aspergillus niger* and *Tricoderma viride* using sugar cane waste." *Journal of Yeast and Fungal Research*. 2: 19-21.

Li L and Huan XL. (2009): "Simultaneous cloning and expression of two cellulose genes from *Bacillus subtilis* newly isolated from Golden Takin (*Budocras taxicolor Bedfordi*)." *Biochemical and Biophysical Research communications*. 383 (4). 397-400.

Lockington RA, Rodbourn L, Barnett S, Carter CJ and Kelly JM. (2002): "Regulation by carbon and nitrogen sources of a family of cellulases in *Aspergillus nidulans*." *Fungal Genetics and Biology*. 37: 190-196.

Mandels M. (1975): "Microbial Sources of Cellulase." *Biotechnology Bioengineering*. 5:81-105.

Miller GL. (1959): "Use of dinitrosalicyclic acid reagent for determination of reducing sugar." *Biotechnology Bioengineering Symposium*. 5: 193-219.

Narasimha G, Sridevi A, Buddolla V, Subhosh CM and Rajsekhar RB. (2006): "Nutrient effect on production of cellulolytic enzymes by *Aspergillus niger*." *African Journal of Biotechnology*. 5: 472-476.

Noomrio MH and Dahot MU. (1992): "Comparative study on the production of cellulases by Aspergillus niger using agricultural wastes as a carbon source." *Proceedings of All Pakistan Science Conference*. 1: 95-98.

Ojumu TV, Solomon BO, Betiku E, Layokun SK and Amigun B. (2003): "Cellulase Production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob." *African Journal of Biotechnology*. 2: 150-152.

Panday A and Soccol CA. (1998): "Bioconversion of Biomass." *Brazilian Archives of Biology and Technology*. 41: 379-390.

Panday A, Selvakumar P, Soccol CR and Nigam P. (1999): "Solid state fermentation for the production of industrial enzymes." *Current Science*. 79:149-162.

Pandey A. (2003): "Solid-state fermentation." *Biochemical Engineering Journal*. 13: 81-84.

Perez-Guerra N, Torrado-Agrasar A, Lopez Macias C and Pastrana L. (2003): "Main Characteristics and Applications of Solid Substrate Fermentation." *Electronic Journal of environmental, Agricultural & Food Chemistry*. 2 (3): 343-350.

Pothiraj C, Balaji P and Eyini M. (2006): "Enhanced production of Cellulases by various fungal cultures in solid state fermentation of cassava waste." *African Journal of Biotechnology*. 5: 1882-1885.

Prasertan S and Prasertan P. (1996): "Biomass residues from palm oil mills in Thailand. Potential use." *Biomass and Bioenergy*. 11: 387-395.

Sherief AA, El-Tanash AB and Atia N. (2010): "Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran". *Research Journal of Microbiology*. 5: 199-211.

Singhania RR, Sukurmaran RK, Pillai A, Prema P, Szakacs G and Panday A. (2006): "Solid state fermentation of lignocellulosic substrates for cellulase production by Trichoderma reesei NRRL 11460." *Indian Journal of Biotechnology*. 5: 332-336.

Soccol CR and Vandenberghe LPS. (2003): "Overview of applied sold state fermentation in Brazil." *Biochemical Engineering Journal*. 13: 205-218.

Sohail M, Siddiqui R, Ahmad A and Khan SA. (2009): "Cellulase production from *Aspergillus niger* MS82: Effect of temperature and pH." *New Biotechnology*. 25: 437-441.

Tengerdy RP and Szakacs GG. (1998): "Perspectives in agrobiotechnology." *Journal of Biotechnology.* 66:91-99.

Villas-Boas SG, Esposito E and Mitchell DA. (2002): "Microbial conversion of lignocellulosic residues for the production of animal feeds." *Animal feed Science and Technology*. 98 :1-12.

Wang X, Bai J and Lian Y. (2006): "Optimization of Multienzyme production by two mixed strains in solid state fermentation." *Applied Microbiology Biotechnology*. 73: 533-540.