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Optimization and Scale up of Cellulase free Endo xylanase Production by Solid State Fermentation on Corn cob and by Immobilized Cells of a Thermotolerant Bacterial Isolate

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Abstract

Different agro-residues were evaluated as substrates in solid state fermentation for xylanase production by a thermotolerent Bacillus isolate. Various fermentation parameters were optimized for enhanced endoxylanase production under solid state fermentation (SSF). Maximum enzyme production of 74.96±5.2 U/gds took place at 45^0C with corn cob (CC) and mineral salt solution (MSS) after 72 h, at pH 6.0 and particle size of 500 µm. A ratio of substrate: moistening agent of 1:2.5 was found to be optimum for CC, a solid substrate rarely utilized for bacterial xylanase in SSF. Continuous xylanase production by recycling immobilized cells could be achieved till 10 cycles with maximum enhancement of 156.05% and 219.21% after 5 and 4 cycles in static and submerged states respectively. Scale up in large trays under SSF yielded 157.12 ± 8.7U/gds in static state and 111.47± 8.1U/gds at 110 rpm.

Keywords: Cellulase free endoxylanase, Bacteria, Optimization, Solid state fermentation, Scale-up, immobilized cells.

1. Introduction

Xylanases (E.C.3.2.1.8) are key enzymes, which play an important role in the breakdown of xylan. Corn cob is a rich source of xylan (28%) and xylose (23%). Therefore it is an attractive substrate for production of xylanase enzyme. Xylan, a major component of hemicellulose, is a heterogeneous polysaccharides consisting of ß-1,4 linked to D-xylosyl residues on the backbone, but also containing arabinose, glucuronic acid, and arabino glucuronic acid linked to D-xylose backbone (Wong et al.,1988). Enzymatic hydrolysis of xylan is catalysed by different xylanolytic enzymes such as endo-1,4-ß-xylanase, ß-xylosidase, ά-glucuronidase, ά-arabinofuranosidase, and esterase. Among these endo-1,4-β-xylanase(E.C. 3.2.1.8) and ß-xylosidase are the most important enzymes where the first attacks the main internal chain linkages, and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides (Bakir et al., 2001).

A variety of microorganisms including bacteria (Archana and Satanarayan, 1997; Poorna and Prema, 2006), fungi (Kheng and Omar, 2004), actinomycetes and yeasts have been reported to produce xylanase under SSF. Solid state fermentation is the growth of micro-organism on moist substrates in the absence of free flowing water. SSF offers distinct advantages over submerged fermentation including economy of space, simplicity of media, no complex machinary, greater product yields, and reduced energy demand (Sanghi et al., 2008). Although xylanase production in SSF from fungi and actinomycetes have been reported, only few reports using bacteria showing low enzyme yields are available (Archana and Satyanarayana 1997; Gessesse and Memo 1999; Heck et al. 2005; Battan et al. 2006; Sindhu et al. 2006).

Corn cob was also used for xylanase production in SmF by the *B. licheniformis* isolate under different conditions. However, xylanase yield was lower compared to the yield under SSF, which is being reported here. Although corn cob is a rich source of xylan and xylose, and is abundantly available in India, it has rarely been utilized for bacterial xylanase production under SSF. This could be due to low yield on corn cob as compared to other solid substrate like wheat bran. The objective of this work was to optimize various fermentation parameters for xylanase production

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by a thermophilic bacterial isolate on corn cob under SSF, and production enhancement by scaling up the solid state system. Since xylanase production was higher under SSF, and corn cob is an insoluble substrate suitable as solid state support, SSF was applied in the study. Improvement in xylanase yield by recycling immobilized cells for prolonged period are also being reported.

2. Materials and Methods

2.1. Materials:

Oat spelt xylan (Himedia Labroratories Pvt. Ltd., India) was used for enzyme assay. Corn cob was prepared by stripping corn of all kernels, drying, grinding, and sieving (500 mm. particle size). Dried corn cob was added to mineral salt medium; and autoclaved as mentioned in 2.4. Other than autoclaving, no other pretreatment was necessary. All other reagents were of analytical grade.

2.2. Microbial Strain:

Bacterial strain used in this study was isolated from decayed woody materials like xylan rich-wood materials, which are potentially good sources of xylanase producing micro-organism (Oliveira et al., 2006). This strain was identified by Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology, Chandigarh, India. And then was deposited as *Bacillus licheniformis* MTCC 9415. The optimum growth temperature of this strain is 45°C and it grows well in the range of 25^0C - 52^0C. The culture was grown and maintained on agar slants of 4% corn cob and mineral salts mentioned in 2.4.

2.3. Inoculum Prepararion:

Culture was maintained on agar slants of 4% corn cob and mineral salt mentioned in 2.4., then stored at 4^0C. And was subcultured routinely after every three four weeks. Inoculum was prepared by transferring one loopful of bacterial cells from a 48h old slant culture into 2 ml of fermentation medium; and incubated at 45^0C for 48h. This was used to inoculate 20 ml of fermentation medium.

2.4. Medium composition and growth condition:

Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 10 g of corn cob and 20 ml of mineral salt solution (MSS g/l : $MgCl_2.6H_2O$, 6.6g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5g; $(NH_4)_2S0_4$, 2.0g (Sanghi et al. 2007). The pH of medium was 6.7, and the medium was sterilized at 121^0C for 20 min. at 15 p.s.i., and was cooled and was inoculated with 10% (v/v) of inoculum (48h old) And was incubated at 45°C. At the desired intervals, the flasks were removed, and the contents extracted with 50 ml of 0.02 M phosphate buffer (pH 7.0).

2.5. Enzyme Extraction:

Enzyme was extracted with 50 ml of 0.02M phosphate buffer (pH 7.0), and squeezed through a wet muslin cloth. The extracted enzyme was centrifuged at 3000 rpm for 10 min. The clear supernatant was used in the enzyme assay.

2.6. Analytical Procedures:

Endoxylanase activity was measured by incubating 0.5ml of 0.4% (w/v) oat spelt xylan in 0.02 M phosphate buffer (pH 7.0). And 0.5 ml of suitably diluted enzyme extract at 45°C for 30 min. The release of reducing sugar was measured as xylose by dinitro salicylic acid method (Miller, 1959). One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/ml/min under the assay conditions. Endoxylanase production in SSF was expressed as U/g dry fermented substrate (gds).

Cellulase activity was not detected in the culture supernatant. Cellulase was assayed according to Mandels et al., 1974 using sodium nitrate buffer (0.1M, pH 7.0) at 45^0C. One unit of cellulose is defined as the amount of enzyme that liberates 1µmol reducing sugar as glucose ml^{-1} min^{-1} under assay conditions.

2.7. Data Analysis:

All the experiments were carried out in triplicates. The analyses were done in duplicates. The mean values are shown in the figures and tables.

2.8. Statiscal Analysis:

Data were expressed as mean ± standard deviation for all experiments and statistical significance was calculated according to student two-tailed t test. Values corresponding to $p<0.001$ were considered statistically significant.

2.9. Endoxylanase Production Using Agro-Industrial Residues:

Bacterial strain was cultivated on different substrates such as wheat bran, corn cob, sugarcane baggase, rice bran, wheat straw, and rice straw. All growth conditions, mentioned in 2.4., were followed in all cases. Only agro-residues (10g in each case) were varied.

2.10. Effect of Incubation Period, Media Ph, And Temperature on Enzyme Production:

Effect of incubation period on endoxylanase production was determined by assays of enzyme after 24h, 48h, 72h, and 96h at 45^0C. Effect of media pH on endoxylanase production was estimated by culturing the strain in media of different pH-3, 4, 5, 6, 7, 8, and10. Effect of temperature on xylanase production was studied by incubating the strain at 30^0C, 35^0C, 40^0C, 45^0C, and 50^0C.

2.11. Effect of particle size:

The effect of particle size of corn cob on enzyme production was evaluated by culturing the organism on corn cob of different particle sizes (350µm, 500µm, 600µm, 710µm and 1000µm).

2.12. Effect of Moisture Contents:

The effect of moisture level on enzyme production was evaluated by varying the ratio of corn cob to mineral salt solution (66.6%-80%).

2.13. Scale up of Xylanase Production in Solid State:

The bacterial strain was cultivated in aluminum tray ($20\times8\times5cm^3$)containing 80g of corn cob moistened with MSS (ratio of 1:2.5), and other conditions as optimized in 250 ml Erlenmeyer flasks as mentioned earlier. The trays were covered with aluminum foil and sterilized at 121^0C for 20min., And then cooled and inoculated with 10% of 48h old inoculum. The trays were incubated at 45^0 for 96h. Samples were withdrawn at desired intervals, and xylanase was assayed as described in sections 2.4 and 2.5.

2.14. Immobilized Cell System:

Poly urethane foam (PUF) $1cm^2$ cubes, and scotch brite, SB,$1cm^2$ cubes were used for whole cell immobilization of bacterial strain. Cubes of PUF and SB were washed with distilled water and dried overnight at 60^0C in an oven, and then placed in a 250 ml flask

containing 50 ml of 1% yeast extract, peptone (YEP) medium supplemented with 1% xylose. After sterilization at 121^{0}C for 30 min, 10% (v/v) inoculum was added to each flask and was incubated at 45^{0}C. After 48h, PUF cubes with immobilized cells were carefully drained and washed with sterile water for eliminating all non adhering bacteria; also the broth was replaced with fresh medium for the next cycle. The process was carried out over 10 cycles.

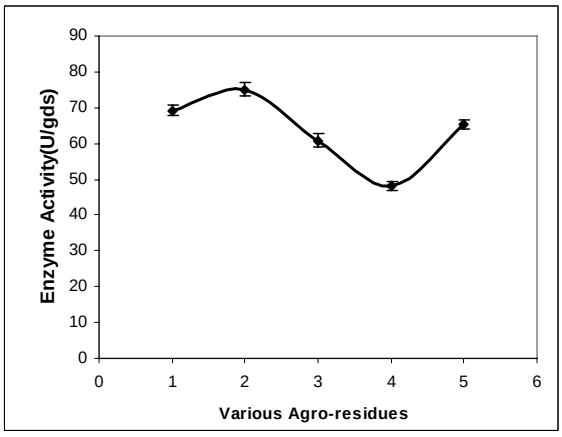

Figure 1 : Production of endoxylanase enzyme by *Bacillus* isolate under SSF on various agro-residues at 72h of incubation. Temperature 45^{0}C; pH 6.7. 1-WB; 2-CC; 3-WS; 4-RS; 5-SB.

3. Results and Discussion:

3.1. Effect of Different Agro Residues:

The effect of various substrates for xylanase production was examined with 10 g of each wheat bran, corn cob, wheat straw, rice straw, and sugarcane baggase in 250 ml Erlenmayer flasks with 20 ml of mineral salt solution. Cultivation was carried out at 450C for 96 h. As indicated in fig.1, corn cob (74.96±5.2 U/gds) was found to be the best substrate for endoxylanase production in the present study. The high level of xylanase production on xylan containg substrate (corn cob) suggest that xylan is necessary for effective induction of xylanase. Xylan may not be the direct inducer since it can not enter cells directly but its initial hydrolysis products like xylobiose, xylotriose, etc. are genrerated by constitutive xylanase action, which may act as inducers. Corn cob contained xylose (23%) and xylan (28 may fulfill the role of inducers, and use of corn cob for xylanase production under SSF by bacteria is very limited. Ninawe and Kuhad (2005) also reported wheat bran and corn cob as an enhancer for xylanase production by *Streptomyces cyancus* SN32. Consequently, corn cob was selected as the substrate for xylanase production in the present work.

3.2. Effect of Incubation Period, Media Ph, and Temperature:

Xylanase production by bacterial isolate under SSF showed that a low level of xylanase activity was detected in earlier stages of incubation and enzyme activity steadily reached a maximum level (74.96±5.2 U/gds) by 72h of incubation (Figure 2) there was a decrease in enzyme activity (64.54±4.8 U/gds) with further increase in incubation period. Similar findings have been reported with *B. lichenifoermis* where enzyme production reached a maximum level by 72 h in wheat bran medium (Archana and Satyanarayan, 1997). The reduction in xylanase yield after optimum period was probably due to the depletion of nutrient available to microorganism or due to proteolysis (Flores at. el., 1997).

effect of media pH on enzyme production is shown in Fig.3. The optimum pH for xylanase production was found to be 6.0. Each microorganism holds a pH range for its growth and activity with optimum value between around this range. Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane (Poorna and Prema, 2006). The results of influence of temperature on xylanase production are shown in Figure 4, showing that the optimum temperature for xylanase production was 45^{o}C at 72h of incubation. Enzyme activity at 50^{0}C was also significant and comparable to that at 40^0 C till 72 h.

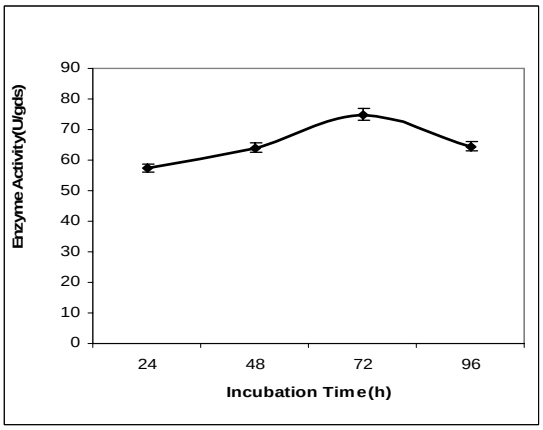

Figure 2. Effect of incubation time on endoxylanase and protein production by *Bacillus* isolate on corn cob as substrate in SSF. Temperature 45^{0}C; pH 6.7.

3.3. Effect of Particle Size:

Effect of particle size of corn cob on enzyme production is shown in Fig.5. Particle size of 500µm was found best for maximum xylanase production (74.96±5.2 U/gds). The results agree with Poorna and Prema, where 500 µm particle size gives maximum xylanase production with wheat bran.

3.4. Effect of Moisture Contents:

The moisture content in SSF is an important factor that determines success of the process. A moisture content higher than optimum moisture level causes decreased porosity of substrate, alternation in particle size, gummy texture, and lower oxygen transfer (RaimBault and Alazard, 1980 and Feniksova et al., 1960). A lower moisture level leads into a reduction in solubility of nutrients of solid substrate. And it leads to a lower degree of swelling and to a higher water tension (Ikasari and Mitchell, 1994). As indicated in fig.6, xylanase production was optimum (84.37±5.9 U/gds) when corn cob and moistening agent was 1:2.5.

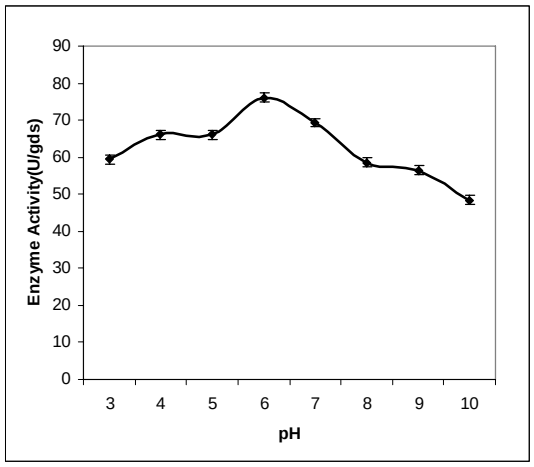

Figure-3. Effect of media pH on endoxylanase production by *Bacillus* isolate on corn cob as substrate in SSF.Temperature 45^0C.

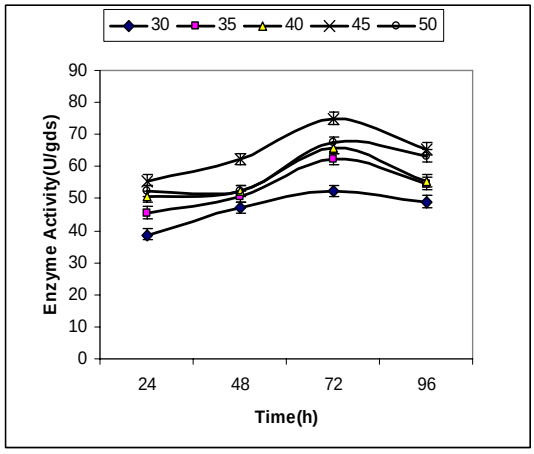

Figure-4. Effect of temperature on endoxylanase production by *Bacillus* isolate on corn cob as substrate in SSF at 72h of incubation. pH 6.7.

3.5. Scale-Up of Xylanase Production Under SSF:

Xyalnase production was enhanced by scaling up the solid state system by using enamel trays containg 80g corn cob. When SSF was performed in trays for xylanase production using bulk quantities of corn cob (80 g), xylanase production was higher if compared with Erlenmayer flasks. Cultivation in large enamel trays yielded 157.12 ±8.7 U/gds in static state and 111.47 ±8.1U/gds at 110 rpm when compared to the value obtained in 250 ml flasks (74.96 ±5.2 U/gds). The improvement of xylanase production in trays, more than flasks, may be due to efficient aeration, better mass, and heat transfer. A slight decrease (12.14%) in enzyme production by scaling up has been reported in *Bacillus megaterium* (Sindhu et al.,2006), while in *Bacillus licheniformis* (Archana and Satyanarayan, 1997), scaling up stimulate xylanase production. It may be possible to further improve the enzyme yield with higher quantities of the substrate - thus making corn cob a potential solid substrate for xylanase production.

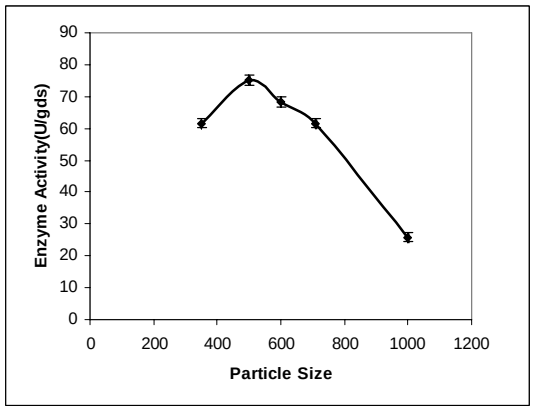

Figure-5. Effect of particle size on endoxylanase production by Bacillus isolate on corn cob as substrate in SSF at 45^0C. pH 6.7.

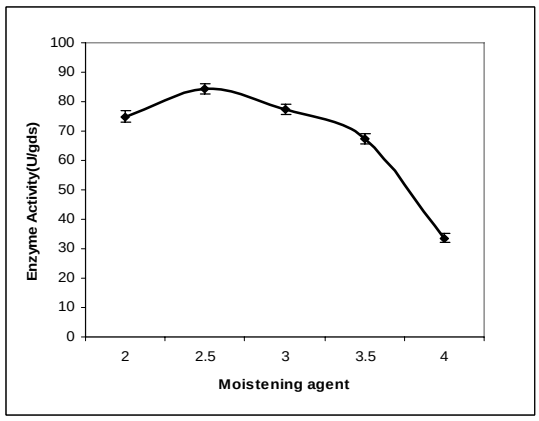

Figure-6. Effect of initial moisture level on endoxylanase production by *Bacillus* isolate on corn cob. Temperature 45^0C.

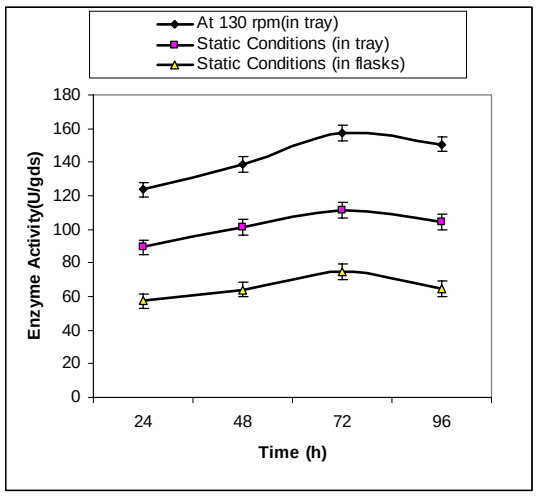

Figure-7. Scale up of xylanase production from bacterial isolate in large trays. Temperature 45^0C; pH 6.7.

3.6. Whole Cell Immobilization:

As indicated by fig.8, PUF was a superior support for immobilization if compared to SB. PUF was selected for further immobilization studies. The porous structure of foam allowed growth of the cells inside the pores, and a non-diffusion limited environment for substrate and

product. As indicated by Fig.9, it was observed that after immobilization of bacterial strain on PUF cubes, endoxylanase production was increased 156.05% in static state and 219.21% in submerged state compared to control value (free cells). Xylanase production by immobilized cells attained maximum level at V^{th} cycle in static state and at IV^{th} cycle in submerged state, After this xylanase production declined slowly up to ten cycle. Similar observations were made by Beg *et al.* (2000) in *Streptomyces Sp. QG-11-3* species, where immobilization on polyurethane foam (PUF) enhanced xylanase production by 2.5 fold. These results also indicated that this type of fiber materials has a significant role in providing a favorable environment for enzyme production. Increase in xylanase production, observed after immobilization, may be due to adherence of bacterial cells to the surface of PUF, as well as into the pores, thus increasing the residence time of cells in the medium.

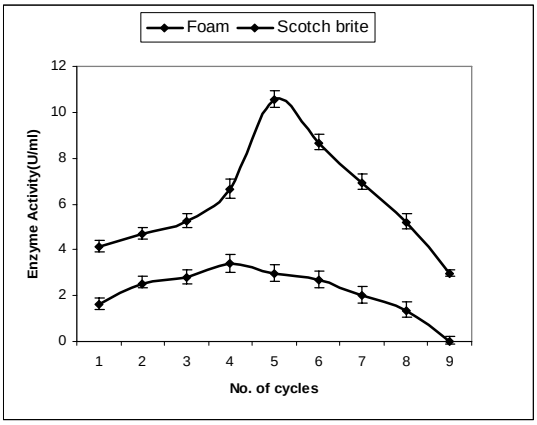

Figure-8. Xylanase production by the bacterial strain immobilized on polyurethane foam and scotch brite. Temperature 45^0C; Medium YEP+Xylose.

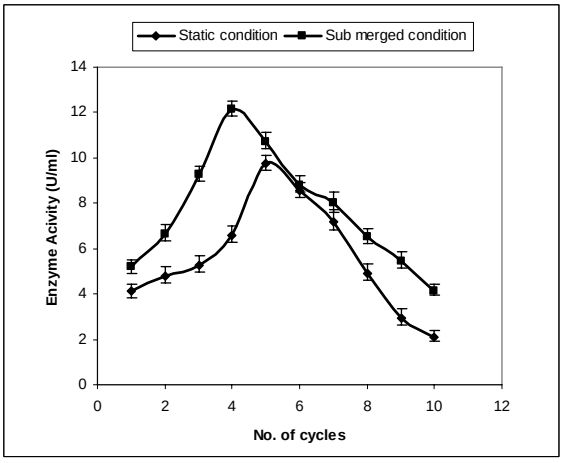

Figure-9. Xylanase production by immobilized cells under liquid surface and submerged conditions.

4. CONCLUSIONS:

Corn cob, an abundantly available agro-residue in India was successfully used as a solid state support by the *B.licheniformis* isolate at 45^0C for xylanase production, and it has not been reported so far.The results presented here show that optimization of process parameters and immobilization of the bacterial cells on PUF resulted in higher production of xylanase and could be continued by recycling the cells till 10 cycles. Scaling up under SSF also resulted in significant improvement in xylanase production.

REFERENCES

Archana, A. and Satyanarayan, T.1997.Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid state fermentation. Enz. Microb. Technol., 21:12-17.

Poorna, AC and Prema, P. 2006. Production of cellulose free endoxylanase from novel alkalophilic thermotolerent *Bacillus pumillus* by solid state fermentation and its application in wastepaper recycling, Bio.Reso.Tech., 98: 485-490.

Bakir, U, Yavascaoglu, S, and Guvenc Fand Ersayin, A. 2001. An endo β-1, 4-xylanase from *Rhizopus oryzae*: Production, partial purification and biochemical characterization. Enz. Microb. Technol., 29:328-334.

Battan, B, Sharma J, and Kuhad, RC. 2006. High level xylanase production by alkalophilic *Bacillus pumilus* ASH under solid state fermentation. World J. Microbiol. Biotechnol. 22; 1281-1287.

Beg, QK, Bhushan, B, Kapoor, M and Hoondal, GS. 2000. Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp. Enz. Microb. Technol., 27: 459-466.

Feniksova, RV, Tikhomrova, AS and Rakhleeve, BE. 1960. Conditions for forming amylases and protease in surface culture of *Bacillus subtilis.* Mikrobiologica, 29:109-117

Flores, ME, Perez, R and Huitron, C. 1997. ß-Xylosidase and xylanase characterization and production by *Streptomyces sp.* CH-M-1035. Lett. Appl. Microbiol., 24:410-416.

Gessesse, A, and Mamo, G. 1999. High level xylanase production by an alkalophilic *Bacillus sp.* by using solid state fermentation. Enz. Microb. Technol., 25: 68-72.

Heck, J, Flores, S, Hertzm, P and Ayub, M. 2005. Optimization of cellulose free xylanase activity by *Bacillus coagulans* BL69 in solid state cultivation. Proc. Biochem., 40:107-112.

Ikasri, I and Mitchell, DA. 1994. Protease production by *Rhizopus oligosporus* in solid state fermentation. Appl. Microbiol. Biotechnol., 10:320-324.

Kheng, PP. and Omar, IC. 2004. Xylanase production by a local fungal isolate *Aspergillus nigar* USM A11 via solid state fermentation using palm kernel cake (PKC) as substrate. Songkhanakarin I. Sci. Technol. 27: 325-336.

Mandels, M, Andreotti, R and Roche, C. 1976. Measurment of saccharifying cellulose, Biotechnol. Bioeng. Symp., 6:21-31.

Miller, GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal.Chem., 31:426-428.

Ninawe, S and Kuhad, RC. 2005. Use of xylan rich cost effective agroresidues in the production of xylanase by *Streptomyces Cyaneus* SN32, J. Appl. Micrbiol., 99:96631141-1148.

Oliveira, LA., Porto, ALF, and Tambourgi, EB. 2006. Production of xylanase and protease by Penicillium janthellum CRC 87 M-115 from different agricultural waste, Biores. Tech. 97:862-867

Raimbault, M and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. Eur. J. Appl. Microbiol. Biotechnol., 9:199-209.

Sanghi, A, Garg, N, Sharma, J, Kuhar, K Kuhad, RC. and Gupta, VK. 2008. Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtlis* ASH in solid state fermentation. World J. Microbiol. Biotechnol., 24:633-640.

Sindhu, I, Chhibber, S, Capalash, N and Sharma, P. 2006. Production of cellulose free xylanase from *Bacillus megaterium* by solid state fermentation for biobleaching of pulp. Curr. Microbiol., 853:167-172.

Wong, KKY, Larry Tan, UL and Saddler, JN**.** 1988. Multiplicity of β-1, 4 – xylanase in microorganisms : functions and applications. Microbiol. Rev., 52:305-317.