Original Research

Endoglucanase and Xylanase Production by *Chryseobacterium* Species Isolated from Decaying Biomass

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Abstract

Lignocellulosic materials are potential sources of isolating bacteria that can be used to produce important value-added products such as cellulase (endoglucanase) and xylanase in industry. Based on the above-mentioned premise, this study aimed to assess endoglucanase and xylanase-degrading potentials of a bacterial isolate from decaying sawdust samples collected from a wood factory at Melani village, Nkonkobe Municipality of Eastern Cape Province, South Africa. The bacteria showed high activity for endoglucanase and xylanase when grown on carboxymethyl cellulose (CMC) and birch wood xylan as sole carbon sources, respectively. The bacterial isolate was identified through 16S rDNA sequencing and the gene sequence was found to have 98% similarity with that of *Chryseobacterium taichungense*. The sequence was deposited in the GenBank as *Chrysobacterium taichungense* SAMRC-UFH2 with accession number KU171370. Optimum culture conditions for endoglucanase and xylanase production included: pH 6, incubation temperature (25ºC), and agitation rates of 50 rpm and 150 rpm for endoglucanase and xylanase, respectively. The high enzyme activities exhibited by this bacterial strain portend it as a potentially relevant candidate as a producer for value-added products of biotechnological importance.

Keywords: lignocellulosic materials, *Chrysobacterium* sp. SAMRC-UFH2, submerged fermentation, endoglucanase, xylanase

Introduction

The existence of pollution problems associated with agro-industrial wastes, scarcity of places for its disposal, costlier treatment options, and increased need to save valuable resources have forced the utilization and bioconversion of waste (lignocellulosic materials) into high-value industrially useful products. The huge amount of residual plant biomass considered as "waste" can be a potential source of isolating microorganisms, which can be used to produce various value-added products like biofuels, animal feeds, chemicals, and enzymes [1]. The demand for industrial enzymes, particularly of microbial

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origin, is ever increasing due to their application in a wide variety of processes [2].

Many bacteria and fungi are capable of utilizing cellulose as their carbon source by synthesizing cellulases as inducible enzymes that are responsible for the degra-dation of cellulose into simple sugars composed predominantly of glucose [3]. The comprehensive hydrolysis of cellulosic matter requires a combined action of a variety of cellulases (1,4-β-D-glucanglucanohydrolase, EC 3.2.1.4) that are multienzyme complexes, comprising three main components that have been shown to act synergistically in the hydrolysis of cellulose to glucose units [4]: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21).

Xylan is known to be the second richest natural polysaccharide of plant biomass and is considered one of the most significant polysaccharides for industrial applications [5-6]. Its primary chain is made up of β-xylopyranose residues and the complete enzymatic hydrolysis of this chain needs a series of enzymes with endo-1,4-β-D-xylanase (EC 3.2.1.8), the most fundamental in the depolymerisation of xylan [Polizeli et al., 2005]. The diversity in chemical arrangements of xylans resulting from the cell walls of different plant materials has led to many different xylanases that have dissimilar physicochemical attributes, structures, and hydrolytic activities [7].

Microorganisms from different environments are prospective sources of cellulase as well as xylanase for biotechnological use. Such environments may have extremes of pH, temperature, and even salt content; hence these organisms produce enzymes that have been developed to operate optimally under some of these harsh conditions [8].

The genus Chryseobacterium is ubiquitous in nature. The taxonomy of these yellow pigmented, gram-negative rods is incorporated in the taxonomy of the *Flavobacterium* genus. Flavobacterial microbes have been documented as the causatives of food spoilage for many food products such as poultry, meat, fish, milk, and other dairy foods [9]. *Flavobacterium* genus is known to be the source of the bitter and fruity flavours in milk, and the slime production and discolouration in cheese [10]. Currently, many *Flavobacterium* species that were once known to cause food spoilage have been reclassified as Chryseobacterium genus. Furthermore, there are quite a number of reports on the pathogenicity of several *Chryseobacterium* species to animals and humans [11]. *Chryseobacterium indologenes* and *Chryseobacterium gleum* have the ability to cause nosocomial infections in humans, and this commonly happens in infants and patients with compromised immune systems with underlying diseases [11].

To the best of our knowledge, nowhere in the literature has *Chrysobacterium taichungense* been implicated in cellulose or xylan degradation. Hence, this present study describes cellulose and xylan-degrading potentials of a *Chrysobacterium* sp. isolated from decaying sawdust in the Eastern Cape Province of South Africa. The ability of

the bacteria to produce endoglucanase and xylanase was assessed and the culture conditions for their optimum production were further investigated.

Materials and Methods

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

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 contained peptone (10.0 g/l), yeast extract (5.0 g/l), and NaCl (5.0 g/l). Basal agar medium contained carboxymethyl cellulose (CMC) (5.0 g), $NaNO_3$ (1.0 g), K_2HPO_4 (1.0 g), KCl (1.0 g), $MgSO_4.7H_2O$ (0.5 g), yeast extract (0.5 g), and agar (15.0 g) in 1 litre of distilled water. For basal medium containing xylan agar, CMC (5.0 g) was replaced with birchwood xylan (5 g) [12].

Isolation of the bacteria was carried out in accordance with the modified method of Jeffrey [13]. Briefly, 1 g of the sawdust was mixed with 100 ml of sterile distilled water (sdH_2O). The sawdust suspension was then stirred vigorously at room temperature (28ºC) on a shaker at 200 rpm for 1 h. A series of dilutions of the suspension from 10^0 to 10^{-6} were prepared in sterile phosphate buffer saline (PBS), and 100 µl of the diluted suspensions was spread onto the surface of the growth media. All the plates were then incubated at 28ºC for about a week. At the end of the incubation period, typical bacterial colonies were selected based on their morphology, size, and colour, and streaked again onto fresh R2A medium plates for purity and incubated further at 28ºC for one week. After purification, the colonies were compared visually to separate those with similar colonial characteristics, and discrete colonies were sub-cultured again and glycerol stock cultures were prepared from the pure colonies and stored at -80ºC for future use.

Test tubes containing 10 ml of sterile nutrient broth (NB) were inoculated with a loop full of the purified isolates and incubated with shaking at 150-200 rpm at 28ºC for 48 h. After incubation, 1 ml of the culture suspension was transferred to sterile Eppendorf tubes and centrifuged at high speed for 5-10 min to sediment the cells. The supernatant was carefully decanted before adding 1 ml of sterile distilled water followed by vortexing. The suspension was transferred into sterile McCartney bottles containing 9 ml of sterile distilled water and mixed by vortexing. Subsequently, the suspension was standardized to optical density of 0.1 at 600 nm.

The method described by Maki et al. [14] was used to screen the bacterial isolates for cellulase activity. Isolates were grown with shaking in 10 ml of LB broth at 28°C for 24 h, and slower-growing isolates were left to incubate for an additional 48 h. All resulting broth cultures were tested for cellulase (endoglucanase) activity using the Gram iodine method [12]. Briefly, the broth culture was standardized as described earlier, 5 µl of each culture suspension was placed onto a Petri dish containing carboxymethyl cellulose (CMC) agar, incubated at 28°C for 48 h, and flooded with Gram's iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH$_2$O) for 5 min to indicate endoglucanase activity. The presence of a halo zone at the point of inoculation with broth culture outwards was indicative of substrate (CMC) degradation by the enzyme present in the fermented broth and hence regarded as a positive reaction. Halo zone diameters were measured for a semi-qualitative comparison of endoglucanase activity among the isolates after 72-96 h of incubation. The evaluation of xylanase activity in the bacterial isolates followed the same method described for endoglucanase activity (above), except that CMC was replaced with birchwood xylan as substrate.

The bacterial isolate was identified by 16S rDNA sequencing. The genomic DNA used for PCR was prepared from the single colonies grown on basal 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 using universal primers (16S forward Primer -5'-GCCTAACACATGCAAGTCGA-3' and 16S reverse primer -3'CGTATTACCGCGGCTGCTGG-5') [15]. The following PCR conditions were utilized: initial denaturation at 94ºC for 3 min followed by 30 cycles of amplification (Denaturation at 94ºC for 60 sec, annealing temperature is 55ºC for 60 sec, and extension at 72ºC for 60 sec) and an addition of 5 min at 72ºC as final extension. The amplification reactions were carried out with a total volume of 50 µl in a gradient PCR. The PCR products were then visualized using 1% agarose gel and purified using a PCR clean-up kit according to the manufacturer's instructions. Direct sequencing was performed by dideoxy chain end strategy using a 3100-Avant Genetic Analyzer at Inqaba Biotec. Finally, the obtained sequences were analyzed for homology using BLAST (Entrez Nucleotide database) [16].

A modified method of Poorna and Prema [17] was used for endoglucanase and xylanase production in a submerged fermentation process. The fermentation medium was prepared using 1% w/v carboxymethyl cellulose (as cellulose substrate) and 1% w/v birchwood xylan (as xylanase substrate), K$_2$HPO$_4$ 0.1% (w/v), MgSO$_4$.7H$_2$O 0.05% (w/v), yeast extract 0.05% (w/v), KCl 0.1% (w/v), and NaNO$_3$ 0.1% (w/v), and was autoclaved at 121ºC for 15 min. After sterilization, the medium was then allowed to cool to room temperature and then inoculated with 1 ml of the bacterial culture and incubated with shaking (160 rpm) at 28ºC for 24 h. Subsequently, the fermented broth was centrifuged at 10,000 rpm, 4ºC for 5 min, and the cell-free supernatant was used as a crude enzyme source for determining enzyme activity.

Endoglucanase activity was determined according to the method of Jecu [18] using carboxymethyl cellulose 1% (w/v). The amount of reducing sugars released was determined using dinitrosalicylic acid (DNS) assay [19]. The activity was expressed in international units (IU), defined as the amount of enzyme required to produce 1 mmol glucose per min. The same method was used 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 min under assay conditions. All the experiments were carried out independently in triplicate and the results presented here are the means of each set.

The effect of temperature on enzyme production was determined by incubating the production media with shaking at temperatures ranging from 20-50ºC for 72 h. The culture filtrates were then collected and assayed for endoglucanase or xylanase activities [12].

The effect of pH on enzyme production was determined by adjusting the initial pH of the production media to pH 6, 7, 8, 9, 10, and 11 using 1 M HCl or NaOH prior to autoclaving. The media were incubated at 30ºC under shaking conditions (160 rpm) for 72 h and assayed for enzyme activity [21].

To assess the effect of agitation speed on enzyme production, the agitation speed of the production media was varied from 0-300 rpm at 50 rpm incremental levels as described by Adhyaru et al. [22].

In order to achieve maximum endoglucanase or xylanase production, incubation time was varied from 0-108 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 Agnihotri et al. [23].

The experimental data was expressed as mean ± standard deviation (SD) of the triplicates. Statistical analysis was done using Statistical Package for the Social Sciences (SPSS). The Friedman test was used to compare the data among the parameters that were studied in the research. $p < 0.05$ (Asymp. significance) was considered statistically significant.

Results and Discussion

Based on the morphological characteristics on R2A agar plates, the bacteria were selected, purified, and stored at 4ºC for further studies. A total of 52 distinct bacterial colonies were recovered from the sawdust samples, and 11 distinct isolates were screened for endoglucanase and xylanase activities. Eight screened isolates were positive for at least one of the enzymes tested while three were negative for both enzyme activities (Table 1). Three isolates (TC3, PO2, and PYO2) were positive for both endoglucanase and xylanase activities. A total of six isolates

Table 1. Enzyme activities of selected isolates based on their ability to degrade cellulose and xylan.

Isolate code	Halo zone diameter on CMC (average±SD)	Halo zone diameter on xylan (average±SD)
TC3	38.3±1.15	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	-
PYO2	**51.0±4.36**	**44.5±9.19**
MW1	-	-
PC1	-	-

(TC3, PO1, PO2, MCO, PYO2, and PYO1) were positive for endoglucanase activity while a total of five (TC3, BC2, PO2, TC1, and PYO2) were positive for xylanase activity (Table 1). Isolate PYO2 exhibited the second-highest endoglucanase activity (51.0 mm halo zone diameter) and xylanase activity (44.5 mm halo zone diameter) (Table 1). Because the appearances of the halo zones for both endoglucanase and xylanase activities for PO2 on the basis of the high activity exhibited by PYO2, the isolate was selected for detailed study.

Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where cellulosic materials are present are the best sources for isolating cellulolytic microorganisms [24]. Several microorganisms have been discovered over the past decades which have capacity to convert cellulose into simple sugars [25]; but the need for a newly isolated cellulose-degrading microorganism still continues [26].

Stability is the most critical factor when considering the suitability of enzymes for applications in industrial processes; hence, greater emphasis is placed on the effect of various parameters such as temperature, pH, and inhibiting irons on enzyme stability [27]. There has been great emphasis on the use of fungi because of their ability to produce large amounts of cellulases and hemicellulases, which are secreted into the medium for easy extraction and purification [28]. Nonetheless, a major obstacle for industrial application is that most cellulases of fungal origin lack thermal stability at high temperature [29]. Since most industrial processes are carried out at high temperature, the industrial applications of fungal origin cellulases are correspondingly limited [30]. Bacteria have high growth rate and enzyme production rate as compared to fungi and have good potential to be used in cellulase production [31]. The biotechnological industries have de-

veloped an alternative way of improving enzymes production by microorganisms through genetic engineering [32]. In addition, submerged fermentation has mainly been exploited for enzymes production purposes in the biotechnological sector [33].

The Basic Local Alignment Search Tool (BLAST) analyses of the 16S rDNA nucleotide sequences of PYO2 showed it to have 98% similarity to a *Chryseobacterium taichungense* strain, and the sequences were deposited in GenBank as *Chryseobacterium taichungense* SAMRC-UFH2 with accession number KU171370. From literature search, *Chryseobacterium* species are well known for the production of proteolytic enzymes. Nevertheless, there is scant literature on their ability to produce ligninolytic enzymes such as cellulase (endoglucanase), xylanase, ligninase, mannanase, laccase, and others. To the best of our knowledge, this is the first study on the production of endoglucanase and xylanase by *Chryseobacterium* species. In this present study, the test bacterial strain was isolated from decaying sawdust samples from a wood factory at Melani village in the Eastern Cape Province of South Africa.

The effect of incubation temperature (25-50ºC) on endoglucanase and xylanase production was investigated and the results are presented in Fig. 1. The highest activities for both endoglucanase (90 U/ml) and xylanase (420 U/ml) were recorded at 25ºC (Figs 1-2).
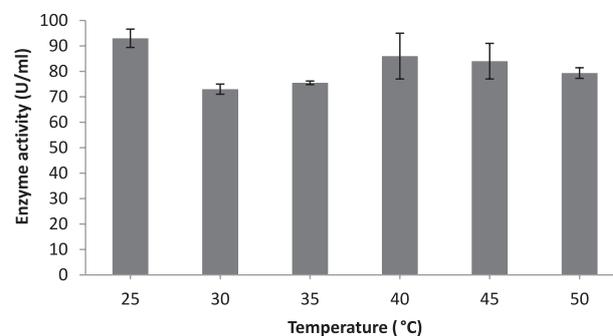
Fig. 1. Effect of incubation temperature on cellulase produced by *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates (p>0.05).
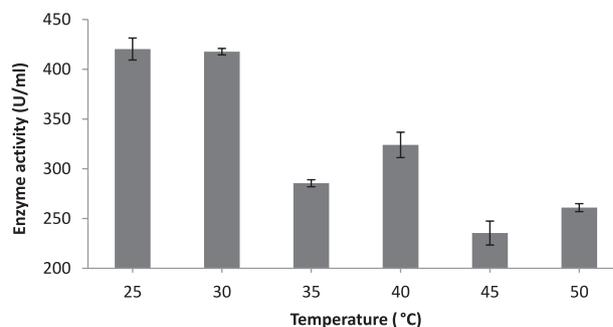
Fig. 2. Effect of incubation temperature on xylanase produced by *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates.

Xylanase activity decreased slightly at 30ºC, followed by a drastic decline at 35ºC and a slight recovery at 40ºC with the lowest activity of 235.5 U/ml recorded at 45ºC (Fig. 2). A non-parametric Friedman test of differences between endoglucanase and xylanase was conducted and showed an asymptotic significance of 0.306, which was not significant (p>0.05). The bacteria displayed high activity of endoglucanase and xylanase under favourable growth conditions. The test bacteria produced both endoglucanase and xylanase optimally at 25ºC (Figs 1-2). Sharma and colleagues [34] reported two bacterial isolates – *B. megaterium* R_5 and *B. subtilis* N_{11} – which displayed minimum xylanase activity at 25ºC, contrary to the results obtained in our study. In another study conducted by Bakare et al. [35], they found that the cellulase produced by *Pseudomonas fluorescence* was activated from 30 to 35ºC, showing the optimum temperature at 35ºC, whereas Odeniyi et al. [36] documented 37ºC as a favourable temperature for cellulase production by *Bacillus coagulans*. A *Streptomyces ruber* was found to show good cellulase production between 35-45ºC with maximum activity at 40ºC [37]. According to the report of Immanuel et al. [38], maximum endoglucanase activity from *Cellulomonas*, *Bacillus*, and *Micrococcus* sp. was recorded at 40ºC and neutral pH. *Geobacillus* sp. T1 could not grow at temperatures less than 40ºC; but at temperatures ranging from 40 to 60ºC this bacterium grew well and produced high amounts of CMCase activity [39]. Among the tested temperatures, the optimum temperature for cellulase production by *Geobacillus* sp. T1 was 50ºC. Similarly, Abdel-Fattah et al. [40] using optimization methods achieved maximum cellulase activity at 55ºC for a thermophilic *Geobacillus* strain isolated from soil.

Murugan et al. [41] and Kumar et al. [42] documented that xylanase produced by *Arthrobacter* sp. MTCC 6915 and *Bacillus pumilus* VLK-1 was found to be optimum at 30ºC. However, an increase in temperature beyond 30ºC led to a decline in the production of the enzyme. The optimum temperature for enzyme production by an organism may vary since it is likely to affect growth of the organism. Irfan et al. [43] reported that the three bacterial strains used in the study did not grow well at 25ºC and thus resulted in decreased enzyme production. *B. subtilis* BS04 showed highest enzyme production at 35ºC. Higher incubation temperature also is 50-60ºC, which has been reported by other researchers in the literature [44-49].

The impact of initial pH of growth medium on endoglucanase and xylanase production was assessed (Figs 3-4). The bacteria could produce endoglucanase at a wide pH range from 3-11; however, significantly high production was achieved at acidic conditions (pH 3-5), with the maximum activity of 101.3 U/ml at pH 6. From pH 7, the activity started to drop and slightly picked up at pH 8 and dropped again as pH increased with the lowest pH of 65 U/ml being observed at pH 11 (Fig. 3). As illustrated in Fig. 4, xylanase activity was investigated as well and the results showed that the highest xylanase activity of 384.3 U/ml was recorded at weak acidic pH of 6, which was similar to the pH for optimal
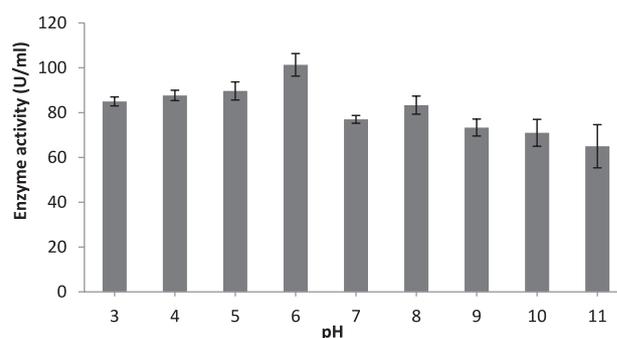
Fig. 3. Effect of initial culture medium pH on cellulase produced by *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates.
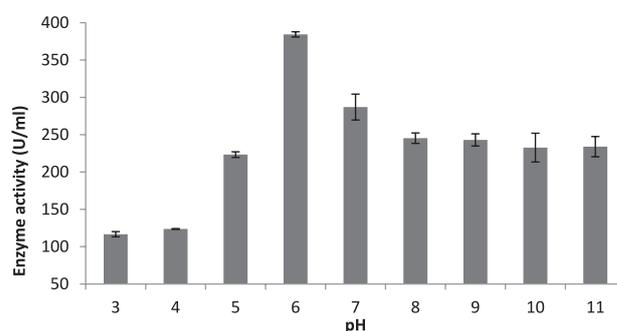
Fig. 4. Effect of initial culture medium pH on xylanase produced *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates.

endoglucanase production previously reported. After pH 6, a steady decrease in xylanase production was observed (Fig. 4). The alkaline conditions (8-11) supported higher xylanase production compared to acidic conditions (3-5).

A non-parametric Friedman test of differences between endoglucanase and xylanase activities was conducted and had an asymptotic significance of 0.460, which was not significant (p>0.05). Enzyme production by microorganism can be influenced by the pH of the medium in two ways; firstly, it can affect the growth of the microorganism, which in turn affects enzyme production; secondly, the unfavourable ionic environment which is caused by initial pH of the medium may lead to denaturation or inactivation of the produced enzyme [50-51]. The initial pH also affects the transportation of different enzymes across the cell membrane [52]. In our present study, the bacteria displayed the highest activity for enzyme production at pH 6 for both endoglucanase and xylanase (Figs 3-4). This indicates that slightly acidic conditions favoured both bacterial growth and enzyme production.

Xylanase production by various bacteria has been shown to be predominantly dependent on the pH of the medium. Acidic pH (4.0–6.0) generally favours endoglucanases while higher pH usually favours xylanases [27]. *Bacillus licheniformis* K_{22} has been reported to significantly exhibit high cellulase production

at pH 6.5 [34], an observation that is in agreement with the results reported in this study. Jaradat et al. [53] found that *Streptomyces* sp. (strain J2) displayed cellulase activity at a pH range of 4-7 with maximum activity at pH 6. In the case of an Ire and Berebon [54] report, maximum endo-glucanase pro-duction by *P. aeruginosa* was observed at pH 5.5 and least enzyme production was observed at pH 3 when corncob was used as a carbon source. According to a general observation of the effect of pH on enzyme production, optimum pH is required to maintain the three-dimensional shape of the active site of enzyme, and the change in pH results in loss of functional shape of enzyme due to alterations in the ionic bonding of the enzyme [55]. In addition, each microorganism requires an optimum pH for its growth and activity. Thus, the optimum pH for enzyme production varies from one bacterial strain to another [42]. The initial pH of the medium may influence many enzymatic systems and the transport of enzymes across the cell membrane.

Remarkably, *Geobacillus* sp. T1 was able to grow and produce cellulase over a wide range of pH (4.5-9.5). The highest enzyme production by *Geobacillus* sp. T1 was obtained at pH 6.5 [39]. Similarly, this observation is in collaboration with the report of Rastogi et al. [56], who found pH 7 for maximum cellulase production by another thermophilic *Geobacillus* strain with most similarity with *Geobacillus thermoleovorans*. In the case of the report of Han et al. [57], Sepahy et al. [45], and Bajaj and Manhas [58], and xylanase production by *Bacillus* NT 9, *Bacillus mojavensis* AG137, and *B. licheniformis* P11(C) it was observed over a wide pH range in acidic, neutral, and alkaline conditions with maximum production at medium between pH 8-10. Thus, these organisms have not only grown successfully at acidic and alkaline pH, but also generated considerable enzyme titre.

Most of the bacteria are dependent on pH and produce xylanase under high pH [17, 32]. For the production of cellulase by *Bacillus subtilis* and *Bacillus circulans*, pH in the range of 7.0-7.5 was found to be optimum by Ray et al. [59]. According to the report of Irfan et al. [43], pH 8 was the best among the pH values of the medium for xylanase production by *B. subtilis* BS04 and *B. megaterium* BM07 in submerged fermentation. In addition, lower pH, i.e., 4 and higher pH, i.e., 10 of the medium retards xylanase secretion drastically. These results indicated that both bacterial strains can tolerate alkaline conditions.

The effect of agitation speed on both endoglucanase and xylanase production was conducted using a shaking speed range of 0-200 rpm with 50 rpm incremental levels. The highest endoglucanase activity was observed at 50 rpm. Endoglucanase production was observed at agitation speed of 100-200 rpm and subsequently dropped slightly in a fluctuating pattern. The lowest endoglu-canase activity of 75.5 U/ml was observed at 0 rpm (Fig. 5). With respect to xylanase, activity increased steadily from the static conditions with the lowest xylanase activity of 118 U/ml, peaking at 150 rpm with 270.5 U/ml re-corded and then followed by a noticeable decline at 200 rpm (Fig. 6). A non-parametric Friedman test of dif-
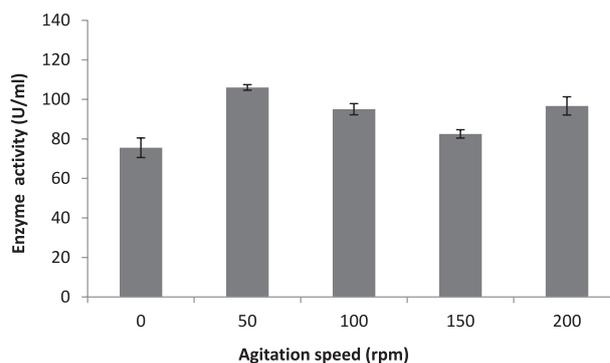
Fig. 5. Effect of agitation speed on cellulase produced by *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates.

ferences among enzyme activities for endoglucanase and xylanase was conducted and had an asymptotic significance of 0.406, which was significant ($p<0.05$). The results obtained from this present study showed that the bacteria produced endoglucanase optimally at agitation speed of 50 rpm, above which a fluctuating decrease in enzyme activity was observed (Fig. 5). The reduction in enzyme activity with increasing agitation speed may be due to shearing of the cells [20]. The report of EI-Refai et al. [60] concurs with our findings, in which the best agitation rate for cellulase was found to be between 0 and 50 rpm. For xylanase, production was optimal at agitation speed of 150 rpm.

The reason for low enzyme activity at low agitation speed may also be attributed to the limitation of the dissolved oxygen and to the inadequate mixing of the medium components due to the low speed used. However, the strain displayed a very good pattern for the production of xylanase (Fig. 6). The highest xylanase production by *B. pumilus* VLK-1 was observed at an agitation rate of 200 rpm after 48 h of incubation [42]. The enzyme yield was low when the agitation rate was increased or decreased beyond 200 rpm. This was consistent with
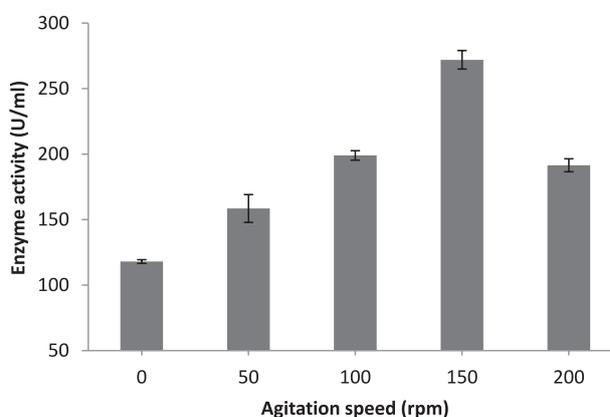
Fig. 6. Effect of agitation speed on xylanase produced by *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates.
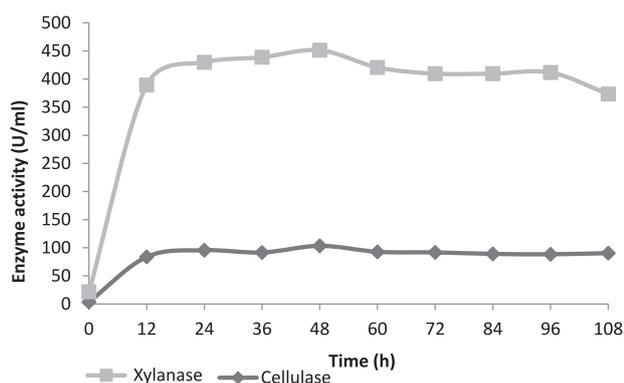
Fig. 7. Time course of cellulase and xylanase produced by *Chryseobacterium* sp. The values are means of the enzyme activities±SD of the triplicates.

earlier reports [45, 61]. The lower enzyme level under low agitation conditions may be attributed to the dissolved oxygen limitation for cell growth, improper mixing of media components, and cell clumping.

Time course experimentation was conducted using the optimum culture conditions obtained for the endoglucanase and xylanase production and the results obtained are illustrated in Fig. 7. The bacteria showed a steady increase in endoglucanase production and attained a peak in 48 h with enzyme activity of 103.3 U/ml. Subsequently, endoglucanase production declined to about 92 U/ml and remained relatively constant at this level for the rest of the fermentation period. Moreover, xylanase production increased steadily, attaining a peak in 48 h with an activity of 348 U/ml and thereafter declined to 283 U/ml at 108 h (Fig. 7). A non-parametric Friedman test of differences among enzyme activities for endoglucanase and xylanase was conducted and had an asymptotic significance of 0.071, which was considered as weakly significant (p slightly>0.05). In order to obtain efficient enzyme production, it is critical to consider optimum cultivation conditions for a specific microorganism. For time course assay, the optimum culture conditions discussed above were used for the experiment. Time course experimentation was used to determine the incubation time at which the enzymes will be maximally produced under optimized culture conditions in order to know what may be the bacterial strain can produce the enzyme of interest within a shorter period of time. The advantage of a shorter incubation period for enzyme production is that the bulk production of the enzymes may be achieved over a short period of time, which implies cost-containing possibilities on an industrial production scale. Fermentation time for maximum enzyme production varies among different bacteria and is dependent upon the type of the organism, culture/environmental conditions, and genetic makeup of the organism.

In this present study, *Chryseobacterium* sp. was observed to display highest enzyme production for both endoglucanase and xylanase after 48 h of cultivation period and, subsequently, enzyme production started to

decrease uniformly, resulting in almost a constant pattern for endoglucanase, and the lowest enzyme activity was observed after 108 h for xylanase (Fig. 7). It has been suggested that the depletion of nutrients in the fermentation medium causes the reduction in enzyme production and this usually occurs after the log phase or at the beginning of the death phase of the incubation period [20]. On the contrary, the study on *B. pumilus* EB3 by Ariffin et al. [62] showed lesser CMCase activity (0.079 IU/ml) after 24 h of incubation. Nagendra et al. [63] noticed that the optimum incubation period for cellulase was also within 96 h in late stationary phase of growth. Lugani et al. [55] reported that maximum cellulase production by *Bacillus* sp. was observed at 96 h of incubation, and that the activity was significantly reduced after 96 h due to depletion of nutrients or accumulation of other by-products in the fermentation media, which lead to a decrease in cellulase activity [64]. The maximum cellulase production by thermophilic *Bacillus* sp. was observed after 96 h of incubation [24]. In collaboration with our findings in this present study for xylanase, optimal production after 48 h of incubation, several researchers have documented this cultivation time for their xylanase production. For example, *Bacillus* sp. NZ produced maximum enzyme titre near the end of the exponential phase and early stationary phase [65]. *B. cereus* [66] and *B. halodurans* PPKS-2 [67] showed maximum xylanase production after 48 h. Gupta and Kar [68] studied xylanase production by *Bacillus* sp. and reported that maximum xylanase production was observed in 48 h and 72 h using wheat bran and corn cob as a substrate, respectively. Mrudula and Shyam [69] reported 48 h of fermentation time for maximum production of protease from *B. megaterium* MTTC 2444. Sepahy et al. [45] reported a fermentation period of 48 h by *Bacillus mojavensis* AG137 in submerged fermentation using oat bran as a substrate. On the other hand, contrary to our findings, a shorter cultivation period was recorded for maximum xylanase production by *Bacillus* sp. after 24 h using digested bran as a substrate [46].

Conclusions

The optimization of media is an important parameter for the development of fermentation technology because a cost-effective media results in the reduction of cost of enzyme. Hence, the optimization of all the process parameters are being considered as pre-requisites to make the process of enzyme production cost-effective on a large scale. Findings from this study indicated that nutrients and cultural properties played a pivotal role in enzyme production. *Chryseobacterium* species isolated from decaying sawdust samples collected from a wood factory in a village in South Africa's Eastern Cape Province displayed similar optimum growth conditions for both endoglucanase and xylanase production for almost all the parameters that were investigated in this present study, with the exception of agitation speed. A similar pattern for time course assay for both cellulase and xylanase

production was observed. This study confirms the potential of the test bacteria in lignocellulosic materials degradation and that the detailed characterization of the purified enzymes and development of process conditions for their pilot scale production is an on-going study in our research group. In addition, the molecular biology on the genetic basis of enzyme production and how their yields can be improved through genetic engineering is currently under investigation in our laboratory.

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