Comparative study of potential cellulolytic and xylanolytic bacteria isolated from compost and their optimization for industrial use

Nivedita Sharma[*], Preeti Buragohain, DivyaTandon[*], Richa Kaushal

Microbiology Research Laboratory, Department of Basic Sciences, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP) – 173 230 India

_____

Abstract

Compost a nutrient rich organic fertilizer and soil conditioner is a product of humification of organic matter. Compost formation is aided by combination of living organisms including bacteria, fungi and worms which transform lignocellulosic waste into humic like substances. Interest in microorganisms responsible for degrading compost has increased due to potential commercial applications which include biodegradation and production of their bioactive compounds such as enzymes. So the present study was carried out to isolate celluloytic and xylanolytic microorganisms from rural compost and their screening for potential hydrolytic microorganisms. In total, 49 isolates were isolated from compost, out of which 43 were bacteria. Among all the isolates, four bacterial isolates were selected for enzyme production studies depending upon their higher cellulase (CMCase, FPase and β-glucosidase) and xylanase producing capability. Out of selected isolates two were K_{22}, K_{23}, R_5 & N_{11} were identified as *B. licheniformis*, *B. sps.B.megaterium* and *B. subtilis* N_{11} respectively by physiological, biochemical and 16 S rRNA PCR technique.

Keywords: *Bacillus*, Cellulase, Xylanase, Fermentation

_____

1. Introduction

Production of industrial enzymes has been carried out extensively using microbial isolates, which exhibited higher productivity compared to the plants and animalsources. Enzymes are proteins and are considered as potential biocatalysts for a large number of reactions. Commercial applications of industrial enzymes, microorganisms are the most important source of various enzymes [54]. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such enzymes those are important for industrial utilization because of the possible economic benefits of being able to degrade plant residues at elevated temperatures [55].

Each organism or strain has its own special conditions for maximum enzyme production.

Hence, optimization of medium composition has to be carried out in order to maintain a balance among various medium components, thus minimizing the amount of non-utilized components at the end of fermentation.[35].

Composting is the biological conversion of solid organic waste into usable end products such as fertilizers, substrates for mushroom production and biogas. Moreover, their high organic matter content and biological activity make composts effective in a variety of applications, including erosion control, re-vegetation, biofiltration and bioremediation [11].

Cellulases have versatile applications in textile, laundry, pulpand paper, fruit juice extraction, and animal feed additives [9]. In addition, they find use in saccharification of lignocellulosicagroresidues to fermentable sugars which can be used for production of bioethanol, lactic acid, and single-cell protein [43-

_____

Corresponding author: *e-mail:* pniveditashaarma@yahoo.co.in/divya_mbg@yahoo.co.in

48]. Bacteria have been widely explored for cellulose production owing to their high growth rate, expression of multienzyme complexes, stability at extreme temperature and pH, lesser feedback inhibition, and ability to withstand variety of environmental stress.

There is a wide spectrum of microorganisms which can produce the variety of enzymes like cellulases, hemicellulases, ligninases, pectinases, esterases, oxidoreductases and proteases [4,6,7] under appropriate culture conditions. Although a large number of microorganisms can degrade cellulose, only a few them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose [53].

2. Materials

Isolation of cellulolytic and xylanolytic microorganisms from rural compost

Collection of compost samples. Compost samples were collected from different villages of Distt. Solan and Distt.Sirmour of Himachal Pradesh [http://cpcb.nic.in/divisionsofheadoffice/hwmd/lead.pdf.]

Enrichment of compost samples. Samples were pooled together and were enriched by addition of 2 % of cellulose powder to 100g of sample and water was sprinkled in the petriplate and mixed thoroughly. The petriplates were incubated at 28±2 °C for 3 days.

Isolation of cellulolytic and xylanolytic microorganisms. One gram of enriched compost sample was serially diluted from 10^{-2} to 10^{-8} times using sterilized 9 ml dilution blanks. The diluents (0.1 ml) were placed on the surface of Rivieres' medium . Rivieres', 1961 in a petriplate and evenly spreaded with the help of a spreader and the plates were incubated at 28±2°C for 5 days [33]. The pure line cultures were obtained by streaking method and preserved on the same agar medium at 4°C with periodic sub-culturing.

Morphological and PhysiologicalCharacteristics of isolates. The cellulolytic and xylanolytic microorganisms isolated from different compost samples were characterized on the basis of morphological, cultural and physiological characteristics. Isolates were classified into different bacteria accordingly.

Phenotypic characterization. The conventional morphological and biochemical tests as described by Aneja (2003) were performed which include [2]:

Morphological test. Morphological properties were investigated by using 24 h old culture of bacteria for observing cell morphology, Gram reaction, shape and culture conditions.

Physiological and Biochemical test.   The isolates were identified by the use of conventional methods for presumptive identification by biochemical tests. These were: catalase, urease, H_2S production, MRVP test, gelatin hydrolysis, fermentation of carbohydrates, citrate utilization, casein hydrolysis and indole test.

Screening of cellulolytic and xylanolytic microorganisms. The microbial isolates were screened for the production of extracellular enzymes i.e. cellulase - CMCase, FPase and β-glucosidase and xylanase.

Production of extracellular enzymes - cellulase and xylanase by microbial isolates. Each bacterial isolate was grown in 100 ml of nutrient broth at 35±2°C for 24 hrs. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 using autoclaved distilled water.

5 ml of inoculum was added to each 45 ml of Riviere's broth containing 1% cellulose for cellulase and 1% xylose for xylanase in 250 ml of Erlenmeyer flasks and the flasks were incubated for 5 days at 35±2°C [33]. After incubation, the culture contents were centrifuged at 10,000 rpm for 15 min (4°C). The supernatant was collected. The following quantitative tests were performed with the supernatant to screen out the hypercellulolytic and hyperxylanolytic producers among different isolates.

Enzyme assays. The sub enzymes of cellulase were measured by following standard assays. CMCaseandFPasewas determined by Reese & Mandel, 1963 [31], ß-Glucosidase assay [1,5]. Xylanase activity was measured according to Miller method [22].

Production and optimization of cellulase and xylanase under submerged fermentation

Various fermentation variables *viz.* medium, pH, temperature, inoculum size, substrate concentration and incubation time were studied to monitor their effect on cellulase and xylanase production.

1. Effect of different media

A. Optimization of cellulase

From K_{22} and K_{23} Five different medias viz. PYC Medium [17], Basal Salt Medium [20]. Modified Basal Salt medium. Okoshi *et al.*, medium [29] Li &Gao medium [17] were studied to monitor their effect on cellulase production.

Inoculum preparation. Each bacterial isolate was grown in 100 ml of nutrient broth and was incubated at $35\pm2°C$ for 24 hrs. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 using sterilized distilled water.

Production of extracellular cellulose. 5 ml of 1.0 O.D inoculum was added to each 45 ml of each different media in 250 ml of Erlenmeyer flasks and the flasks were incubated at $35\pm2°C$ under shaking conditions for 5 days.

After incubation, the culture contents were centrifuged at 10,000 rpm for 15 min (4°C).The supernatant was collected.The following quantitative tests were performed to find out the best media for cellulase production.

Enzyme assays. Cellulase activity. Endoglucanase activity (carboxymethylcellulase activity) in the culture supernatant was determined as described by Reese and Mandel, 1983. FPase activity (filter paper activity) was determined by the method of Reese and Mandel β-glucosidase was assayed by the procedure of Bergham and Petterson,1973. Units (IU) of endoglucanase and FPase were defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions. One unit of β-glucosidase was defined as the amount of enzyme liberating 1 micromole of p-nitrophenol per minute.

Estimation of reducing sugar and soluble protein. Reducing sugar was estimated as glucose by Miller method and soluble protein was measured by Lowry's method[19].

Percent reducing sugars released is the reducing sugars formed after biodegradation of lignocellulosic biomass. Percent proteins formed are the microbial biomass produced by utilizing simple sugars in the supernatant after biodegradation.

*Statistical Analysis.*All experiments were conducted in triplicates along with equal number of control.

The data obtained were subjected to analysis of variance technique using completely randomized block design (CRD) [14, 15].

Basal salt medium showing highest enzyme units was selected for the next experiment.

B Optimization of xylanase

From R_5 and N_{11} Five different medium viz Bacillus Xylose Salt Medium andXylan Medium [3], Basal Salt Medium [20], TGY Medium [13] and Emmerson Medium [13] werestudied to monitor the effect of xylanase.

Inoculum preparation: Sameas described in previous section.

Production of xylanase: Same as described in previous section.

Enzyme assay

 Xylanase activity [22]. Xylanase activity in the cultural filtrate was determined according to the method of Miller [22].One unit of xylanase activity is the amount of enzyme in 1 ml sample solution producing 1 μg/ml of xylose after incubation for 1 min under the given experimental conditions [22-25].

1.Protein estimation. The protein content was estimated in the culture supernatant according to Lowry's Method (1951) [19]. TGY medium showed maximum enzyme production for both *B. megaterium* R_5 and *B. subtilis*N_{11} and thus were selected for the next experiment.

2.Effect of pH. Effect of pH on enzyme production was examined at different pH ranging from5.0, 5.5,………., 8.5 and 9.0 for both cellulolytic and xylanolytic strains.

Cellulase production was highest at pH 6.5 and 7.5 for K_{22} and K_{23} respectively. Maximum enzyme production for R_5 and N_{11} were found to be 7.5. and 8.0 respectively and thus were selected for the next experiment.

3. Effect of temperature. The optimum temperature for cellulase and xylanase production was determined by incubating the cultures at different temperature ranges *viz.* 25°C, 30°C,….., 45°C and 50°C using optimized pH from the previous experiment and rest of the conditions were same.

Cellulase production was showed highest at temperature 35°C and 30°C for *B. licheniformis* K_{22} and *B.* sp. K_{23} respectivelywhile maximumxylanase production for R_5 and N_{11} were found to be at

temperatures 45°C and 40°C respectively and thus were selected for the next experiment.

4.Effect of inoculum size. Inoculum size was optimized for maximum cellulase and xylanase production by varying the sizes *viz.* 5%, 7.5%, 10%, 12.5% and 15% using optimized conditions of the previous experiments.

Inoculum size of 5 % and 7.5 % showed maximum cellulase production Based on maximum xylanase production at 10 % inoculum size from both R_5 and N_{11} this was selected for the next experiment.

5. Effect of incubation time. Optimization of incubation time for cellulase and xylanase production from K_{22} and $K_{23}$was done by varying the incubation days*viz.*1, 3, 5, 7 and 10 days keeping rest of the conditions optimized in the previous experiment.

Highest cellulase production was showed on day $7^{th}$and $5^{th}$from K_{22} and K_{23} respectively Maximum xylanase production was on day 7th for both R_5 and N_{11} and was selected for the next experiment.

Percent (%) increase in enzyme activity after optimization of different parameters

% increase in enzyme activity =

$$\frac{\text{Final enzyme activity} - \text{Initial enzyme activity}}{\text{Initial enzyme activity}} \times 100$$

Identification of cellulolytic and xylanolytic enzyme producing isolate

Among all the bacterial isolates K_{22}, K_{23}, N_{11} and R_5 were found to be the highest cellulase and xylanase producers respectively. These were tentatively identified as *Bacillus sp.* on the basis of their morphological, physiological and biochemical characteristics. Their molecular characterization using 16 s rRNA was performed for confirmed identification.

Molecular characterization using 16s rRNA PCR technique

Four isolates K_{22}, K_{23}, N_{11} and R_5 showing high enzyme production were identified at genomic level using 16S rRNA techniques.

Isolation of genomic DNA

The genomic DNA of the strain was isolated according to Genei DNA isolation kit.

PCR amplification of 16s rRNA region: PCR amplification was done to confirm the identity of the bacterial strains, the small sub-unit 16s rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGCTCAG3') and 16SR (5'TACCTTGTTACGACTT3') primers to get an amplicon size of 1500 bp. Amplifications were carried out in 20 µl reaction mixture consisting of 10 x buffer, 2.0 µl ; 2.0 µl; 2.0 mMdNTPs, 2.0 µl ; 3.0 U/µl Taq DNA polymerase, 0.2 µl ; 100 ng/µl of each primer, 1.0 µl ; template DNA, 1.0 µl and sterilized distilled water 12.8 µl in a Biorad (USA) thermalcycler using the PCR conditions 95°C for 2 min (denaturation), 53.8°C (Strain R_5), 52.3°C (Strain K_{22}) and 60.4°C (Strain N_{11}) for 1 min (annealing) and 72°C for 3 min (extension). The total number of cycles was 40, with the final extension of 72°C for 10 min. The amplified products (50 µl) were size separated on 1 % agarose gel prepared in 1% TAE buffer containing 0.5 µg/ml ethidiumbromide and photographed with the gel documentation system (Biorad, USA). A 100 bp DNA ladder (Genei) was used as molecular weight size markers.

Purification of the PCR product: The PCR product (1500 bp) was purified from contaminating products by electroelution of the gel slice containing the excised desired fragments with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µl of nuclease free water.

Nucleotide sequencing: Sequencing pattern − The PCR amplicons obtained by amplifying PCR products was diluted in Tris buffer (10 mM, pH 8.5). The dilution used was 1:1000 in order to obtain the DNA concentration required for sequencing (30 ng/µl), the sequencing reaction required 8 µl DNA. The primer used in all sequencing reactions was 16 SF (5'AGAGTTTGATCCTGGCTCAG3') at a concentration of 3 µM. Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA).

BLASTN Analysis: Translated nucleotide sequence was then analyzed for similarities by using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/).

3. Results and discussion

Isolation of cellulolytic and xylanolytic bacteria from compost: Bacteria isolated from compost showed a great variation in their morphological characteristics i.e. in color, texture and shape.

In total 43 bacteria were isolated and their morphological, physiological and biochemical properties were analyzed. The color of the colonies also exhibited a variation amongst the different strains. They varied from cream, pink, yellow, brown, white to off white. Gram staining of all these bacterial isolates differentiated them into gram +ve and gram-ve. Amongst these 43 isolates, 27 were found to be gram +ve and the rest 16 were found to be gram – ve. Out of 43 isolates 24 were rod shaped and 19 were round in shape.

Different biochemical tests were performed such as catalase test, H_2S production test, urease test, citrate utilization test, fermentation of carbohydrates, hydrolysis of gelatin, casein test and indole test for the characterization of all the 47 bacterial isolates. Out of 43 bacterial isolates, 28 showed positive catalase test and rest were negative. Urease test was negative for all the bacterial isolates. Out of 43 bacterial isolates, 21 showed positive H_2S production test, 23 for citrate utilization test, 27 for MRVP test and 10 showed positive tests for fermentation of carbohydrates. 11 strains showed positive test for casein hydrolysis. For gelatin hydrolysis, all strains showed positive test. All strains showed negative test for urease test. Depending upon their morphological, physiological and biochemical characteristics, these bacteria were tentatively identified as *Bacillus* spp. and *Coccus*spp.

Compost is a rich habitat of potential biodegrading microorganisms as it passes through a different succession of phases including drastic temperature variations to complete the process. The isolation of hypercellulolytic and hyperxylanolytic microorganisms has been done in the present study from the compost considering it a valuable niche of microorganisms.

Nik and Rosario (2010) [26] isolated two-hundred and twenty-five mangrove bacterial strains from several mangrove areas in the Philippines and were qualitatively screened for cellulase production and 154 were found to be capable of cellulase production. Among them, ten strains showed very strong positive cellulase activities and these were identified as *Cellulomonas* sp., *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus* sp.[26,28]

Krairitthichai and Thongwai (2007) [52] isolated one hundred and twenty-five isolates of bacteria from soils, decomposing logs and composts collected from the Northern part of Thailand. McCaig *et al*. (2001) [51] and Song *et al.* (2001) [42] isolated compost microorganisms which included *Bacillus* sp.*,* etc. Mayende *et. al*. (2006) [21] isolated thermophilic microorganisms from a compost system followed by screening for cellulase and polyphenol oxidase activity.

Interest in these microorganisms has been increased recently due to potential commercial applications, which include biodegradation and the production of bioactive compounds such as enzymes, biofuels and antibiotics.

Screening of cellulolytic and xylanolytic bacteria: The production of extracellular enzymes i.e. cellulase and xylanase from different bacterial isolates were investigated. Among them, K_{22} showed the highest total cellulase activity of 1.928 IU/ml (CMCase 0.783 IU/ml, FPase 0.223 IU/ml and 0.992 IU/ml β-glucosidase) followed by K_{23} showing enzyme activity of 1.753 IU/ml (CMCase 0.672 IU/ml, FPase 0.345 IU/ml and β-glucosidase 0.736 IU/ml) whereas K_2 showed the lowest cellulase activity i.e. 0.052 IU/ml. Maximum xylanase activity was exhibited by N_{11}- 12.590 IU/ml closely followed by R_5 - 12.189 IU/ml and the least xylanase activity was noted in K_{20} - 0.758 IU/ml.

Sajani *et al*. (2010) [34] isolated four bacterial colonies (*Bacillus* sp., two *Pseudomonas* sp.and *Proteus* sp*.*) using spread plate technique and were checked for cellulase production with the help of congo red and NaCl/NaOH. High level of cellulase production was achieved by *Bacillus* sp. (0.09 IU/ml and 0.08 IU/ml) for coir waste and saw dust as substrate. Tae Il *et al*. (2000) isolated a bacterium producing extracellular cellulase was isolated from cow dung compost and has been identified as *Bacillus* sp. When the isolate was cultured in CMC media at 37°C for 24 hrs, CMCase, FPase and Avicelase activity was 1.65 IU/ml, 0.13 IU/ml and 0.18 IU/ml whereas β-glucosidase activity was not detected. The optimum pH and temperature for enzyme induction was 7.5 and 50°C. The maximum CMCase activity was observed at pH 7.5 and 75°C.

Annamalai *et al.* (2009) [3] isolated a bacterium, *Bacillus subtilis* from the estuarine environment and was grown in shake flasks to derive the optimum culture conditions and also cultured in lab scale fermenter to obtain more xylanase. Maximum enzyme production (128 IU/ml) was recorded in stationary phase (36hrs) of the culture.

Production and optimization of cellulase and xylanase from hypercellulolytic and xylanolytic microorganisms under SmF

Different growth parameters like effect of media, pH, temperature, time of incubation and substrate concentration have drastic role to play in enzyme production. So in the present study these factors have been evaluated to enhance the production of cellulase and xylanase from hypercellulolytic and xylanolytic bacterial isolates.

Effect of different media: The effect of different media for cellulase and xylanase production was examined and it was found that the highest cellulase activity was found in the Basal salt medium yielding 3.314 IU/ml for *B. licheniformis* K_{22} and 3.435 IU/ml for *B.* sp. $K_{23.}$ Least enzyme production i.e. 2.136 and 2.317 IU/ml by *B.licheniformis* K_{22} and *B.* sp. K_{23} was on Okoshi *et al.* medium (Table 1) [29].

Whereas in case of xylanase, best medium was TGY medium yielding 22.647 and 17.984 IU/ml of xylanase activity for *B.megaterium* R_5 and *B.subtilis* N_{11} respectively which are significantly higher than others. Least enzyme production i.e. 7.327, 6.661 and 1.798 IU/ml by *B.megaterium* R_5 and *B.subtilis* N_{11} was on Xylan medium (Fig1).
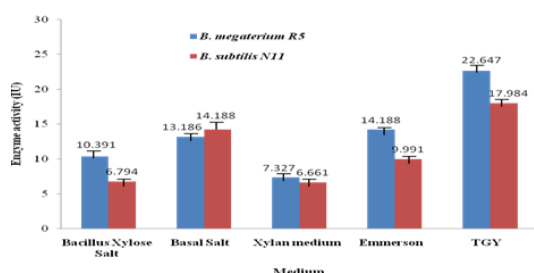

Figure 1. Effect of media on xylanase production from R5 and N11

In case of cellulase production, Basal salt medium has been found best for all hypercellulolytic microbes. Basal salt medium is having $(NH_4)_2SO_4$ as its major ingredient which seems to play a vital role in cellulase production from bacteria as well as fungi. Presence of tryptone in TGY medium (best medium for xylanase production by *B.megaterium* R_5 and *B. subtilis* N_{11}). Since nitrogen is a major constituent of protoplasm and building block of the enzymes, the above mentioned three nitrogen sources have helped directly in higher amount of enzyme synthesis.

Maximum cellulase productivity was exhibited when molasses broth medium was supplemented with cellulose as a carbon source and $(NH_4)_2SO_4$ as a nitrogen source [37]. Poorna & Prema (2007) [30] and Chellapandi & Himanshu (2008) [49] reported that maximum cellulase activity was obtained by *Bacillus pumilus, Ruminococcusalbus,* etc.,when ammonium sulphate was added to the production media. Dhillon and Khanna (2000) [10] reported that the best nitrogen source for xylanase production by *B. circulans*AB16 was tryptone and also for *Geobacillusthermoleovorans* [38]. Yeast extract, beef extract, peptone or soybean meal have been reported as most balanced and conditioned nitrogen sources in terms of accessibility and composition for getting high yield of xylanase from different bacteria [37].

Effect of pH: Table 2 and fig. 2 depicted effect of pH of medium on cellulase and xylanase production by $K_{22}$and *B.* sp. K_{23}, R_5 and N_{11} pH of the medium (already optimized) was varied between 5.0, 5.5, ………8.5 and 9.0 for hypercellulolytic and xylanolytic bacteria Significantly highest cellulase production from K_{22} was observed at pH 6.5 i.e. 4.151 IU/ml while it was maximum i.e. 3.565 IU/ml for *B.* sp. K_{23} at pH 7.5 which is significantly higher than others. Whereas least production – 1.886 and 2.997 IU/ml of cellulase were observed at pH 9 and 5 respectively for K_{22} and *B.* sp. K_{23}.
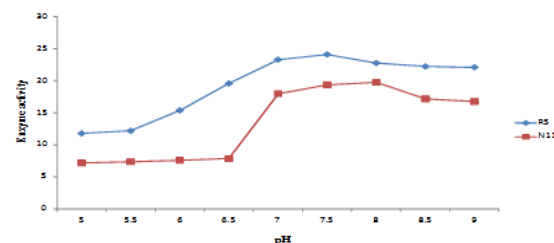

Figure 2. Effect of pH an xylanase production from R5 and N11

As far as xylanase production is concerned, R_5 and N_{11} have shown alkalophilicnature exhibiting maximum enzyme units at pH 7.5 i.e. 24.111 IU/ml and pH 8.0 i.e.19.782 IU/ml respectively which are significantly higher than others while least enzyme titres of 11.790 and 7.194 IU/ml respectively were observed at pH 5.0 for both of them.

Out of four hypercellulolytic and xylanolytic bacteria, majority has shown mild to moderate alkalophilic range for hydrolytic enzyme production (pH 7.5 to 8.0) except K_{22} (pH 6.5) which is one of the desirable attributes in industry.

pH is an   important parameter that influence enzyme activities and production [27]. The extracellular pH has a strong influence on the pathways of metabolism and product formation by microorganism. Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus lowering their overall metabolic activity [12].

Effect of temperature: Effect of temperature on cellulase and xylanase production from hypercellulolyticandxylanolytic bacteria has been shown in Tables 3. Maximum cellulasetitres were produced at 30°C from K_{23}(3.985 IU/ml) while K_{22} showed maximum cellulase units at 35°C (4.244 IU/ml) which are significantly higher to others. Least cellulase (3.571 and 3.485 IU/ml) production was observed for both K_{22} and K_{23} at 50°C respectively

Table 3 revealed data on effect of temperature on xylanase production bypotential bacteria. R_5 and N_{11} exhibited optimum temperature of 45°C (25.311 IU/ml), 40°C (25.300 IU/ml) respectively for xylanase production which are significantly higher than others. Minimum enzyme was produced at 25°C for both of bacterial isolates (2.598 and 6.394 IU/ml)

Optimum temperature range obtained for potential enzyme producing bacteria in the present study has reflected an inclination towards being thermpohilic in nature, thus suggesting to be an asset for commercial use.

Incubation temperature is a critical factor in enzymatic productivity [36]. Maximum enzyme production is produced at optimum temperature and the decrease in enzyme production at lower or higher temperatures is due to the fact thatat these temperatures, growth of the organisms were inhibited, causing a decrease in the synthesis of the enzymes [40].

Odeniyi *et al.* (2009) [27] reported that temperature for cellulase and polygalacturonase production in culture media by *Bacillus coagulans* was 37°C.

Effect of inoculum size: Effect of inoculum size on cellulase and xylanase production was evaluated in Table 4. Different inoculum size used were 5%, 7.5%,…………12.5% and 15%.

Optimum xylanase production from R_5 (25.645 IU/ml)andN_{11} (25.904 IU/ml) which are significantly higher than others. While least production for all them was observed at 15% i.e. 5.862, 17.651 and 14.862 IU/ml of xylanase for R_5 and N_{11} respectively. Optimum inoculum size for cellulase production from K_{22} and K_{23} was found to be 5% (4.360 IU/ml) and 7.5 % (4.621 IU/ml) respectively which are significantly higher than others and enzyme production was dipped down to 4.098 and 3.670 IU/ml for these spp. respectively.

Enzyme activity is maximum at optimal level because at this point an equilibrium is maintained between inoculum size and availability of substrates while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks.

With subsequent increase in inoculum size, competition for carbon source increases and results in rapid depletion of macro and micro nutrients and thus inhibit their growth and enzyme production [50].

Das *et al*. (2010) [9] examined inoculum size for cellulase production from *Bacillus* sp. isolated from cowdung compost and found that 7% inoculum size resulted in maximum enzyme activity 92.747 µgmg^{-1} min^{-1}

Effect of substrate concentration on cellulase and xylanase production: Table 5 showed the data on effect of substrate concentration for cellulase production using concentration range from 0.25%, 0.5%, ………… to 2.00% from hypercellulolytic bacteria. Optimum level of substrate concentration were found 0.75 % for K_{22} showing significantly higher enzyme activity of 5.897 IU/ml, 0.5 % for K_{23} (6.332 IU/ml).Least enzyme activity i.e. 4.004 and 2.701 IU/ml were measured at 2% for both of them respectively. Increase in substrate concentration level other than the optimum showed a consistent decrease in cellulase production.

Whereas effect of substrate concentration   on xylanase production from potential bacteria has been shown in fig. 3 and revealed that R_5 and N_{11} exhibited significantly higher xylanase activity at 2 % substrate concentration i.e. 65.943 and 72.537 IU/ml respectively Minimum xylanase production (3.863 and 9.325 IU/ml) was observed at 0.25% substrate concentration for all of hyperxylanolytic bacteria.
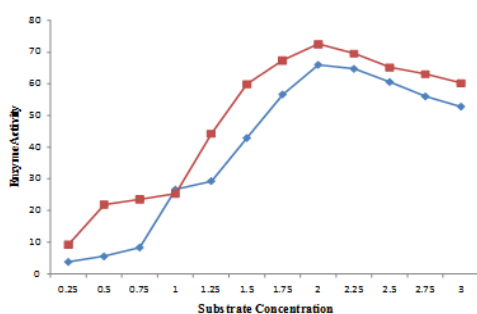
Figure 3. Effect of substrat concentration on xylanase production from R5 and N11.

Substrate concentration is an influencing factor that affects the yield and initial rate of hydrolysis of cellulose [8]. Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis [32]. However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme production [18,41].

Effect of incubation time on cellulase and xylanase production: Effect of incubation time on cellulase and xylanase production was evaluated in Table 6 and figure 4. Enzyme activity was measured at regular intervals for a period of 10 days *viz.* 1st, 3rd, 5th, 7th and 10th. Highest cellulase activity was produced on 7th day (5.992 IU/ml) and 5th day(6.356 IU/ml) from $K_{22}$and K_{23} respectively which are significantly higher than others followed by a gradual decline with further increase in incubation time.

Hyperxylanolyticisolates*viz.* R_5, N_{11} exhibited an increasing pattern of enzyme production with increase in incubation time showing highest xylanase activity on day 7th yielding 70.766 and 75.320 IU/ml respectively which are significantly higher than others and then resulted in a considerable decrease with further increase in incubation time. While minimum enzyme titres were produced on 1st day of incubation (50.155 and 56.655 IU/ml) from all of them.
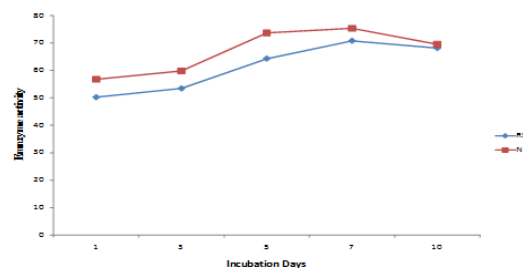
Figure 4. Effect on incubation days on xylanase production from R5 and N11

Optimum incubation time for enzyme production was found to be 7 days for all the potential bacterial except for K_{23}. A decline afterwards might be due to the depletion of macro and micro nutrients in the fermentation medium alongwith course of time, which stressed the microbial physiology resulting in the inactivation of secretary machinery of the enzymes [40].

There was a considerable increase in cellulase and xylanase production escalating up to 587.670 % was observed after optimizationof the different growth parameters. In case ofcellulase, highest increase of 169.553 % and 181.346 %K_{22} and K_{23} respectively. Much higher % increase was shown in case of xylanase production by all the three hyperxylanolytic microbes used in the study. R_5 and N_{11} showed an increase of 428.997 % and 464.512 % in extracellular enzyme level.

Table 1. Effect of media on cellulase production from K_{22} and K_{23}

Media	K_{22}					K_{23}				
	Protein conc. (mg/ml)	CMCase activity	FPase activity	β-glucosidase activity	Total enzyme activity	Protein concn. (mg/ml)	CMCase activity	FPase activity	β-glucosidase acitvity	Total enzyme activity
PYC Medium	0.780	1.104* (1.415)**	1.029* (1.319)**	1.147* (1.471)**	3.280* (4.205)**	0.600	1.104* (1.840)**	1.024* (1.707)**	1.261* (2.100)**	3.389* (5.640)**
Basal Salt Medium	0.900	1.193 (1.326)	1.024 (1.138)	1.097 (1.219)	3.314 (3.682)	0.760	1.263 (1.662))	1.029 (1.354)	1.143 (1.504)	3.435 (5.830)
Modified BSM	1.200	1.222 (1.018)	1.038 (0.865)	0.000 (0.000)	2.260 (1.883)	0.160	1.104 (6.900)	1.031 (6.444)	1.138 (7.113)	3.273 (20.456)
Okoshiet al	1.760	1.096 (0.623)	1.040 (0.591)	0.000 (0.000)	2.136 (1.214)	0.760	1.259 (1.657)	1.058 (1.392)	0.000 (0.000)	2.317 (3.040)
Li &Gao	0.290	1.089 (3.755)	0.000 (0.000)	1.460 (5.034)	2.549 (8.790)	0.530	1.222 (2.306)	0.988 (1.864)	1.085 (2.047)	3.295 (6.217)
$CD_{0.05}$	0.207	0.004 (0.582)	0.003 (0.194)	0.003 (0.745)	0.010 (1.380)	0.177	0.004 (1.236)	0.004 (1.150)	0.003 (1.293)	0.010 (3.682)
S.E.(Mean)	0.093	0.002 (0.261)	0.001 (0.087)	0.001 (0.334)	0.004 (0.620)	0.079	0.002 (0.555)	0.002 (0.516)	0.001 (0.583)	0.005 (1.653)

* Enzyme activity (IU) ; µmoles of reducing sugars released / min / ml of enzyme

**Values in parentheses depict specific activity i.e. enzyme activity / mg of protein

Table 2. Effect of pH on cellulase production from K_{22} and K_{23}

pH	K_{22}					K_{23}				
	Protein conc. (mg/ml)	CMCase activity	FPase activity	β-glucosidase activity	Total enzyme activity	Protein concn. (mg/ml)	CMCase activity	FPase activity	β -glucosidase activity	Total enzyme activity
5.0	0.550	1.148* (2.087)**	0.764* (1.385)**	0.998* (1.815)**	2.908* (5.287)**	0.470	0.990* (2.106)**	0.876* (1.864)**	1.131* (2.406)**	2.997* (6.377)**
5.5	0.600	1.215 (2.025)	0.877 (1.462)	1.235 (2.058)	3.327 (5.545)	0.500	1.089 (2.178)	0.899 (1.798)	1.157 (2.314)	3.145 (6.290)
6.0	0.580	1.133 (1.953)	0.912 (1.572)	1.209 (2.084)	3.254 (5.610	0.510	1.096 (2.149)	1.027 (2.014)	1.235 (2.422)	3.358 (6.584)
6.5	0.580	1.156 (1.993)	1.029 (1.774)	1.966 (3.390)	4.151 (7.157)	0.580	1.111 (1.916)	1.029 (1.774)	1.238 (2.134)	3.378 (5.824)
7.0	0.570	1.148 (2.014)	1.027 (1.802)	1.209 (2.121)	3.384 (5.937)	0.500	1.112 (2.224)	1.027 (2.054)	1.392 2.784)	3.431 (6.862)
7.5	0.600	1.111 (1.852)	1.027 (1.712)	1.105 (1.842)	3.243 (5.405)	0.500	1.090 (2.180)	1.031 (2.062)	1.444 (2.888)	3.565 (7.130)
8.0	0.550	1.096 (1.993)	0.823 (1.496)	1.470 (2.673)	3.389 (6.162)	0.370	1.089 (2.943)	1.029 (2.781)	1.418 (3.832)	3.536 (9.557)
8.5	0.470	1.089 (2.317)	0.712 (1.515)	1.026 (2.183)	2.827 (6.015)	0.510	1.086 (2.129)	1.012 (1.984)	1.366 (2.678)	3.464 (6.792)
9.0	0.214	0.989 (4.621)	0.000 (0.000)	0.897 (4.192)	1.886 (8.813)	0.500	0.993 (1.986)	1.009 (2.018)	1.261 (2.522)	3.263 (6.526)
$CD_{0.05}$	0.043	0.003 (0.148)	0.003 (0.117)	0.003 (0.187)	0.010 (0.449)	0.063	0.003 (0.313)	0.003 (0.290)	0.003 (0.387)	0.010 (0.989)
S.E.(Mean)	0.021	0.002 (0.070)	0.002 (0.056)	0.002 (0.089)	0.005 (0.213	0.030	0.002 (0.149)	0.002 (0.138)	0.002 (0.184)	0.005 (0.471)

*

**

Table 3. Effect of temperature on cellulaseproductionfromK$_{22}$andK$_{23}$

Temperature (°C)	K$_{22}$					K$_{23}$				
	Protein conc. (mg/ml)	CMCaseacxivity	FPaseactivt y	β - glucosidase activity	Total enzyme activity	Protein conc. (mg/ml)	CMCaseacxivit y	FPase activity	β - glucosidase activity	Total enzyme activity
25	0.160	1.193* (7.456)**	1.029* (6.431)**	1.366* (8.538)**	3.588* (22.425)**	0.200	1.163* (5.815)**	1.031* (5.155)**	1.523* (7.615)**	3.717* (18.585)**
30	0.180	1.326 (7.367)	1.031 (5.728)	1.366 (7.589)	3.723 (20.683)	0.290	1.148 (3.959)	1.027 (3.541)	1.810 (6.241)	3.985 (13.741)
35	0.260	1.259 (4.842)	1.031 (3.965)	1.954 (7.515)	4.244 (16.323)	0.260	1.281 (4.927)	1.027 (3.950)	1.597 (6.142)	3.905 (15.019)
40	0.240	1.378 (5.742)	1.031 (4.296)	1.339 (5.579)	3.748 (15.617)	0.170	1.193 (7.018)	1.036 (6.094)	1.366 (8.035)	3.595 (21.147)
45	0.180	1.222 (6.789)	1.027 (5.706)	1.654 (9.189)	3.903 (21.683)	0.178	1.193 (6.702)	1.027 (5.770)	1.340 (7.528)	3.560 (20.000)
50	0.200	1.252 (6.260)	1.031 (5.155)	1.288 (6.440)	3.571 (17.855)	0.160	1.193 (7.456)	1.031 (6.444)	1.261 (7.881)	3.485 (21.781)
CD$_{0.05}$	0.004	0.004 (0.104)	0.004 (0.083)	0.004 (0.125)	0.011 (0.312)	0.004	0.004 (0.101)	0.004 (0.085)	0.004 (0.123)	0.011 (0.307)
S.E. (Mean)	0.002	0.002 (0.048)	0.002 (0.038)	0.002 (0.057)	0.005 (0.143)	0.002	0.002 (0.085)	0.002 (0.039)	0.002 (0.056)	0.005 (0.141)

*
**

Table 4. Effectof inoculum size on cellulaseproduction fromK$_{22}$ and.K$_{23}$

Inoculum size (%)	K$_{22}$					K$_{23}$				
	Protein conc. (mg/ml)	CMCase activity	FPase activity	β - glucosidase acitvity	Total enzyme activity	Protein concn. (mg/ml)	CMCase activity	FPase activity	β - glucosidase acitvity	Total enzyme activity
5	0.900	1.244* (1.382)**	1.044* (1.160)**	2.072* (2.302)**	4.360* (4.844)**	0.660	1.244* (1.885)**	1.047* (1.586)**	2.176* (3.297)**	4.467* (6.768)**
7.5	0.720	1.241 (1.724)	1.047 (1.454)	1.810 (2.514)	4.098 (5.692)	1.340	1.284 (0.958)	1.056 (0.778)	2.281 (1.702)	4.621 (3.449)
10	0.720	1.237 (1.718)	1.038 (1.442)	1.706 (2.369)	4.213 (5.851)	1.180	1.230 (1.042)	1.038 (0.880)	1.706 (1.446)	3.974 (3.368)
12.5	0.660	1.200 (1.818)	1.038 (1.573)	2.019 (3.059)	4.257 (6.450)	0.660	1.178 (1.785)	1.036 (1.570)	1.915 (2.902)	4.129 (6.256)
15	0.670	1.170 (1.746)	1.036 (1.546)	1.967 (2.936)	4.173 (6.228)	0.590	1.118 (1.895)	1.033 (1.751)	1.519 (2.575)	3.670 (6.220)
CD$_{0.05}$	0.004	0.004 (0.004)	0.004 (0.002)	0.004 (0.009)	0.011 (0.014)	0.004	0.004 (0.004)	0.004 (0.003)	0.004 (0.008)	0.010 (0.015)
S.E. Mean)	0.002	0.002 (0.002)	0.002 (0.001)	0.002 (0.004)	0.005 (0.006)	0.002	0.002 (0.002)	0.002 (0.001)	0.002 (0.004)	0.005 (0.007)

*
**

Table 5. Effect of substrate concentration on cellulaseproduction fromK_{22} and K_{23}

Substrate conc. (%)	K_{22}					K_{23}				
	Proteinconc. (mg/ml)	CMCase activity	FPase activity	β - glucosidase Acitvity	Total enzyme activity	Proteinconcn. (mg/ml)	CMCase activity	FPase activity	β - glucosidase Acitvity	Total enzyme activity
0.25	0.460	1.333* (2.898)**	1.038* (2.257)**	1.936* (4.209)**	4.307* (9.363)**	0.540	1.141* (2.113)**	1.033* (1.913)**	1.119* (2.072)**	3.293* (6.098)**
0.50	1.000	1.289 (1.289)	1.033 (1.033)	2.145 (2.145)	4.467 (4.467)	0.660	1.526 (2.312)	1.038 (1.573)	3.768 (5.709)	6.332 (9.594)
0.75	0.750	1.898 (2.531)	1.034 (1.379)	2.965 (3.953)	5.897 (7.863)	0.700	1.112 (1.589)	1.038 (1.483)	1.980 (2.829)	4.930 (6.573)
1.00	0.740	1.274 (1.722)	1.039 (1.404)	1.998 (2.700)	4.311 (5.826)	0.560	1.141 (2.0380	1.997 (3.566)	1.910 (3.411)	4.548 (6.146)
1.25	0.980	1.274 (1.300)	1.036 (1.057)	1.761 (1.835)	4.108 (4.192)	0.570	1.139 (1.998)	1.987 (3.486)	1.915 (3.360)	4.041 (8.844)
1.50	0.700	1.289 (1.841)	1.030 (1.471)	1.784 (2.549)	4.103 (5.861)	0.540	1.141 (2.113)	1.657 (3.069)	1.436 (2.659)	3.234 (7.841)
1.75	0.790	1.259 (1.594)	1.036 (1.311)	1.701 (2.229)	4.056 (5.134)	0.590	1.126 (1.908)	1.650 (2.797)	0.981 (1.663)	2.757 (6.368)
2.00	0.740	1.265 (1.709)	1.038 (1.403)	1.798 (2.299)	4.004 (5.411)	0.530	0.141 (2.153)	1.876 (3.540)	1.684 (3.177)	2.701 (8.870)
$CD_{0.05}$	0.003	0.003 (0.006)	0.003 (0.004)	0.003 (0.010)	0.010 (0.020)	0.003	0.003 (0.006)	0.003 (0.004)	0.003 (0.012)	0.010 (0.020)
S.E. (Mean)	0.002	0.002 (0.003)	0.002 (0.002)	0.002 (0.005)	0.005 (0.010)	0.002	0.002 (0.003)	0.002 (0.002)	0.002 (0.006)	0.005 (0.009)

*
**

Table 6. Effect of incubation time on cellulase production fromK_{22}andK_{23}

Incubation time (Days)	K_{22}					K_{23}				
	Proteinconc. (mg/ml)	CMCase activity	FPase activity	β - glucosidase Acitvity	Total enzyme activity	Proteinconcn. (mg/ml)	CMCase activity	FPase activity	β - glucosidase Acitvity	Total enzyme activity
1st	0.786	1.156* (1.471)**	1.030* (1.310)**	2.568* (3.267)**	4.754* (6.048)**	0.823	1.141* (1.386)**	1.030* (1.252)**	2.673* (3.248)**	4.844* (5.886)**
3rd	0.864	1.156 (1.338)	1.031 (1.193)	2.961 (3.427)	5.148 (5.958)	0.892	1.222 (1.370)	1.031 (1.156)	3.630 (4.070)	5.883 (6.505)
5th	0.998	1.222 (1.224)	1.031 (1.033)	3.614 (3.621)	5.867 (5.879)	0.986	1.444 (1.465)	1.098 (1.114)	3.814 (3.868)	6.356 (6.446)
7th	1.120	1.237 (1.104)	1.036 (0.925)	3.719 (3.321)	5.992 (5.350)	0.912	1.267 (1.389)	1.031 (1.130)	2.778 (3.046)	5.076 (5.566)
10th	1.100	1.163 (1.057)	1.031 (0.937)	3.509 (3.190)	5.703 (5.185)	0.872	1.222 (1.401)	1.031 (1.182)	2.751 (3.155)	5.004 (5.739)
$CD_{0.05}$	0.004	0.005 (0.007)	0.007 (0.005)	0.005 (0.005)	0.006 (0.006)	0.005	0.005 (0.006)	0.007 (0.007)	0.006 (0.007)	0.007 (0.006)
S.E. (Mean)	0.001	0.002 (0.002)	0.002 (0.002)	0.002 (0.002)	0.002 (0.002)	0.002	0.001 (0.002)	0.002 (0.002)	0.002 (0.002)	0.002 (0.002)

*
**

Identification of screened bacteria: The identification of screened bacteria was done at molecular level by using 16S rRNA PCR technique and these were identified as *Bacillus licheniformis* K_{22}, *Bacillus* sp. K_{23}, *Bacillus megaterium* R_5 and *Bacillus subtilis* N_{11} respectively. Genomic DNA was isolated from all the four strains by using protocol of Genei which resulted in a decent amount of DNA, good quality and quantity.

The isolated genomic DNA (50-100 ng) was used in PCR to amplify small units of 16S rRNA using specific primers (16SF and 16SR). Expected size (in 1500 bp) amplification product in both the isolates was obtained in PCR. The PCR product (1500 bp) was purified from contaminating products by electroelution of the gel slice containing the excised desired fragment with Qiaquick gel extraction kit. The elution was carried out in 300 µl of nuclease free water. The purified and amplified gel fraction were

used for sequencing and sequencing was performed in ABI automated sequencer using 16SF primer. Finally these isolates i.e. K_{22}, K_{23}, R_5 and N_{11} were identified as *Bacillus licheniformis* K_{22}, *Bacillus* sp. K_{23}, *Bacillus megaterium* R_5 and *Bacillus subtilis* N_{11} respectively using BLASTN analysis (Plate 1- a, b).
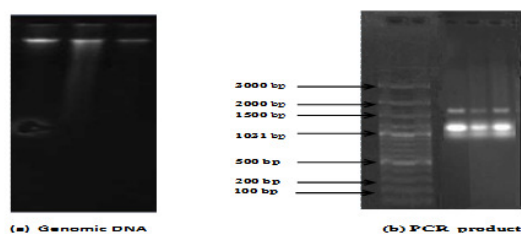

Plate 1. Isolation of genomic DNA b Molecular characterization using 16s rRNA technique

4. Conclusion

The significant increase in cellulase and xylanasetitres is a major achievement as it was one of the main objectives of the present study. The relevant increase in extracellular cellulase and xylanase from hypercellulolytic and xylanolytic bacteria was because of providing best congenial conditions i.e. nutrients, pH, temperature, time, concentration of substrate, etc. for them.

Acknowledgements

Compliance with Ethics Requirements

Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

References

1. Amouri, B; Gargouri, A; .Characterization of a novel β-glucosidase from *Stachybotrys*strain, *Biochemical Engineering,* **2004**, *32*, 191-197

2. Aneja K R. 2003. Experiments in Microbiology, Plant pathology and Biotechnology, 4th rev. ed. New Age International Limited Publishers. New Delhi. 607 p.

3. Annamalai, N;Thavasi, Jayalakshmi S., and Balasubramanian,Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment, *Indian Journal of Biotechnology,* **2009**, *8*, 291-297

4. Aslam, N., Sheikh, M A.,Asraf M., and Jamil A; Expression pattern of *Trichoderma*cellulases under different carbon sources,*Pakistan Journal of Botany* **2009**, *42*, 2895-2902.

5. Berghem L E R., and Pettersson L G., Mechanism of enzymatic cellulose degradation and purification of a cellulolytic enzyme from *T. viride* active on highly ordered cellulose, *Journal of Biochemistry,* **1973**, *37*, 21-30.

6. Chandra, M.,Kalra, A., Sharma, P K.; Kumar, H and Sangwan, R S; Optimization of cellulaseproduction by *Trichodermacitrinoviride*on marc of *Artemisia annua* and its application for bioconversion process. *Biomass and Bioenergy,* **2010**, *34*, 805-811.

7. Chidi S B., Godana B., Ncube I., Rensburg E J V.,Cronshaw A., and Abotsi E K.., Production, purification and characterization of cellulase-free-xylanase from *Aspergillusniger*UL4209; *African Journal of Biotechnology,* **2008**, *7*, 3939-3948

8. Da, Silva R., Lago, E S., Merheb, C W., Macchione, M M., Park, Y K., and Gomes E; Production of xylanase and CMCase on solid state fermentation in different residues by *Thermococcusaurantiacusmiehe*. *Brazilian Journal of Microbiology,* **2005**, *36*, 235-241

9. Das A., Bhattacharya S., and MuraliL.Production of cellulase from a thermophilic *Bacillus* sp. isolated from cowdung, *American-Eurasian Journal of Agriculture and Environmental Sciences,* **2010**,*.8*, 685-691

10. Dhillon, A., and Khanna, S., Production of a thermostable alkali-tolerant xylanase from *Bacillus circulans* AB16 grown on wheat straw. *World Journal of Microbiology & Biotechnology* **2000**, *16*, 325-327

11. Farinas, C S., Loyo, M M., Junior, A B.,Tardioli, P W.,Neto V B., and Couri S; Finding stable cellulase and xylanase : evaluation of the synergistic effect of pH and temperature, *New Biotechnology,* **2010**, *27*, 810-815.

12. Frazier W C. 1958. Food Microbiology (McGraw Hill Book Company. Inc. New York)472 p.

13. Garg, S.,Al,i R., and Kumar A; Production of alkaline xylanaseby an alkalo-thermophilic bacteria, *Bacillus halodurans*, MTCC-9512 isolated from dung, *Current trends in Biotechnology and Pharmacy.* **2009**, *3*, 90-96

14. Gomez K A., and Gomez AA., Statistical procedures for agricultural research, 2ndedn, John Wiley and Sons, 1976 Singapore.

15. Khan, A.,Ikram-Ul-Haq.,But,t W A., and Ali, S;Isolation and screening of *Aspergillusniger*isolates for xylanase biosynthesis, *Biotechnology*, **2000**, *2*, 185-190

16. Kim, J., Hur, S., and Hong, Purification and characterization by alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810. *BiotechnologyLett.ers,* **2005**, *27*, 313-316

17. Li X., and Gao P; CMC-liquefying enzyme, a low molecular mass initial cellulose-decomposing cellulase responsible for fragmentation from *Streptomyces* sp. LX, *Applied Microbiology* **2010**, *83*, 59-66

18. Liu, J., and Yang, Cellulase production by *Trichodermakoningii* A83.4262 in solid state fermentation using lignocellulosicwaste from the vinegar industry,*Food Technology & Biotechnology* **2007**, *45*, 420-425.

19. Lowry, O H.,Rosebrough, N J., Farr,, A L., and Randall R J., Protein measurement with the folin phenol reagent,*Journal of Biology & Chemical Sciences* **1951**,*193*, 265-275

20. Maniatis, T., Fritsch, E F., and Sambrock, J; Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory. 1982 New York

21. Mayende, L.,Wilhelmi B S., and Pletschke B I., Cellulases (CMCases) and polyphenol oxidases from thermophilic*Bacillus* spp. isolated from compost. *Soil Biology &Biochemistry,* **2006**, *38*, 2963-2966

22. Miller G L. Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry,* **1959**, *31*, 426-428

23. Miranda,M, Kam T L and Wensheng, Q., Prospects of cellulase producing bacteria for the bioconversion of lignocellulosic biomass., *International Journal of Biological Sciences,* **2009**, *5*, 500-516

24. Myat,Thu.,Mya, M O.,Myo M., and Saw S M; Screening on cellulase enzyme activity of *Aspergillusniger* strain on cellulosic biomass for bioethanol production. GMSARN 2008, *International Conference on Sustainable Development* : Issues and Prospects for the GMS.

25. Nair, S G.,Sindhu, R., and Shashidhar, S., Fungal xylanase production under solid state and submerged fermentation conditions, *African Journal of Microbiology,* **2008**, *2*, 82-86

26. Nik, S C T., and Rosario, G M; .Screening and optimization of cellulase production of *Bacillus* strains isolated from Philippine Mangroves,, *Philipines Journal of Systemic Biology,* **2010**, *4*, 79-87

27. Odeniyi, OA; Onilude AA., and Ayodele, M; Production characteristics and properties of cellulase/polygalacturonase by a *Bacillus coagulans* strain from a fermenting palm-fruit industrial residue, *African Journal of Microbiology Research,* **2009**, *3*, 407-417

28. Okafor, U A.,Okachi, V I., Oxyegen-okerenta B M.,Nwodo-Chinedu S. Xylanase production by *Penicillium chrysogenum* (PCL 501) fermented on cellulosic wastes, *African J of Biochem Res.,* **2007**, *1*, 48-53.

29. Okoshi, H., Ozaki, K., Shikate, S.,Oshoni, Kawai S., and Ito S.., Purification and characterization of multiple carboxymethylcellulases from *Bacillus* sp. KSM-522, *Agricultural Biological Chemistry,* **1990**, *54*, 83-89.

30. Poorna, CA; and Prema, P, Production of cellulase-free xylanase from novel alkalophilicthermostable *Bacillus pumilus*by solid state fermentation and its application in waste paper recycling, *Bioresouce Technology*, **2007**, *98*, 485-490

31. Reese, E T., and Mendels, M. Enzymatic hydrolysis of cellulose and its derivatives *In*: Methods Carbohydrate Chemistry (ed. Whistler R L) 3rdedn., Academic Press, London, 1963, 139-143

32. Regina, M., Broetto, F.,Giovannozzi-Sermanni., Marabatini R., and Peranni, C., Influence of stationary and bioreactor cultivation on *Lentinulaedodes*(bark) Pegler lignocellulolytic activity,*Brazilian Archieves of Biology&Technology,* **2008**, *51*, 223-233

33. Riviere J., Isolation of purification des bacteries cellulolytiques aerobies du sol, *Annales de L'institut Pasteur*, **1961**, 253-258

34. Sajani, S., Muthukkaruppan, S M., Gayatri, S N and Senthil, K P K.; Cellulase production by *Bacillus* spp. and *Aspergillusniger* using coir waste and saw dust and partial purification. *International Journal of Current Research,* **2010**, *10*, 31-34

35. Beg, QK; Kapoor, M., Mahajan, L., Hoonda,l GS. Microbial xylanases and their industrial applications: A review *Applied Microbiology and Biotechnology*, **2001**, *56*, 326-338

36. Seyis,I; and Aksoz, N; Determination of some physiological factors affecting xylanase production from *Trichodermaharzianum* 1073-d3, *Microbiology* **2003**, *26***,** 75-81

37. Shabeb, M S A., Younis, M A M.,Hezayen, FF., and Nour-Eldein, M A.Production of cellulase in low-cost medium by *Bacillus subtilis* KO strain, *World Applied Science* **2010**, *8*, 35-42

38. Sharma, A., Adahikari, S., and Satyanarayana, T. Alkali-thermostable and cellulase-free xylanase production by an extreme thermophile *Geobacillusthermoleovorans*. *World Journal of Microbiology Biotechnology* **2007**, *2*, 483-490

39. Sharma, N.,Bansal, K L., and Neopaney B. Comparison of forest waste degradation under solid state fermentation and submerged fermentation by co-culture of *Bacillus coagulans* and *Bacillus licheniformis*, *Asian Journal of Microbiology Biotechnology and Environmental Sciences* **2005**, 7, 51-54

40. Simoes, M L G.,Tauk-Tornisielo, S M., and Tapia, D M T; Screening of culture condition for xylanase production by filamentous fungi, *Afrcian Journal of Biotechnology* **2009**, *8*, 6317-6326

41. Singhania R R., Sukumaran R K., Patel A K.,Larroche C., and Pandey A., .Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases, *Enzyme and Microbial Technology* **2010**, *46*, 541-549

42. Song, J., Weon, H Y., Yuan, S H., Park, D S., Go, S J., and Suh, T W.; Phylogenetic diversity of thermophilicactinomycetes and *Thermoactinomyces* spp. isolated from mushroom composts in Korea based on 16s rRNA gene sequence analysis, *Microbiology Letters*, **2001**, 97-102.

43. Tae-II, K.; Han, J D., Jeon, B S., Yang,C B., Kim, K N., and Kim, M K; Isolation from cattle manure and characterization of *Bacillus licheniformis*NLRI-X33 secreting cellulase, *Asian-Austral Journal of Animal Science,* **2000***, 13*, 427-431

44. Pérez, J., Munoz-Dorado, J., De la,Rubia T., and Martinez, Biodegradationand biological treatments of cellulose, hemicellulose, and lignin: an overview, *International Journal Of Microbiology,* **2002***, 5*, 53-63

45. Lee, S M.,& Koo, Y M; Pilot-scale production of cellulose using *Trichodermareesei* Rut C-30 in fed-batch mode, *Journal of Microbiology Biotechnology,* **2001**, *11*, 229-233

46. Jaradat, Z; Dawagreh, A., Ababneh, Q;andSaadoun, I. 2008. Influence of culture conditions on cellulase production by *Streptomyces* sp. (Strain J2), *Jordan Journal of Biological Sciences,* **2008**, *1*, 141-146

47. Nath, D; Rao, M, pH dependent conformational and structuralchanges of xylanase from an alkalophilicthermophilic *Bacillus* sp (NCIM 59) *Enzyme Microbial Technology*, **2001**, *28*, 397-403

48. Sanchez, OJ., and C. A, Cardona,Trends in biotechnological production of fuel ethanol from different feedstocks, *Bioresource Technology,* **2008**, *99*, 5270–5295

49. Chellapandi P.; Himanshu M. Jani, Production of endoglucanase by the native strains of *Streptomyces* isolates in submerged fermentation, *Brazilian Journal of Microbiology*, **2008**, *39*, 122-127

50. Omojasola, P.F., O.P. Jilani and S.A. Ibiyemi, Cellulase production by some fungi cultured on pineapple waste. *Nature Sci.*, **2008**, *6*, 64-69.

51. Allison E. McCaig[*], L. Anne Glover, James I. Prosser, Numerical Analysis of Grassland Bacterial Community Structure under Different Land Management Regimens by Using 16S Ribosomal DNA Sequence Data and Denaturing Gradient Gel Electrophoresis Banding Patterns, *Appl. Environ. Microbiol*. October **2001**, *67*(10) 4554-4559, doi: 10.1128/AEM.67.10.4554-4559.2001

52. S. Krairitthichai and N. Thongwai, The 34 th Congress on Science and Technology of Thailand (STT34), Thailand (2008).

53. Koomnok, C., Selection of cellulose producing thermophilic fungi, *31ˢ Congress on Science and Technology of Thailand*, 2005

54. Ibrahim CO, Development of applications of industrial enzymes from Malaysian indigenous microbial sources. *Bioresour. Technol*. **2008**, *99*, 4572-4582.

55. Haki GD, Rakshit SK., Developments in industrially important thermostable enzymes: A review., *Bioresour Technol*. **2003**, *89*(1), 17-34.