Optimization of growth parameters for cellulase and xylanase production by *Bacillus* species isolated from decaying biomass

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The valorization of carbonoclastic materials to economically viable products are imperative to the global economy hence, abundance of lignocellulosic biomass in the biosphere presents an imperative opportunity for use as cheap substrate and/or sources of organisms with novel properties. Consequently, the *Bacillus* specie isolated from decaying wood shavings was evaluated for cellulose and xylan degradation potentials. Preliminary screening of the bacterial isolate revealed substantial activities with the development of 37 mm and 55.3 mm halo zone diameters, an indication of cellulase and xylanase, respectively. The bacteria were identified through partial 16S ribosomal deoxyribonucleic acids (rDNA) nucleotide sequencing, and Basic local alignment search tool (BLAST) analysis of the gene sequence showed that the bacterial isolate had 99% similarity to Bacillus cereus strain MCCC and the sequences were deposited in the GenBank as Bacillus cereus SAMRC-UFH1 with the accession number KU171369. The optimum culture conditions for cellulase production under submerged fermentation were as follow: pH 6 (103 U/ml), temperature 25°C (93 U/ml) and agitation speed 150 rpm (126.3 U/ml) for cellulase; and pH 5 (298.5 U/ml), temperature 30°C (428.5 U/ml), and agitation speed 100 rpm (248.5 U/ml) for xylanase. Under optimized conditions, cellulase was optimally produced (102.7 U/ml) after 84 h of cultivation whereas; xylanase production was peaked (390.5 U/ml) at 72 h. Conclusively, the bacteria exhibit promise as a source of potential cellulase and xylanase that could be useful for degrading lignocellulosic matter into value-added products of economic importance.

Keywords: Lignocellulosic biomass; *Bacillus* sp. SAMRC-UFH1; submerged fermentation; cellulase; xylanase.

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Introduction

Lignocellulosic biomass is an abundant renewable resource, which is inexpensive, and can be utilized for value added products through bioconversion by enzymes produced microorganisms. Lignocellulosic wastes are hugely generated in pulp and paper industries, municipal wastes [1]. It represents the better prospect for bioethanol production compare to starch-containing sources which is compete with food consumption. In addition, the abundance of lignocellulolytic biomass that came from agricultural and forestry waste offers a renewable and in-expensive feedstock for long-term supply of fermentable sugar sources [1, 2].

Several enzymes have been identified to have potential to degrade lignocellulosic biomass to bio-products of economic importance.

Cellulases are synthesized and produced by several microorganisms including bacteria, actinomycetes and fungi, but the latter are of great interest because they excrete their enzymes extracellularly [3]. Different techniques have been employed for production of these enzymes; submerged fermentation technique (used for cultivating bacteria) and solid state fermentation technique (used for cultivating fungi) [4]. Cellulases and hemicellulases have applications in various industries including the textile, paper and pulp, animal feeds, beverages and bakery [5, 6]. It is imperative to explore new microorganisms that are capable of producing enzymes that can sufficiently degraded cellulose and hemicellulose in order to release the abundant glucose trapped as cellulose in lignocellulosic materials. Consequently, this will allow industries to achieve improved bioconversion of these materials into value added products.

Xylanases are produced by wide range of different microorganisms [7, 8]; nonetheless, due to their high metabolic diversity of bacteria, they are usually exploited for xylanase production for different industrial applications [9]. The conditions involved in industrial processes are normally unfriendly in terms of extremes of temperature, pH, presence of inhibitors etc., and the enzymes intended to be utilized for such processes must be robust enough to in order to be able to withstand such conditions. Most of the xylanases reported in the previous studies did not meet these criteria [10, 11]; thus, search for better enzymes which are tough enough to withstand such harsh conditions involved in the industrial processes are focus of research in this field [8].

High cost of cellulase and xylanase production is another key obstacle limiting industrial applications of these enzymes [12]. Pure substrates being highly expensive cannot be afforded at the industrial-level bulk production of enzymes. Consequently, it will be highly propitious to utilize cost-effective substrates for enzyme production. Agricultural–residues are example of cheap raw material for industrial production of enzymes [13]. In order to contribute in the improvement of biodegradation of cellulosic biomass through microbial co-cultures, exploration, selection and characterization of potential cellulase- and xylanase producing microbes may have important roles [14]. Hence, attention has been focused on studying the cellulolytic and xylanolytic activities as well as cellulase and xylanase production by several microorganisms in various products as well as in various environments.

To establish a successful fermentation process, it is necessary to make the environmental and nutritional conditions favorable for the microorganism for overproduction of the desired metabolite. In this study, we evaluated the cellulase and xylanase production potentials of *Bacillus* specie isolated from decaying sawdust from a wood factory in the Eastern Cape, South Africa.

Materials and Methods

Sample collection

Decaying wood shavings were collected from wood factory at Melani village, Nkonkobe Municipality of the Eastern Cape, South Africa. The samples were aseptically collected into sterile plastic bags and transported on ice to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory for analyses.

Media compositions and isolation of cellulase and xylanase-producing bacteria

The growth media used in the experiments included R2A agar supplemented with nystatin (50 mg/l) to retard fungal growth and contained yeast extract (0.5 g/l), protease peptone (0.5 g/l), casamino acids (0.5 g/l), glucose (0.5 g/l),

soluble starch (0.5 g/l), dipotassium phosphate (0.3 g/l), $MgSO_4.7H_2O$ (0.5 g/l), sodium pyruvate (0.3 g/l), and agar (15.0 g/l). Luria Bertani (LB) broth contains peptone (10.0 g/l), yeast extract (5.0 g/l), NaCl (5.0 g/l). Basal medium contains carboxymethyl cellulose (CMC) (5.0 g/l), $NaNO_3$ (1.0 g/l), K_2HPO_4 (1.0 g/l), KCl (1.0 g/l), $MgSO_4.7H_2O$ (0.5 g/l), yeast extract (0.5 g/l) and agar 15.0 (g/l) in 1 liter of distilled water. For basal medium containing xylan the CMC (5.0 g) was replaced with birch wood xylan (5 g) [15]. Isolation of the bacteria was conducted in accordance to the method described by Jeffrey [16], with modifications. One gram (1 g) of the sawdust was mixed with 100 ml of sterile distilled water (sdH_2O). The saw dust suspension was then shaken vigorously at room temperature on a rotary shaker at 200 rpm for 1 h. Serial dilutions of the suspension from 10^0 to 10^{-6} was carried out by using sterile phosphate buffer saline (PBS) and 100 µl of the diluted suspensions were spread onto the surface of the growth media (R2A medium supplemented with nystatin as described above). All the plates were incubated at 28°C for about a week. At the end of the incubation, typical bacterial colonies were selected based on their morphology characteristics, size and color. The colonies were purified by streaking onto fresh R2A medium plates and incubated at 28°C for about 1 week further. After purification, the colonies were compared visually to eliminate those with similar colonial characteristics and discrete colonies were sub-cultured again and stock cultures (20% glycerol) were prepared from the pure colonies and stored at -80°C for further experiments.

The standardization of the inoculums was carried out as follows: 10 ml of sterile nutrient broth (NB) were inoculated with a loop full of the purified isolates and incubated in a rotary shaker at 150-200 rpm, 28°C for 48 h. After incubation period, 1 ml of the culture broth was transferred to sterile Eppendorf tubes and centrifuged at high speed for 5-10 min to sediment the cells. The supernatant was carefully decanted and 1 ml of sterile distilled water was added to the pellets and vortexed afterwards. The suspension was transferred into sterile McCartney bottles containing 9 ml of sterile distilled water and vortexed. The optical density of the suspension was standardized by adjusting the optical density with sterile distilled water to 0.1 OD_{600}.

Cellulase and xylanase activities screening

The cellulase activity screening method was as described by Maki *et al* [1]. Bacterial isolates were grown in 10 ml LB broth for 24 h at 28°C, with shaking at 200 rpm and slower growing isolates were left to incubate for an additional 48 h. All resulting broth cultures were tested for cellulase activity using the Gram' Iodine method [15]. Fermented broths were standardized as described previously and about 5 µl of each culture suspension was inoculated on carboxymethyl cellulose (CMC) agar plates, incubated in a rotary shaker at 200 rpm, at 28°C for 48 h. After incubation period, the plates were flooded with Gram's Iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH_2O) for 5 min to visualize cellulase activity. Halo zones around the growing cellulolytic and xylanolytic bacterial isolates confirmed positive isolates, which also indicated the degradation of the substrate (CMC) or xylan by the enzyme present in the broth. The isolate with the largest clear zone diameter assumed to exhibit the highest activity; the selected isolates were transferred into minimal CMC and xylan agar slants for cellulase- and xylanase-producing bacteria, respectively. The slants were maintained at 4°C for further analysis [17].

Identification of cellulase and xylanase-producing bacteria

The identification of the bacterial isolate was carried out by 16S rDNA sequencing. The genomic DNA used for the PCR was prepared from the single purified colony grown on basal salt medium for 72 h. The total genomic DNA was extracted with the aid of a DNA purification kit according to the manufacturer's protocol. The 16S rDNA gene fragment was amplified by using universal primers (16S forward Primer: 5'-GCCTAACACATGCAAGTCGA-3' and Reverse primer: 3'-CGTATTACCGCGGCTGCTGG-5') [18]. Conditions of the PCR were standardized with an

initial denaturation at 94°C for 3 min followed by 30 cycles of amplification (Denaturation at 94°C for 60 secs, annealing temperature, 55°C for 60 secs and extension at 72°C for 60 secs) and an addition of 5 min at 72°C as final extension. The amplification reactions were carried out with a total volume of 50 µl. The PCR products were then analyzed by using 1% agarose gel and purified by using a PCR clean up kit according to the manufacturer's instructions. The direct sequencing was performed by dideoxy chain end strategy using 3100-Avant Genetic Analyzer at Inqaba Biotec. Finally, the obtained sequences were analyzed for homology by using BLAST (Nucleotide database) [19].

Cellulase and xylanase production in submerged fermentation

Respective cultures were fermented in a sterile media with the following composition: carboxymethyl cellulose (1% w/v) and birchwood xylan (1% w/v), K_2HPO_4 (0.1% w/v), $MgSO_4.7H_2O$ (0.05% w/v), yeast extract 0.05% (w/v), KCl (0.1% w/v) and $NaNO_3$ (0.1% w/v) [4]. The bacterial isolate was resuscitated in basal salt medium supplemented with CMC or birch wood xylan and incubated for 24 h. Two milliliters of the pre-culture were inoculated into 100 ml of production medium contained in Erlenmeyer flasks (250 ml) and incubated on a rotary shaker 160 rpm at 28°C. After the fermentation period, the culture broth was centrifuged at 10,000 rpm, 4°C for 5 min and the cell free supernatant was used as crude enzyme source for the determination of enzyme activity.

Enzyme assay

Cellulase activity was determined in accordance with the method of Jecu [20]. The amount of reducing sugars released was determined by using 3,5-dinitrosalicylic acid (DNS) reagent [21]. The activity was expressed in international units (IU), defined as the amount of enzyme required to produce 1 mmol glucose per minute. The same procedure applied for xylanase activity which was determined by measuring the release of reducing sugars from birchwood xylan (in Tris buffer 50 mM, pH 8). One unit of xylanase activity (IU) was defined as the amount of enzyme necessary to release 1 mmol of xylose equivalent per minute under assay conditions.

Determination of optimum growth conditions for enzymes production

The effect of temperature on enzyme production was determined through fermentation at temperature intervals between 25-50°C for 72 h. Cellulase or xylanase activities were assessed after the fermentation period [22]. The effect of pH on enzyme production was determined by adjusting the initial pH of the growth medium from pH 6 to 11. The cultures were incubated at 30°C under shaking conditions (160 rpm) for 72 h and assayed for enzyme activity [23]. To assess the effect of agitation speed on enzyme production, the agitation speed of the production cultures was varied from 0-300 rpm at 50 rpm incremental levels as described by Adhyaru *et al* [24]. In order to achieve maximum cellulase or xylanase production, incubation time was varied from 0 to 108 h. The crude enzyme was extracted and assayed at regular intervals of 12 h. The supernatant was used as the crude enzyme to determine enzyme activity as described by Mangamuri *et al* [19].

Data analysis

Empirical data were in triplicate experimentation, and all data were subjected to relevant statistical tests. The variations between treatments were analyzed by using the analysis of variance (ANOVA) tool. The statistical package for social scientist (SPSS) using MINITAB Student Release 12 statistical package for Windows 95/98 NT (Minitab Inc., State College, PA, USA, 2007) was used for the data analyses.

Results and discussion

Identification of cellulase and xylanase-producing bacteria

The isolation of the bacteria was based on their morphological characteristics on R2A agar plates. Out of 52 bacterial isolates obtained from decaying wood shavings, 10 isolates were

Table 1. Summary of all isolates based on their ability to degrade cellulose and xylan shown by the average halo zone diameter and standard deviation.

Isolate code	CMC as a substrate	Xylan as a substrate
	Diameter of halo zones (mm) ± SD	Diameter of halo zones (mm) ± SD
TC3	38.3 ± 1.1	11.0 ± 1.41
BC2	-	37.2 ± 5.13
PO1	51.7 ± 6.43	-
PO2	**37.0 ± 3.0**	**55.3 ± 5.13**
MC0	23.3 ± 5.51	-
TC1	-	9.5 ± 0.71
BW0	-	-
PYO1	22.0 ± 7.78	-
MW1	-	-
PC1	-	-

selected and screened for cellulase and xylanase activities in this study (Table 1). Seven of the screened isolates were positive for at least one of these enzymes (cellulase and xylanase) tested, while 3 were negative for both enzyme activities (Table 1). Five isolates (TC3, PO1, PO2, MC0, and PYO1) were positive for cellulase activity, while 4 isolates (TC3, BC2, PO2, and TC1) were positive for xylanase activity (Table 1). Two isolates (TC3 and PO2) were positive for both cellulase and xylanase activities. Isolate PO2 had high cellulase activity (37.0 mm halo zone diameter) and highest xylanase activity (55.3 mm halo zone diameter) (Table 1). Bacterial isolate PO2, exhibited activities for both enzymes, as a result, was selected for detailed study. The Basic Local Alignment Search Tool (BLAST) analyses of the 16S rDNA nucleotide sequences of PO2 showed 99% similarity to *Bacillus cereus* strain MCCC and the sequences were deposited in GenBank as *Bacillus cereus* SAMRC-UFH1 with the accession number KU171369.

For production of high levels of any enzyme, optimizing the growth parameters is of major importance in industrial enzymology [25]. Several studies are focusing on attaining enzyme stability under various fermentation conditions which are usually temperature, pH, and cations in order to meet the basics of the industries [26]. Many *Bacillus* species have been studied for their capability and efficiency to utilize lignocellulosic biomass as well as for the production of ligninolytic enzymes. However, most reports on *Bacillus* species are documented on the production of xylanase by using plant biomass such as agricultural wastes, sugar bagasse, and wheat bran as sole carbon sources. *Bacillus subtilis* is the most exploited *Bacillus* specie in this area of study and there are very few on *Bacillus cereus*.

In this present study, *Bacillus sp.* SAMRC-UFH1 exhibited cellulase and xylanase activity when subjected to a qualitative test using simple sole carbon sources. There are several studies on *Bacillus genus* recovered from a diversity of natural sources that were reported to be producer of hemicellulases [23-25]. Quite a lot of bacterial isolates belonging to the genera *Bacillus*, *Paenibacillus*, *Streptomyces*, and *Microbacteria* have been reported as cellulase-producing bacteria [1]. For example, Mayende and colleagues reported on the production of cellulase (CMCase) by a thermophilic *Bacillus sp*. [27].

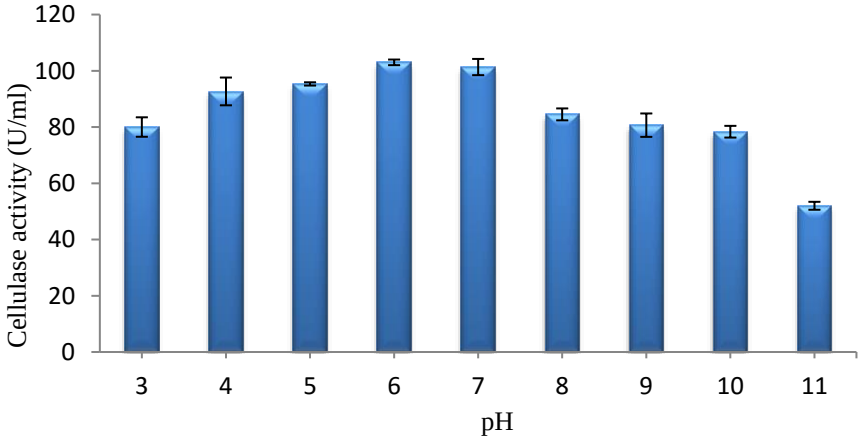

Figure 1. Effect of initial pH of growth medium on cellulase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

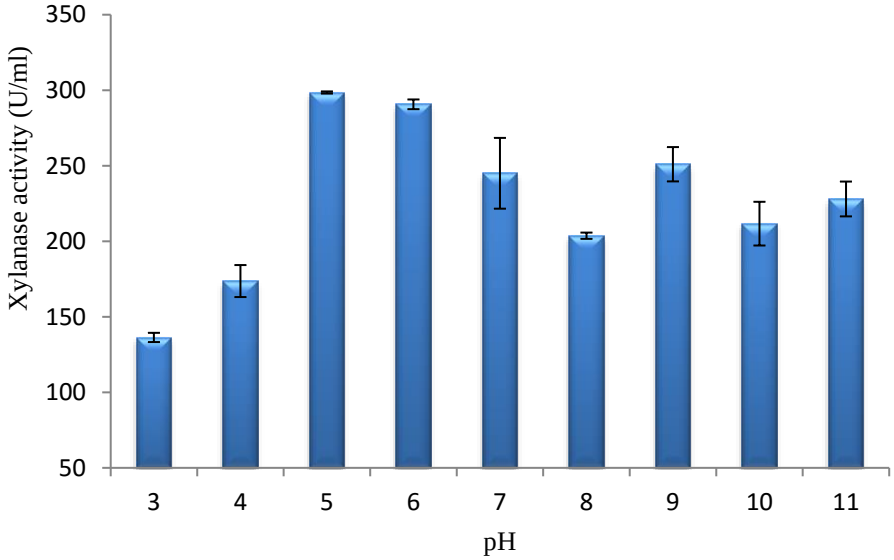

Figure 2. Effect of initial pH of growth medium on xylanase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

Evaluation of cellulase production by bacterial isolates can be a challenge due to the fact that bacteria can produce a number of different forms of cellulases (endoglucanase, β-glucosidase, and exoglucanase) and these enzymes can be found existing as extracellular enzymes that are free as well as an enzyme complexes also known as cellulosomes which are found on the cell membrane [29]. As a result, a qualitative test based on CMC degradation in CMC-supplimented agar was used to discern cellulase activity that represents primarily endoglucanase and β-glucosidase activities; xylanase activity was discerned by using birch wood xylan as a supplement. Derivatives of xylan are commonly utilized as inducers in xylanase production by microbes in fermentation processes (either on a submerged fermentation

or solid state method) [26]; and in the present study, submerged fermentation was used. Enzyme production was carried out by using carboxymethyl cellulose (1% w/v) for cellulase and birchwood xylan (1% w/v) for xylanase as sole carbon sources and the bacteria showed activity for both cellulase and xylanase.

Determination of optimum growth conditions for enzymes production

1. Effect of initial pH of growth medium

The effect of initial pH of growth medium on cellulase and xylanase production was investigated at pH range 3 to 11 (Figures 1 and 2). It was observed that cellulase activity was significantly higher under weakly acidic pH conditions as well as neutral conditions. An optimal activity was obtained at pH 6 (103 U/ml) beyond which cellulase activities began to decline (Figure 1). For xylanase, activity was optimal at pH 5 (298.5 U/ml) and more negatively affected at pH values below 5 compared to the activity observed at pH values above 5 (Figure 2). A non-parametric Friedman test of differences among enzyme activities for cellulase and xylanase was conducted and had an asymptotic significance of 0.157 which was not significant ($p > 0.05$). Initial pH of the medium has been reported to strongly influence many enzymatic processes by affecting the transport of a number of chemical products and enzymes across the cell membrane [29]. The pH growth range and pH growth optimum vary according to different microorganisms. Optimal initial pH for cellulase and xylanase production varies between bacterial groups due to differences in interactions of media compositions and microbial specificities [30]. According to the observation of Norsalwani *et al* [31], microorganisms have the ability to grow over wide range of pH. Nevertheless, extreme pH can damage microorganisms by distracting the plasma membrane as well as inhibiting the enzymatic activity and membrane transport proteins [32]. In addition, synthesis and expression of certain genes, as well as microbial metabolic activities, are thought to be influenced by the internal pH, in response to that of the external environment [33]. Consequently, bacterial synthesis and activity of proteins associated with various processes are adjusted in response to internal and external pH levels [33]. The results of our present study indicated that the bacteria produced these enzymes well at weak acidic conditions. The results of other researchers collaborate with our findings, for example, Fatokun *et al* [34] reported optimal cellulase production by *S. albidoflavus* strain SAMRC-UFH5 at pH 6. Similarly, according to the report of Yang *et al* [35], a *Bacillus sp*. maximally produced cellulase at a pH range of 6-8. Other researchers also have documented different pH value for cellulase production from the previous studies. For example, optimum pH 6.5 and 8 for cellulase production by *Streptomyces* strain C188 and *Streptomyces sp.*, respectively, have been reported [36, 37]. In another study carried out by Mmango-Kaseke *et al* [38], cellulase and xylanase production by *Micrococcus sp*. SAMRC-UFH3 under submerged fermentation conditions was maximal at pH 5 and 10 which contradict our findings. On the other hand, in our present study, for xylanase production, even though the optimum pH was slightly acidic, the bacterium was observed to be alkali-tolerant with spikes in enzyme production observed at pH values of 9 and 11 (Figure 2). Furthermore, the maximum xylanase production by *Cellulosimicrobium sp*. CKMX1 was observed at pH 8 [39]. In another study, xylanase produced by *Bacillus sp*. PKD-9 was found to increase with increasing medium pH from 5-8 and maximum production was attained at pH 8 [40]. Maximum yield of xylanase has also been reported at pH 9.0 by *Bacillus sp.* [41], and also with alkalophilic *Bacillus* strain MK001 [42]. Das *et al* [43] obtained optimum xylanase production at pH 7. Several other researchers [44, 45] have reported pH 7 as the optimal pH for many bacterial xylanases and cellulases. Likewise, some other previous studies on xylanase production by bacteria also reported marked dependence on an initial pH 7.0 of the medium [9, 46]. In addition, other authors reported optimal initial pH 7 for xylanase and endocellulase production by *Geobacillus sp*. WSUCF1 and *Cellulomonas sp*., respectively [47,

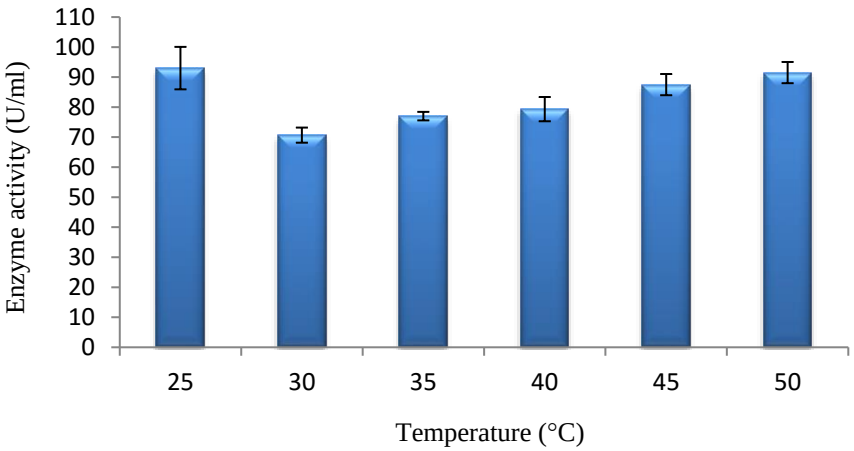

Figure 3. Effect of incubation temperature on cellulase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

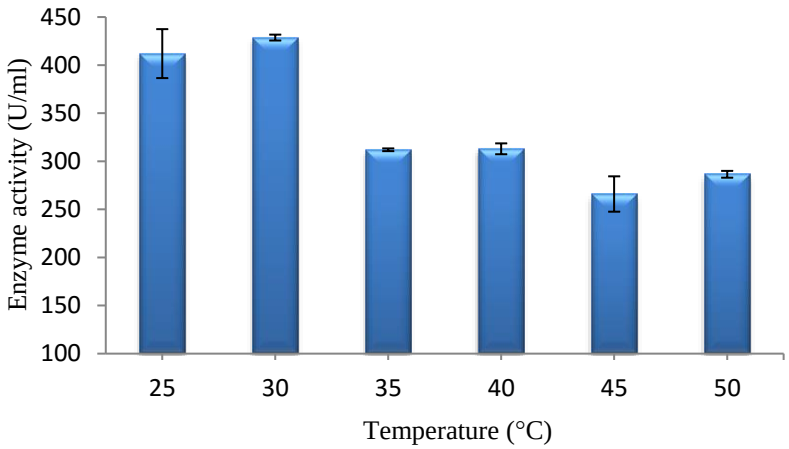

Figure 4. Effect of incubation temperature on xylanase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

48]. A *Bacillus subtilis* has been reported to exhibit xylanase production under neutral to moderately alkaline conditions [49]. Likewise, an enzyme produced by *B. pumilus* was found to exhibit high xylanase activity over an extensive pH range of 6-10 [50]. However, there are xylanases produced by other *Bacillus* species with expansive pH range (5–11) that have been documented in the previous studies [51, 52]. The optimal pH for xylanase production by *B. subtilis* was 9, and also *Enterobacter sp*. MTCC 5112 [53] and *Staphylococcus sp.* [54]. Enzyme production can be affected by pH of the medium in various ways and one of which is the inhibitory effect on the organism's growth which consequently affects enzyme production. On the other hand, the enzyme produced may be exposed to denaturation by ionic environment that is unfavorable due to the pH of the medium [51]. In accordance with the report of Pandey [51], the pH impacts the solubility of some major media constituents, and also transportation of some enzymes across the cell membrane. Suitable pH is required for the enzyme to maintain the three-dimensional shape of the active site. Changes in the pH might alter the ionic bonding of enzyme

that contributed to the functional shape of the enzyme [55]. Industrially desirable characteristics like thermostability and alkalophilic nature are main requirements for any potentially commercially important enzyme in industries [56].

2. Effect of incubation temperature

The effect of incubation temperature on both cellulase and xylanase activities was examined at different temperatures ranging from 25-50°C. The results shown in figure 3 revealed that the test bacteria exhibited high cellulase activity of 93 U/ml at 25°C. However, the lowest enzyme activity of 70.7 U/ml was observed at 30°C; after which activities increased with increasing temperature up to 50°C. This trend suggests that the bacteria might have multiple optimum temperatures for cellulase production. With regards to xylanase, the bacteria exhibited the highest xylanase activity (428.5 U/ml) at the incubation temperature of 30°C; beyond which a decline in activity was noticeable (Figure 4). A non-parametric Friedman test of differences among enzyme activities for cellulase and xylanase was conducted and had an asymptotic significance of 0.634 which was not significant ($p > 0.05$). Cultivating the bacteria below the environmental temperature where the bacteria was isolated might negatively influence the response of a microorganism either directly or indirectly. Directly effects include decreased growth rate, enzyme activities, alteration of cell composition, and differential nutritional requirements. Indirect effects are usually observed on the solubility of solute molecules, diffusion of nutrients, osmotic effects on membranes and cell density. As temperature falls, the lag phase that proceeds growth extends, leading to a decrease in a growth rate and the final cell number. During the lag phase that precedes growth in mesophiles, many physiological changes occur, including a decrease in the saturation of fatty acids and inhibition of DNA, RNA, and protein synthesis. Lower the temperature below the environmental temperature largely effect on the solute transport system. The lipid bilayer which is basic structure of the microbial membranes must have proper fluidity to maintain the cell permeability and movement of essential solutes. According to the report of Juturu and Wu [57], incubation temperature is paramount for optimal enzyme production due to alterations in microbial protein structure and properties with temperature variations. At temperatures below or above the optimum, metabolic activities are reduced, with consequent inhibition in growth and enzymes synthesis [58]. Enzyme production can also be influenced by the cultivation temperature; hence, it is desirable to obtain the optimum temperature for bacterial growth in order to attain high enzyme yields. Thermostable microorganisms are the potential sources of thermostable enzymes. Thermophiles can tolerate high temperature by using increased interaction than non-thermotolerant organisms, because of the presence of hydrophobic, electrostatic, and disulphide interaction [59]. Specialized proteins such as chaperonins are produced to refold the protein to their native form and restore their function [60]. Cell membrane of thermophiles is made up of saturated fatty acids. Thermal stability of xylanase is an important property due to its potential application in several industrial processes. Use of such enzymes has been expected to greatly reduce the need for pH and temperature adjustments before the enzyme [56]. Our findings from this present study revealed that the test bacterial strain is a mesophilic organism. According to the report of Techapun *et al* [61], differences in optimal temperature for enzyme production could be attributed to strain variations between bacterial groups and adaptability to temperature dynamics. Nandimath and colleagues [62] reported that *Bacillus* specie had higher cellulase production at 30°C. In our study, the test bacteria had another peak at 50°C for cellulase production and this suggests that the bacterial strain has multiple optimum temperatures. One of the reasons for this may be because the cellulase produced by this strain exists in several isoforms that are active at different temperature ranges [63]. Fagade and Bamigboye reported

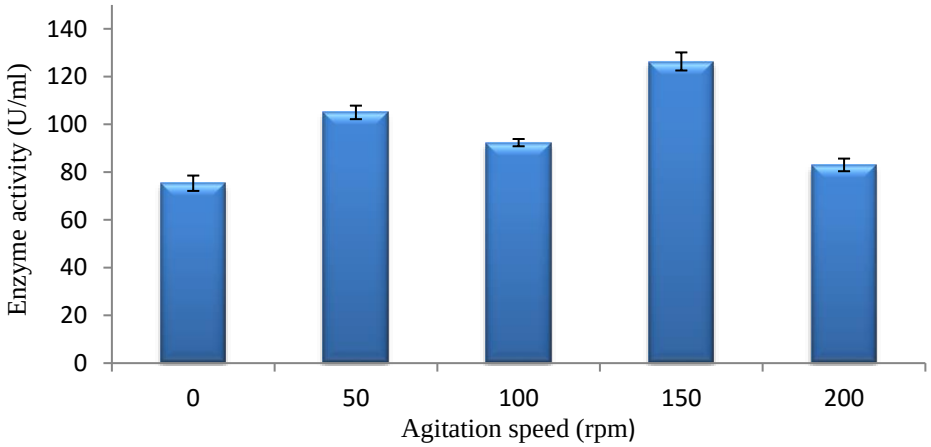

Figure 5. Effect of agitation speed on cellulase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

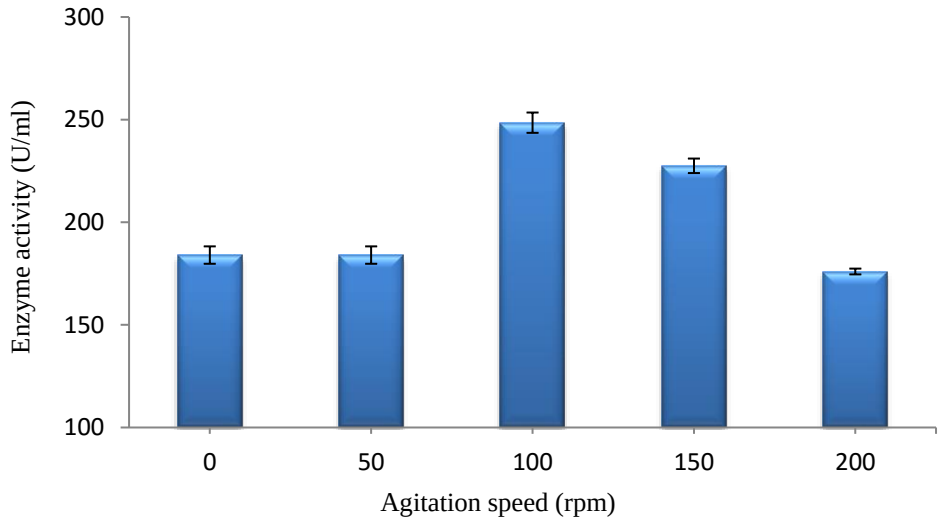

Figure 6. Effect of agitation speed on xylanase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

optimum cellulase activity for three *Bacillus* species when incubated at temperature of 40°C [45]. Similarly, Ire *et al* [64] reported that *Bacillus cereus* GBPS9 and *Bacillus thuringiensis* serovar kurstaki HD1 preferred temperature of 40°C for cellulase production. Higher temperature optimal for both cellulase and xylanase production have been recorded for some *Bacillus* species. In case of Ibrahim and El-diwany [65] report, a *Bacillus sp*. was found to have an optimum temperature of 75°C for cellulase production. Poorna and Prema [4] documented that a *B. pumilus* showed a stable activity for xylanase at a wide temperature range of 25-40°C which corroborates with our findings in this present study. Comparatively, a higher temperature of 50°C for production of CMCase by *B. licheniformis* MVS1 and *Bacillus sp*. MVS3 was reported [66]. Several studies carried out on different *Bacillus* species by some researchers revealed that different optimum temperatures were observed for maximum xylanase and cellulase production [67]. Optimum incubation temperature for xylanase production was found

to be 37°C for *Bacillus pumilus* ASH [22]. Lee *et al* [68], Rahman *et al* [69], and Sharma and Chand [70] documented an optimal incubation temperature of 30°C for cellulase production by *B. subtilis* A-53, *Bacillus sp*. SHC1 and *Pseudomonas sp.* XPB-6. The production of xylanases by *Cellulosimicrobium sp*. CKMX1 decreased drastically at temperature values lower or higher than the optimum temperature of 35°C [39]. Studies of Oakley *et al* [71] and St. John *et al* [72] reported the xylanase production by *B. subtilis* B230 and *B. subtilis* 168 at 37°C whereas, *Bacillus sp*. and *Thermomyces lanuginosu* were capable of producing maximum level of xylanase at 40°C [25, 46]. Incubation temperature is a critical factor in enzymatic productivity [73]. Maximum enzyme production is obtained at optimal temperature and the decrease in enzyme production at lower or higher temperatures may be due to the fact that at these temperatures, growth of the organisms was inhibited, causing a decrease in the synthesis of the enzymes as suggested by Simoes *et al* [74]. In addition, increase in production at optimum temperature might be due to faster metabolic activity, increase in protein content and extracellular enzyme production in culture. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity [75]. Pathania *et al* [56] reported that the fermentation temperature appeared to have a dramatic effect on xylanase production. *Paenibacillus sp.* N1 produced maximum xylanase activity at elevated temperature of 50°C, while displaying minimum activity at 30°C.

3. Effect of agitation speed

The effect of agitation speed on both cellulase and xylanase production was investigated by using shaking speeds ranging from 0-200 rpm with 50 rpm incremental levels. It was observed that the lowest cellulase activity of 75.3 U/ml was at static condition but increased at 50 rpm and slightly decreased again at 100 rpm. The highest cellulase activity of 126.3 U/ml was observed at agitation speed of 150 rpm (Figure 5). On the other hand, with respect to xylanase, activities increased from static condition and peaked at 100 rpm speed with activity of 248.5 U/ml (Figure 6) and thereafter, the enzyme activity declined as agitation speed increased. A non-parametric Friedman test of differences among enzyme activities for cellulase and xylanase was conducted and had an asymptotic significance of 0.211 which was not significant ($p > 0.05$). For the duration of a submerged fermentation, one of the most important parameter that was used for uniform distribution of nutrients and satisfying oxygen demand requirements is agitation [24]. Agitation affects the rate of oxygen mass transfer, and is crucial for maximal production in microbial enzyme fermentation [76]. However, high agitation speed may also result in high shear stress and impact negatively on mycelia growth [77]. Adhyaru and colleagues [24] reported a *B. altitudinis* DHN8 that displayed maximum xylanase production at 250 rpm and displayed significant xylanase production from a range of 150-300 rpm of agitation speed. However, at static and lower agitation (50 rpm) conditions, lower cellulase and xylanase activities were observed, which may be due to improper mixing of the medium components and limitation of the dissolved oxygen. On the other hand, the reduction in enzyme activity when the agitation speed was increasing may be due to the shearing of the cells [22]. Several studies have been reported for maximum xylanase and cellulase production at an agitation speed of 200–250 rpm [50, 68]. An actinomycete isolate designated as *Streptomyces sp*. RCK-2010 was found to have an optimum xylanase activity at 200 rpm when grown under variable shaking conditions [22]. Sepahy *et al* [78] reported 200 rpm as optimal for xylanase production by *Bacillus mojavensis* AG137 cultured on agricultural waste. *Bacillus amyloliquefaciens* DL-3 was investigated for the effect of agitation at variable shaking speeds that ranged from 200-500 rpm and it was found that 300 rpm was for achieving maximum production of cellulose [68]. An optimal agitation of 300 rpm was recorded for cellulase production by *Streptomyces sp*. T3-1, a transformant strain

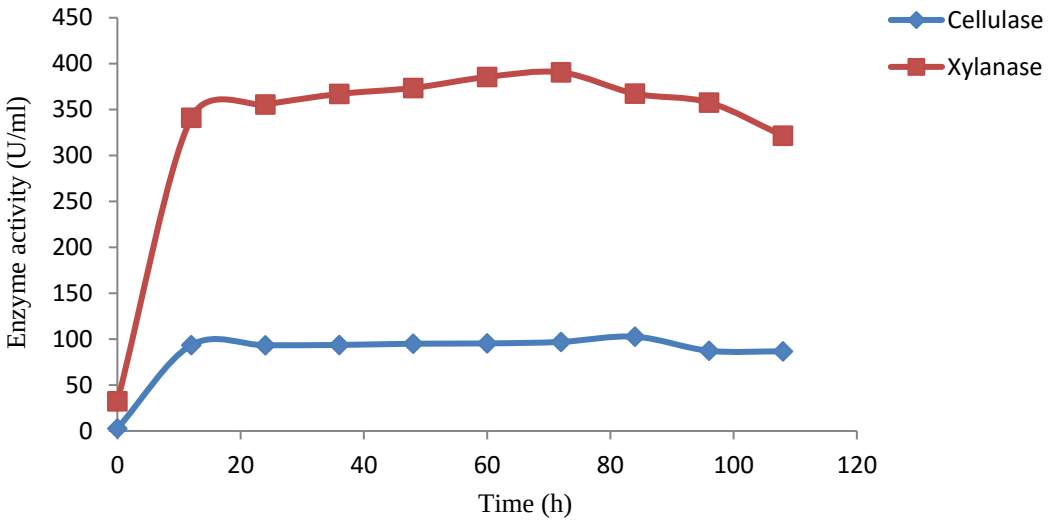

Figure 7. Time course of cellulase and xylanase production by the test bacteria. The mean values of the enzyme activities ± SD are presented.

cultivated in 50 L fermenter [76]. On the other hand, Saratale *et al* [79] reported static condition as optimal for endocellulase production by *Streptomyces sp.* MDS using carboxymethyl cellulose as carbon source.

Time course of cellulase and xylanase production

Optimum culture conditions were applied for time course study of cellulase and xylanase production (Figure 7). Time course assay revealed that cellulase production was maximal after 84 h (102.7 U/ml) of cultivation. Subsequently, the cellulase activity dropped drastically to 87.3 U/ml at 96 h and later remained constant up to 108 h (86.7 U/ml). Time course of xylanase production in basal medium containing birchwood xylan revealed that maximal activity was observed after 72 h (390.5 U/ml) of cultivation and thereafter, a decrease in activity was recorded (Figure 7). A non-parametric Friedman test of differences among enzyme activities for cellulase and xylanase was conducted and had an asymptotic significance of 0.050 which was significant ($p \leq 0.05$). The cultural conditions set for an organism during the cultivation period and also the genetic makeup and the nature of the microorganism determine its maximum enzyme production time [51]. In our present study, it was observed that cellulase and xylanase are produced maximally during the exponential phase because they are part of primary metabolites and a decrease in enzyme secretion starts at the beginning of death phase [80]. There are several factors that may account for the decrease in the activity during the fermentation. These may include inhibition of the enzyme by end products of the fermentation process or some proteases may be activated or the enzyme may interact with some other components that the cell secreted [81]. The reduction in enzyme production may also be due to the depletion of nutrients in the fermentation medium [22]. Various *Bacillus* species exhibiting maximum enzyme production over a cultivation period of 48-96 h have been documented [82]. Mmango-Kaseke *et al* [38] reported that *Micrococcus sp*. SAMRC-UFH3 produced cellulase maximally under optimum growth conditions after 96 h of cultivation period. Similarly, Nagendra *et al* [83] documented that the optimum incubation period for cellulase was also within 96 h in the late stationary phase of growth. On the other hand, Mrudula and Shyam [84] reported a shorter incubation period of 48 h for maximum production of xylanase from *B. megaterium* MTTC 2444. Sepahy *et al* [78] reported a fermentation period of 48 h by *Bacillus mojavensis* AG137 in submerged fermentation

using oat bran as a substrate. Maximum production of xylanase was observed by Wahyuntri *et al* [85] in a culture incubated at 72 h by *Bacillus sp*. AQ-1. Subramaniyan and Prema [86] reported a *Bacillus* SSP-34 that produced xylanase maximally after a fermentation period of 96 h. Likewise, *Bacillus* SSP-34 produced maximum xylanase activity when grown for 96 h [87].

Conclusions

The potentials of *Bacillus cereus* SAMRC-UFH1 isolated from the sawdust samples was investigated on its ability to utilize carboxymethyl cellulose (CMC) and birch wood xylan as sole carbon sources for cellulase and xylanase production. The bacterial strain exhibited enzyme production at 25°C and 30°C; pH of 6 and 5 with an optimum shaking speed of 150 rpm and 100 rpm for cellulase and xylanase, respectively. The results obtained in this study for *Bacillus cereus* SAMRC-UFH1 suggest that it may have great potential to be used for degradation of lignocellulosic materials. Although, further studies on the purification and characterization of the enzymes as well as testing the efficiency of the bacterial strain to degrade natural lignocellulosic material such as sugar cane or agricultural wastes are parts of our on-going studies in our research group. Finding naturally occurring cellulase and xylanase degrading bacteria from the environment is imperative in the field of bio-refining. Hence, this study endorses the notion that utilization of the vast microbial biodiversity could actually lead to identification of novel sources that can produce biotechnologically important products.

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Conflict of interest

The authors declare no conflict of interest.

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