ISOLATION OF CELLULOLYTIC BACTERIA FROM SOIL AND VALORIZATION OF DIFFERENT LIGNOCELLULOSIC WASTES FOR CELLULASE PRODUCTION BY SUBMERGED FERMENTATION

MISBAH GHAZANFAR,[*] MUHAMMAD IRFAN,[*] MUHAMMAD NADEEM,[**] HAFIZ ABDULLAH SHAKIR,[***] MUHAMMAD KHAN,[***] SHAUKAT ALI,[****] SHAGUFTA SAEED[*****] and TAHIR MEHMOOD[*****]

[*]*Department of Biotechnology, University of Sargodha, Sargodha, Pakistan*
[**]*Food and Biotechnology Research Center, PCSIR Laboratories Complex, Ferozpur Road, Lahore, Pakistan*
[***]*Department of Zoology, University of Punjab, New Campus, Lahore, Pakistan*
[****]*Department of Zoology, Government College University, Lahore, Pakistan*
[*****]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
✉ *Corresponding author: M. Irfan, irfan.ashraf@uos.edu.pk, irfan.biotechnologist@gmail.com*

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Cellulases are known to convert cellulose into monomeric or dimeric structures, hence playing an important role in bioethanol production, along with their applications in textile and paper industries. This study was directed towards the isolation and screening of cellulase producing bacteria from different soil samples on CMC (carboxymethyl cellulose) agar plates, followed by Gram's iodine staining. Six strains showed clear zones of hydrolysis on CMC agar plates. Isolates were identified as *Bacillus megaterium*, *Pseudomonas stutzeri*, *Bacillus aerius*, *Bacillus paralichniformis*, *Bacillus flexus*, and *Bacillus wiedmanni* by 16S rRNA gene sequencing. These strains were cultivated by submerged fermentation for cellulase production using various lignocellulosic wastes, such as corn cob, rice husk, wheat straw, seed pods of *Bombax ceiba* and eucalyptus leaves. Results showed that *Pseudomonas stutzeri* is the best cellulase producer among these strains. It offered the highest cellulase activity of 170.9±4.1 (IU/mL/min) in media containing eucalyptus leaves after 24 h of incubation at 37 °C, followed by *Bacillus paralichniformis*, *Bacillus wiedmanni*, *Bacillus flexus*, *Bacillus aerius* and *Bacillus megaterium*. These bacterial strains and lignocellulosic wastes could be potentially used for industrial exploitation, particularly in biofuels and textiles.

Keywords: cellulase, bacterial strains, substrate, *Bombax ceiba*, submerged fermentation

INTRODUCTION

Agricultural and forestry biomass – the major source of cellulose – is a renewable and low-cost feedstock that is abundantly found in nature, being produced in amounts of 1.3 billion tons annually.[1,2] Cellulose is made up of linearly arranged glucose units linked via β-1,4-glycosidic bonds. The wide availability of cellulose makes it an appealing raw material for developing many industrial key products.[3] Cellulases play an important role in the conversion of lignocellulosic biomass into fermentable sugars. Cellulases mainly consist of the following synergistic enzymes: endoglucanases, which break the bare chains of the polymer, exoglucanases, which act to liberate cellobiose from the reducing and non-reducing ends, and β-glucosidases, which assist in the hydrolysis of the cellobiose and short-chain cello-oligosaccharide into glucose.[4,5]

Various microorganisms that can hydrolyze cellulose have been isolated and identified. However, many researchers have emphasized fungal cellulases due to their abundance, easy extraction, and commercial use.[6,7] However, the slow growth rate of fungi and substrates used in production makes cellulases expensive. Fungi, such as *Penicillium*, *Fomitopsis*, *Aspergillus*, *Trichoderma* and *Phanerochaete*, have been broadly explored in recent years, but numerous

bacteria that produce cellulases also deserve the attention of researchers due to their fast growth, resistance to harsh environments, and expression of multi-enzyme complexes.[8] Bacterial cellulases are often more effective catalysts and may also be less influenced by feedback inhibition. The ease with which bacteria can be genetically engineered mainly to intensify the production of cellulase makes them highly important.[9] It is essential to develop microbial strains, media composition, and process optimization for enhanced yields of extracellular accumulation of cellulase.[10] Many aerobic bacterial strains, such as *Cellulomonas fimi*, *C. flavigena*, *C. uda*, *Bacillus cereus*, *B. subtilis*, *B. megaterium*, *B. circulans*, *Pseudomonas fluorescens*, and a few anaerobic bacteria, such as *Ruminucoccus albus*, *Bacteroides cellulosolvens*, *Fibrobacter succinogenes* and *Clostridium thermocellum*, have been used for the production of cellulase.[11]

Lignocellulosic wastes containing a large amount of raw cellulose may cause environmental pollution. Nowadays, several agricultural wastes, such as wheat bran, sugarcane bagasse, rice bran and others, are employed as carbon source for cellulase production. Agricultural wastes are inexpensive, abundantly available, and easily approachable. These carbon sources in the fermentation medium affect the cellulase production ability of bacteria. Generally, two different approaches have been used for cellulase production, *i.e.* solid-state fermentation (SSF) and submerged fermentation (SmF). SSF occurs on solids in the absence of free water, whereas SmF involves free-flowing nutrient media with microbes. SmF is mostly used for the production of bacterial cellulases on the pilot scale, as this type of fermentation is easy to handle and provides easy product recovery.[12-16]

With several applications in different fields, such as paper/pulp, detergents, cattle feed, textiles, pharmaceutical industry, nutrition, food, agriculture industry, and specifically in biofuel industries, cellulases are valuable industrial enzymes. Numerous cellulosic substrates have been hydrolyzed by cellulases and led to the formation of different products, such as ethanol, organic acids, and some important chemicals.[4,7,17] Even though the conventional chemical process of obtaining simple sugars from the degradation of cellulose is easy, the enzymatic process has the advantages of being eco-friendly, cost-effective, and economical.[18] The present study aimed to isolate cellulase producing bacterial strains from soil and to obtain maximum enzyme production from these isolates using different lignocellulosic biomass as a carbon source.

EXPERIMENTAL

Isolation and screening of bacteria

Soil samples from four different places (*e.g.* soil near garden waste, cow dung, old paper, and old wood) of Sargodha were screened for cellulose-degrading bacteria by the method described by Gohel *et al.*[19] Gram's iodine was used for staining purposes.

Identification of cellulase producing bacteria

Cellulase producing isolates were identified by 16S ribosomal RNA (rRNA) gene sequencing.[20] The obtained 16S rRNA gene sequences were submitted to the NCBI GenBank database.

Cultivation of vegetative cells

A total of six isolates were selected for enzyme production. Each strain (24 h old) was inoculated in a different glass vial containing 5 milliliters of autoclaved broth and was kept for 24 h in the incubator at 37 °C at a shaking speed of 120 rpm.[21] These vegetative cells were used as a source of inoculum and were freshly prepared each time.

Fermentation technique

The media contained (g/L): yeast 2.5 g, $(NH_4)_2SO_4$ 2.5 g, $MgSO_4$ 1 g, and 2% substrate as carbon source. Six different crude lignocellulosic wastes, including eucalyptus leaves, corn cob, sugarcane bagasse, rice husk, wheat straw and seed pods of *Bombax ceiba*, were used as substrates in this study. All the substrates were dried and milled to powder form. Twenty-five milliliters of medium were taken in each of the six conical flasks (100 mL). The flasks were then autoclaved for 15 min, and after cooling, each of the six flasks with the same substrate was inoculated with 2% of 6 different bacterial strains. The inoculated medium was incubated at 37 °C in a shaker incubator (JSSI-100T, JSR South Korea) for four days, and samples were taken every 24 h aseptically. These samples were centrifuged at 7840 g for 10 min to obtain the crude extract for further analysis and use. The whole process was repeated for all the substrates.

Cellulase assay

Carboxymethyl cellulase (CMCase) and filter paper activity (FPase) was determined by the method described in earlier reports.[13] Glucose was taken as standard and one unit of CMCase or FPase activity was defined as the amount of enzyme needed to release one µmole of glucose from the substrate per milliliter per minute under standard assay conditions.

Statistical analysis

All the data was analyzed statistically and the values presented are the mean standard deviation of the triplicates (N=3); significance level: $p > 0.05$.

RESULTS AND DISCUSSION

This study dealt with the isolation and identification of bacterial strains and production of cellulase enzyme by these strains using different agricultural and forest wastes as carbon sources. We examined 24 isolates from different soil samples for cellulase production. Out of these 24 isolates, 6 strains showed potential for cellulase production, as confirmed by the appearance of clear zones on the CMC agar plate when flooded with iodine stain (Fig. 1). Kumar and coworkers[22] confirmed the cellulolytic activity of bacteria by observing the zones of hydrolysis by Gram's iodine staining method. Gohel *et al*.[19] also suggested Gram's iodine for plate assay to determine cellulase activity. By the 16S rRNA gene sequencing technology, these strains were identified as *Bacillus megaterium* (MG597037), *Pseudomonas stutzeri* (MG597035), *Bacillus aerius* (MG597041), *Bacillus paralichniformis* (MG597036), *Bacillus flexus* (MG597039) and *Bacillus wiedmanni* (MG597040). The phylogenetic tree of these strains confirms their identification and their evolutionary relationship with other types of *Bacillus* and *Pseudomonas* strains (Fig. 2).

This study found that in media containing eucalyptus leaves, *B. megaterium* showed its maximum FPase activity of 116.8±3.5 (IU/mL/min) and CMCase activity of 34.96±0.5 (IU/mL/min) after an incubation period of 24 h, *P. stutzeri* was also found to produce maximum cellulolytic activity (FPase 170.9±4.1 IU/mL/min and CMCase 44.45±0.7 IU/mL/min) after 24 hours of fermentation. Meanwhile, in the same medium, *B. aerius* exhibited the highest FPase activity (134.6±2.7 IU/mL/min) and CMCase activity (40.93±0.5 IU/mL/min) at 96 h and 72 h of incubation, respectively. Figure 3 illustrates that *B. paralichniformis* has the highest FPase activity (168.7±3.5 IU/mL/min) at 72 h and CMCase activity (45.21±0.5 IU/mL/min) at 24 h of fermentation. *B. flexus* has the highest activities of 143.9±3.0 IU/mL/min (FPase) and 43.66±0.3 IU/mL/min (CMCase) at 96 and 48 h of incubation, respectively.

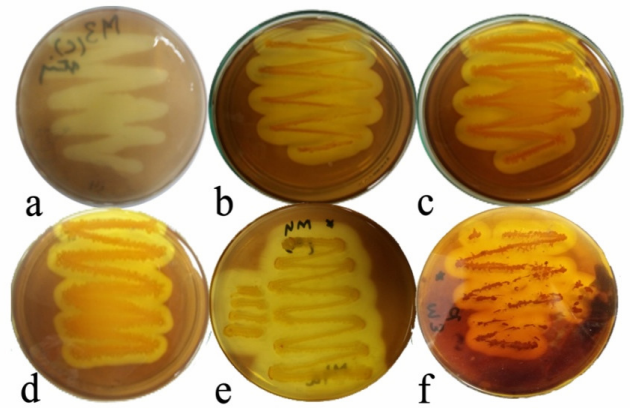

Figure 1: Clearance zone on cellulose agar plates after staining with Gram's iodine identified as corresponding to (a) *Bacillus megaterium*, (b) *Pseudomonas stutzeri*, (c) *Bacillus aerius*, (d) *Bacillus paralichniformis*, (e) *Bacillus flexus* and (f) *Bacillus wiedmanni*

After an incubation period of 72 h, *B. wiedmanni* showed the highest FPase (162.0±4.0 IU/mL/min) and CMCase (37.91±0.3 IU/mL/min) activities.

Figure 4 demonstrates that *B. aerius* has the highest FPase activity (114.2±2.0 IU/mL/min) after 24 h of incubation in media containing seed pods of *Bombax ceiba*, but maximum FPase activities of *B. megaterium*, *P. stutzeri*, *B. paralichniforms*, *B. flexus* and *B. wiedmanni* are 49.71±0.4, 46.41±0.8, 28.33±0.3, 62.38±0.6, and 46.57±0.4 IU/mL/min, respectively, for an incubation period of 72 h. The CMCase activities of these strains are different in the same media. *B. flexus* indicated the highest CMCase activity of 13.56±0.2 IU/mL/min after 96 h of fermentation, while after 24 h, *B. megaterium* (12.21±0.3 IU/mL/min), *P. stutzeri* (14.41±0.2 IU/mL/min), *B. aerius* (11.18±0.2 IU/mL/min), *B. paralichniforms* (10.99±0.2 IU/mL/min) and *B. wiedmanni* (18.33±0.4 IU/mL/min) showed their maximum cellulolytic activities.

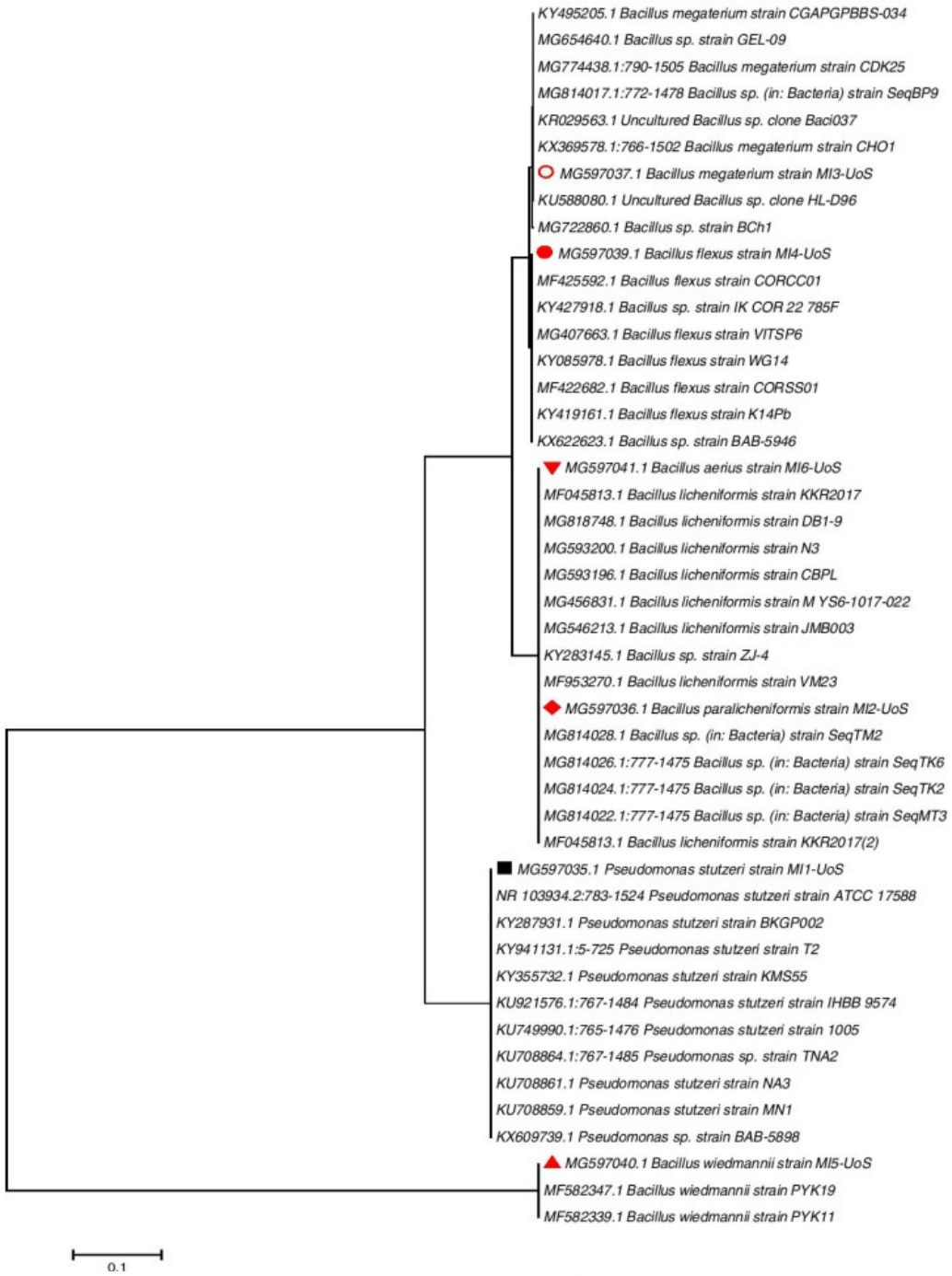

Figure 2: Phylogenetic analysis of identified bacterial strains

In media containing rice husk, the highest FPase activity (103.1±5.0 IU/mL/min) was seen in *B. flexus* after 24 h of fermentation, while its CMCase activity was observed to be 17.50±0.5 IU/mL/min after 96 h. *B. megaterium* exhibited maximum cellulase activities (CMCase 32.89±2.0 IU/mL/min and FPase 4.572±0.1 IU/mL/min) for an incubation period of 48 h in media containing rice husk. *P. stutzeri* showed maximum FPase activity of 60.45±3.0 IU/mL/min after 24 h of fermentation, and maximum CMCase activity of 15.64±1.0 IU/mL/min after 72 h in the same media. *B. aerius* showed the highest cellulase activity (FPase 17.41±1.0 IU/mL/min and CMCase 4.763±0.3 IU/mL/min) after 24 h of incubation, as indicated in Figure 5.

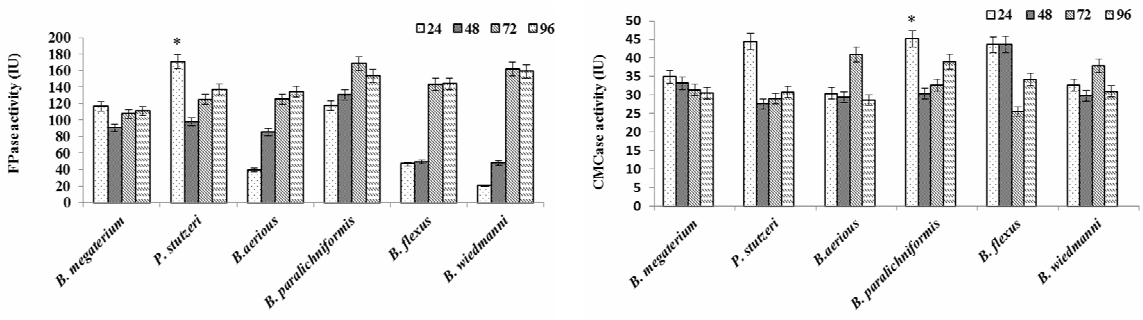

Figure 3: Cellulase enzyme production from different bacterial strains in SmF using eucalyptus leaves as substrate (24, 48, 72 and 96 – fermentation time in h; significant difference at $p > 0.05$)

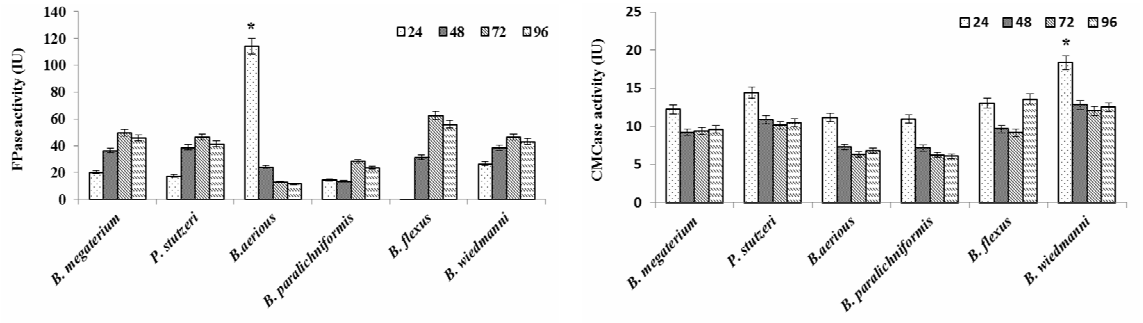

Figure 4: Cellulase enzyme production from different bacterial strains in SmF using seed pods of *Bombax ceiba* as substrate (24, 48, 72 and 96 – fermentation time in h; significant difference at $p > 0.05$)

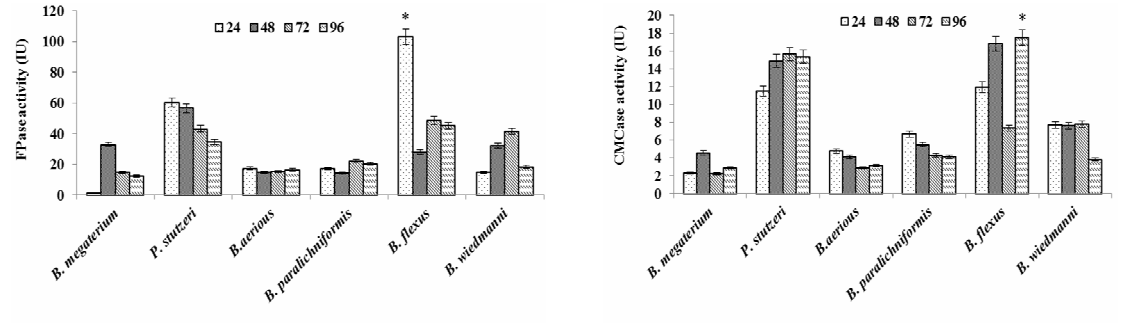

Figure 5: Cellulase enzyme production from different bacterial strains in SmF using rice husk as substrate (24, 48, 72 and 96 – fermentation time in h; significant difference at $p > 0.05$)

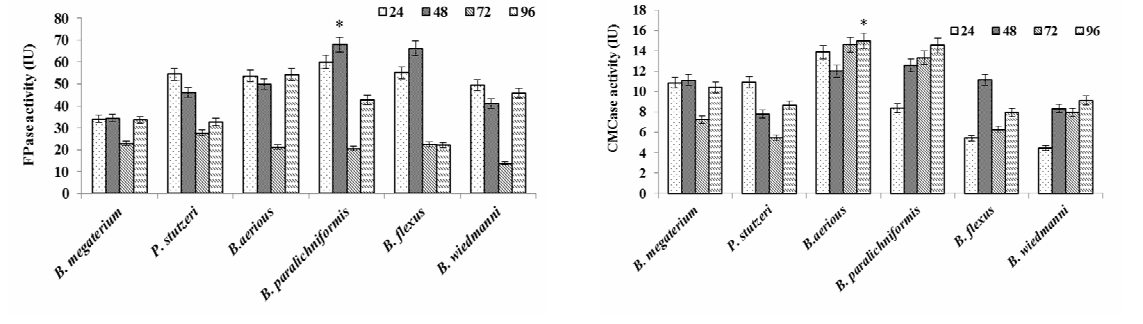

Figure 6: Cellulase enzyme production from different bacterial strains in SmF using wheat straw as substrate (24, 48, 72 and 96 – fermentation time in h; significant difference at $p > 0.05$)

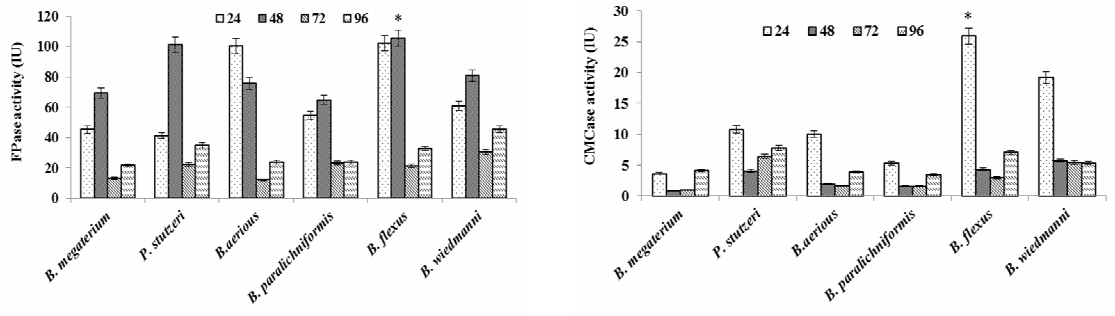

Figure 7: Cellulase enzyme production from different bacterial strains in SmF using corn cob as substrate (24, 48, 72 and 96 – fermentation time in h; significant difference at $p > 0.05$)

B. paralichniformis showed the highest CMCase activity of 22.02±1.0 IU/mL/min and FPase of 6.673±0.7 IU/mL/min after 72 and 24 h, respectively. *B. wiedmanni* offered maximum cellulolytic activity of 41.59±2.0 IU/mL/min (FPase) and 7.808±0.5 IU/mL/min (CMCase) after 72 h of fermentation.

In media comprising wheat straw, these strains show the highest cellulolytic activity at different incubation periods. *B. megaterium* (FPase 34.36±2.0 IU/mL/min and CMCase 11.12±1.0 IU/mL/min) and *P. stutzeri* (FPase 54.5±3.0 IU/mL/min and CMCase 10.89±1.0 IU/mL/min) were observed to show their maximum cellulase activity after 48 h and 24 h of incubation, respectively. *B. aerius*, *B. paralichniformis*, *B. flexus* and *B. wiedmanni* have maximum CMCase activity of 14.93±0.7, 14.52±1.0, 7.93±0.5 and 9.118±0.8 IU/mL/min, respectively, in wheat straw media, while their maximum FPase activities are 54.32±2.0 IU/mL/min (96 h), 68.05±3.0 IU/mL/min (48 h), 66.15±4.0 IU/mL/min (48 h) and 49.41±2.5 IU/mL/min (24 h), respectively, as shown in Figure 6.

Figure 7 reveals that *B. aerius* has maximum FPase activity (100.1±5.0 IU/mL/min) after an incubation of 24 h, while after the incubation period of 48 h, *B. megaterium* (69.34±1.5 IU/mL/min), *P. stutzeri* (101.1±5.0 IU/mL/min), *B. paralichniformis* (64.48±2.0 IU/mL/min), *B. flexus* (105.3±4.3 IU/mL/min), *B. wiedmanni* (80.76±2.7 IU/mL/min) have maximum FPase activities in media containing corn cob. *B. megaterium* exhibited maximum CMCase activity of 4.106±0.4 IU/mL/min after 96 h of fermentation. *P. stutzeri* (10.79±0.8 IU/mL/min), *B. aerius* (10.04±1.0 IU/mL/min), *B. paralichniformis* (5.336±0.3 IU/mL/min), *B. flexus* (25.93±1.0 IU/mL/min), *B. wiedmanni* (19.20±1.0 IU/mL/min) have maximum CMCase activities after 24 h of incubation in the same media.

Different substrates have different proportion of cellulose, so the production of cellulase varies with the substrate. The substrates that give the highest yield of enzyme are assumed to be less complex and hence easily assimilated by the isolated microbe.[23] Our results showed that *P. stutzeri* is the best cellulase producer, among the strains analysed, in SmF in media containing eucalyptus leaves, followed by *B. paralichniformis*, *B. wiedmanni*, *B. flexus*, *B. aerius* and *B. megaterium*. Thus, *P. stutzeri* showed the highest cellulase activity of 170.9±4.1 (IU/mL/min) after 24 h of incubation in eucalyptus leaves media. *B. paralichniformis* and *B. wiedmanni* exhibited maximum cellulase activity of 168.7±3.5 and 162.0±4.0 (IU/mL/min), respectively, after 72 h of incubation in the same media. Meanwhile, *B. flexus* and *B. aerius* recorded the highest enzyme activity of 143.9±3.0 and 134.6±2.7 (IU/mL/min), respectively, in the same media after an incubation period of 96 h. *B. megaterium* showed maximum enzyme activity of only 116.8±3.5 (IU/mL/min) after 24 h of incubation in eucalyptus leaves media. All these maximum cellulase activities of the six strains under study were observed in the FPase assay. Figures 3-7 reveal that these strains also exhibit potential enzyme activity when using other substrates, with different incubation periods.

Liang *et al.*[24] isolated 245 strains from natural reserves of China, out of which 22 produced zones of hydrolysis on CMC agar plates when stained with Congo red. Among these 22 strains, *Paenibacillus terrae* exhibited the highest CMCase activity in liquid culture at 50 °C and pH 5.5. Irfan *et al.*[11] explored a novel strain of *Bacillus subtilis* K-18 (KX881940), which produced the highest enzyme concentration of

3.50 IU/mL in SmF under fermentation conditions of 2% inoculum size, 2% substrate (potato peels) concentration, 1% yeast extract, temperature 50 °C, pH 5.0 and incubation period of 24 h. Different substrates, such as rice straw, corn cob, pretreated bagasse, CMC and filter paper at 0.5% (w/v), were employed separately in the media to increase the cellulase production. CMC was found best among these substrates for cellulase production.[25] Sethi *et al*.[26] isolated cellulase degrading bacteria, namely *Bacillus subtilis*, *Pseudomonas fluorescens*, *Serratia marcescens* and *E. coli*, and found *P. fluorescens* as the best cellulase producer among the four, followed by *Bacillus subtilis*, *E. coli* and *S. marscens* in the media containing glucose as carbon source.

In another study, five different bacterial strains were isolated from soil samples and identified by 16S rRNA gene sequencing as *Paenibacillus dendritiformis*, *B. pumilis*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Bacillus licheniformis.* In SmF, these were grown on sugarcane bagasse. The *B. pumilis* strain produced maximum cellulase.[27] Arshad *et al*.[21] investigated different wastes from agriculture, such as wheat straw, rice husk, defatted soybean meal, sugarcane bagasse, wheat bran, and corn cobs, for production of CMCase in SmF, employing *Bacillus subtilis*-BS06. Among all these substrates, they found sugarcane bagasse as the most satisfactory substrate for a fermentation period of 48 h at 37 °C, with an agitation speed of 140 rpm, for the production of CMCase.

In this work, we used different agricultural and forest wastes as substrate, while Ariffin *et al*.[9] employed *Bacillus pumilus EB3* for cellulase production using CMC as substrate. The cellulase produced by *B. pumilus* showed the maximum enzyme activity of 0.079, 0.011, and 0.038 U/mL for CMCase, FPase, and β-glucosidase, respectively. Padilha *et al*.[28] investigated the production of cellulase from *Bacillus* sp. C1AC5507 in SmF using sugarcane bagasse as substrate and observed that the activity of the produced CMCase varied between 0.14 and 0.37 IU mL^{-1} at 70 °C and pH 7.0.

It has been reported that *Bacillus licheniformis* 2D55 (accession no. KT799651) produced the highest FPase activity of 0.09 U/mL and CMCase activity of 0.33 U/mL in shake flasks at 50 °C after 18-24 h of fermentation, when propagated on microcrystalline cellulose (MCC) as a carbon source. In the same study, untreated and crude empty fruit bunch, rice straw, rice husk, and sugarcane bagasse were used for cellulase production. It was found that the mixture of untreated bagasse and pretreated rice husk offered enhanced CMCase (3.7 and 1.4 times) and FPase activities (2.5 and 11.5 times), as compared to the untreated bagasse and pretreated rice husk, respectively.[29] Also, a study by Ghazanfar *et al*.[30] found maximum cellulose (64%) in pretreated seed pods of *B. ceiba*, as compared to crude seed pods. The pretreatment conditions were as follows: 5% KOH, 10% substrate concentration, and 8 h soaking time, followed by autoclaving at 121 °C, 15 psi, and 15 min. Hence, *B. ceiba* seed pods can be used as a better substrate for cellulase production.

A recent study showed that sulphuric acid pretreated *Sacharum spontaneum* could be utilized as the best substrate for cellulase production by *B. subtilis* K-18 in SmF of 24 h. Maximum FPase activity of 1.389 IU/mL/min was observed under conditions of 1% acid concentration, 10% biomass loading with soaking time of 4 h at room temperature, followed by autoclaving for 15 min.[31] Poplar biomass has been reported as a probable substrate for production of cellulase by *B. cereus* and the best production was achieved after 24 h of fermentation.[32] Iqbal *et al*.[33] reported better cellulase production with pretreated eucalyptus leaves in SmF. The highest FPase production of 2.526 IU/mL/min was obtained with a pretreatment set-up of 0.6% alkali solution, 10% substrate concentration, and 4 h soaking time, while CMCase production of 2.803 IU/mL/min was achieved at 1% alkali solution, 15% substrate concentration and 6 h of soaking time. Gupta *et al*.[34] observed extracellular cellulase activities ranged from 0.012 to 0.196 IU/mL for FPase and 0.162 to 0.400 IU/mL for endoglucanase assay.

CONCLUSION

This study has explored six different bacterial strains having the potential to produce cellulase. Five different, cheap and ubiquitous agricultural and forestry wastes were used. The use of lignocellulosic biomass as substrate for cellulase production lowers the overall cost of the process. All of these six novel strains showed maximum enzyme activity in media consisting of eucalyptus leaves at different incubation periods. Thus, these strains and media could be exploited for the production of cellulase in industrial-scale operations. Further optimization of process

conditions will help in improving enzyme production.

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