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Optimization of Cellulase and Xylanase Production by *Micrococcus* Species under Submerged Fermentation

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Abstract: This paper reports on the optimization of culture conditions for cellulase and xylanase production by bacterial isolate from lignocellulosic biomass. The bacterial isolate was screened for cellulase and xylanase production on carboxyl methyl cellulose (CMC) and birch wood xylan as substrates, respectively. One bacterial isolate showing the highest halo zone diameter (isolate PLY1) was selected for detailed studies. The analysis of the 16S ribosomal ribonucleic acid (rRNA) gene nucleotide sequence of PLY1 revealed it to have 98% similarity to *Micrococcus luteus* strain Fse9 and the sequence was deposited in the GenBank as *Micrococcus luteus* strain SAMRC-UFH3 with accession number KU171371. Cellulase production was achieved in the presence of CMC (1% *w*/*v*) under an incubation temperature of 25 °C (198 U/mL), pH 5 (173 U/mL), agitation speed 50 rpm (173 U/mL) and incubation period of 96 h (102 U/mL). Xylanase was produced maximally when birch wood xylan (1% *w*/*v*) was used as the substrate at 25 °C (1007 U/mL), pH 10 (2487 U/mL), 200 rpm (1814 U/mL), and under an incubation period of 84 h (1296 U/mL). Our findings showed that *Micrococcus* sp. SAMRC-UFH3 appears to be a potentially important candidate for lignocellulosic waste degradation and other relevant industrial applications.

Keywords: lignocellulosic biomass; *Micrococcus* sp. SAMRC-UFH3; culture conditions; cellulase; Xylanase

1. Introduction

Lignocellulose is the main structural constituent of woody plants and non-woody plants, such as grass, that constitutes a major source of renewable organic matter, comprised of lignin, hemicellulose, and cellulose [1]. The constituents of lignocellulosic materials are suitable substrates for biotechnological products of economic importance [2]. The bulk of lignocellulosic wastes are obtained from horticulture, industries of paper-pulp, timber, and other agro allied industries. Large quantities of lignocellulosic wastes are frequently disposed through burning, which is not a viable and sustainable disposal means [3].

Lignocellulolytic enzymes have numerous industrial applications in brewing and wine, chemicals, food, fuel, animal feed, pulp and paper, textile and laundry, and agriculture [3]. Cellulase is a complex enzyme system that plays an important role in the environment in the degradation of cellulose with subsequent conversion into useful products. It can also be utilized as a part of textile industry for "bio-cleaning" of fabrics and making stone washed look of denims, as well as in the household cleansers for enhancing fabric softness and brightness. It can also be used as part of cotton arrangements, wool,

and coloring treatments, in effluent treatment, and pharmaceutical industries. Xylanase, just as with cellulase, plays a critical role in the degradation of plant biomass; hence, its production has attracted the attention of many researchers. It has various applications, incorporating biodegradation of lignocelluloses in food, animal feed, textiles, bio-deinking of waste paper, kraft pulp bleaching, and bio-pulping in the pulp and paper industry [4]. Xylanase is very important to pulp and paper industries because the hydrolysis of xylan encourages the removal of lignin from pulp and consequently diminish the utilization of chlorine as a bleaching agent [5]. Pulps derived during kraft pulping and waste paper deinking processes at high temperature and alkaline pH need novel microbial isolates with the ability to produce thermo-tolerant and alkalophilic xylanases [6,7].

The search for potential sources of cellulolytic enzymes is continuing in the interest of successful bioconversion of lignocellulosic biomass. Several enzymes of fungal and bacterial origin have been evaluated for their ability to degrade cellulosic substrates into glucose monomers [8]. Cellulase-producing bacteria can be isolated and characterized from a variety of sources, such as soil, decayed plant materials, hot springs, organic matter, and feces of ruminants and composts [9]. Researchers are, however, continually searching for new microorganisms with higher cellulase activity [10]. Cellulase-producing microorganisms include; bacteria, yeast, and fungi [11–15]. One of the bacterial species that has been implicated in lignocellolytic and xylanolytic enzymes production is the genus of Micrococcus. Among the *Micrococcus* species, *Micrococcus luteus* is a Gram-positive, non-motile cocci, arranged in tetrads, non-spore forming, pigmented bacteria found in air, water, soil, and even on our skin. It can be a saprophytic or commensal microbe and sometimes acts as an opportunistic pathogen [16,17]. *Micrococcus luteus* has unique ways to remediate sites co-contaminated with metal and organic pollutants by compacting with these contaminants [18,19]. It also has the ability to degrade olefinic compounds and hydrocarbons [20].

The effective bio-conversion of cellulosic materials to value-added products mainly depends on the nature of cellulose, basis of cellulolytic enzymes and cultivation conditions [21]. There are several factors that positively influence the production of cellulase and such factors include; cellulose quality, air circulation, temperature, incubation period, carbon sources, composition, and pH of the medium [22]. In this study, we report on cellulase and xylanase production potential by a *Micrococcus* sp. SAMRC-UFH3 isolated from decaying biomass (sawdust) in the Eastern Cape Province, South Africa, as part of a larger study on the development of a bacterial consortium for the use in bioethanol production from biomass.

2. Materials and Methods

2.1. Study Area and Sample Collection

The decaying plant biomass (sawdust) samples were randomly and aseptically collected from different depths of a sawdust waste dump in a wood factory in Melani village in the Eastern Cape Province of South Africa. The samples were aseptically collected and placed in sterilized containers and later transported on ice packs to the laboratory for processing.

2.2. Isolation of Cellulose and Xylan-Degrading Bacteria

Isolation of cellulose and xylan-degrading bacteria was conducted, as described by Jeffrey et al. [23] with some modifications. About 1 g of the sawdust was mixed with 100 mL of sterile distilled water (sdH_2O). The sawdust suspension was pre-treated at 55 °C for 15 min and later agitated vigorously below room temperature (25 ± 20 °C) on an orbital shaker at 200 rpm for 1 h. A serial dilution of the suspension was carried out using sterile phosphate-buffered saline (PBS) and 100 µL of the diluted suspension was spread on agar containing the growth media (M1 medium supplemented with nystatin (50 mg/L) and nalidixic acid (25 mg/L), and the plates were later incubated at 30 °C for 7–14 days depending on the colonies development. Subsequently, the bacterial colonies were selected based on their morphology, size, and colour and streaked again onto fresh

M1 medium agar plates for purity and incubated further at 30 °C for 7–14 days. After purification, the colonies were compared visually to eliminate those with similar colonial characteristics and discrete colonies were sub-cultured again and the stock cultures were prepared from the pure colonies and later stored at −80 °C in 20% glycerol stock until further use.

2.3. Growth Media Compositions

The growth media used in the study included M1 agar supplemented with nystatin (50 mg/L) and nalidixic acid (25 mg/L) to retard fungal growth and Gram-negative bacteria, respectively. M1 agar contains yeast extract 5.0 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, magnesium sulfate $7H_2O$ 0.5 g/L, sodium pyruvate 0.3 g/L, and agar 15 g/L. Luria Bertani (LB) broth contained tryptone 10 g/L, NaCl 10 g/L and yeast extract 5 g/L. Basal medium was composed of carboxymethyl cellulose (CMC) 5 g/L, $NaNO_3$ 1 g/L, K_2HPO_4 1 g/L, KCl 1 g/L, $MgSO_4$ 1 g/L, yeast extract 0.5 g/L and agar 15 g/L in distilled water. For basal medium containing xylan, CMC (5 g) was replaced with birch wood xylan (5 g) [24].

2.4. Screening for Cellulase and Xylanase-Producing Bacteria

The selected purified colonies were cultured on carboxymethyl cellulose (CMC) and xylan-containing basal media (XBM) prepared in accordance with the description of Pointing et al. [25]. Cellulolytic and xylanolytic bacteria were qualitatively identified using Gram's iodine stain (0.25% *w*/*v* aqueous I_2 and KI) solution [26]. The bacterial isolates were grown in 10 mL of LB broth for 24 h at 30 °C, with agitation at 200 rpm and slow growing isolates were left to incubate for an additional 48 h. The inocula were prepared from the fermented broths after standardizing to optical density 0.1 at OD_{600} and the activity of cellulase was determined by Gram's Iodine method [24]. M1 agar medium or basal media (BM) supplemented with CMC (4% *w*/*v*) or xylan (1.6% *w*/*v*) agar was prepared and sterilized by autoclaving at 121 °C for 15 min. An aliquot (5 µL) of standardized cultures were spread onto the agar plates containing carboxymethyl cellulase (CMC) or xylan basal medium and then incubated at 30 °C for 48 h. The plates were flooded with Gram's iodine solution (KI (2 g) and I_2 (1 g) in 300 mL of distilled water) for 5 min and left in a sterilized laminar flow for 5 min in order to visualize cellulase and xylanase activities. After 72–96 h of incubation period, the formation of halo zone from the point of spot inoculation of the broth culture outwards indicates substrate (CMC or Xylan) degradation by the enzyme produced by the bacterial isolate. Degradation around the colonies appeared as a yellow-opaque zone against a blue colour for un-degraded CMC or xylan [26].

2.5. Identification of the Bacterial Isolate by 16S rRNA Gene Sequencing

The bacterial isolate (PLY1) was identified by 16S rRNA gene nucleotide sequencing. The genomic DNA (deoxyribonucleic acid) used for the PCR was prepared from pure culture of the isolate PLY1 grown on nutrient agar at 30 °C. The collective genomic DNA from the microscopic organisms was isolated utilizing the DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification pack, Helini Bio atoms, India), as indicated by the manufacturer's protocol. The 16S rDNA gene fragment was amplified using general universal primers (Actino specific forward primer 5′-GCCTAACACATGCAAGTCGA-3′ and Actino-specific reverse primer 3′-CGTATTACCGCGGCTGCTGG-5′. The conditions of the PCR were initialised with starting denaturation at 94 °C for 3 min removed after 30 cycles of enhancement (denaturation at 94 °C for 60 s, annealing at 5 °C for 60 s, and extension at 72 °C for 60 s) trailed by certain expansion at 72 °C for 5 min. The amplification reactions were carried out in a total volume of 50 µL in a gradient PCR. The PCR product was visualized using 1% agarose gel and the fragment was purified (Helini Pure Fast PCR tidy up unit, Helini Bio particles, India) as indicated by the maker's guidelines and the band was visualized under UV Transilluminator (XD-79.WL/26MX, UVitec, Cambridge, UK). The direct sequencing of PCR product was performed by dideoxy chain end strategy utilizing ABI PRISM®

3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at Inqaba Biotech South Africa. The results obtained were aligned with published 16S rDNA sequences in the GenBank through a BLAST sequence tool from the National Centre for Biotechnology Information (NCBI) database (Bethesda, MD, USA) [27].

2.6. Cellulase and Xylanase Production

Enzyme production was carried out by submerged fermentation procedures in accordance with the method of Sharma and Bajaj [28], using basal salt medium containing CMC and birch wood xylan as carbon sources at a final concentration of 1% (*w*/*v*). The bacterial isolate was refreshed in basal salt medium supplemented with CMC or birch wood xylan and incubated for 48 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 200 rpm at 45 °C. The enzyme activity was assayed periodically at different time intervals. Two milliliters of the fermented broth was centrifuged at 10,000× g for 5 min at 4 °C to obtain the supernatant that was for assaying cellulase and xylanase activities after appropriate dilutions [29].

2.7. Cellulase and Xylanase Activity

Cellulase and xylanase activities were measured as described by Swe [30]. After centrifugation, 0.2 mL of culture supernatant was added to 1.8 mL of 1% CMC/xylan prepared in 0.05 M phosphate buffer (pH 7) in a test tube and incubated at 40 °C for 30 min. Three milliliters of dinitrosalicylic acid (DNS) reagent (1 g of 3,5-dinitrosallicylic acid in 20 mL 2 M NaOH) was added to terminate the reaction and the tubes were placed in a water bath at 100 °C for 15 min. Rochelle's salt solution (1 mL) was added to stabilize the color before determining absorbance at 575 nm with 0.05 M phosphate buffer used as a blank. One unit of enzyme activity was defined as the measure of enzyme that released 1 µM of glucose/xylose per min.

2.8. Optimization of Culture Conditions for Enzyme Production

2.8.1. Effect of Temperature

The effect of incubation temperature on cellulase and xylanase production was assessed at different temperatures, ranging from 25–50 °C in accordance to the method of Adhyaru et al. [10,31].

2.8.2. Effect of Initial pH of Growth Medium

The effect of initial pH of the growth medium on cellulase or xylanase production was examined by varying the pH from 3–11. The pH was adjusted by using 1 M HCl or NaOH prior to autoclaving [31].

2.8.3. Effect of Agitation Speed

The effect of the rate of agitation on cellulase or xylanase production was assessed at 0–200 rpm at 50 rpm intervals [31].

2.9. Time Course for Cellulase and Xylanase Production

In order to achieve maximum cellulase or xylanase production, optimum growth conditions were used over an incubation period of 108 h. The crude enzyme was extracted and assayed at regular intervals of 6 h [31].

2.10. Data Analysis

The experimental data was expressed as mean ± standard deviation (SD) of the three replicates. Statistical analysis was done by using MINITAB program (version 12 for windows (Minitab Inc., State

College, PA, USA). One-way analysis of variance (ANOVA) was used to compare the data among the cellulase and xylanase fractions with the control. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and Screening for Cellulase and Xylanase Activity

In this study, cellulase and xylanase-producing bacterial isolates were obtained from sawdust samples/wood shavings from Melani village around Alice Town in the Eastern Cape Province of South Africa. Cellulolytic and xylanolytic bacteria were presumptively identified by the qualitative plate assay method. The sample was pre-heated and, in addition, M1 agar was supplemented with nalidixic acid and nystatin to reduce the growth of other microorganisms. Although phenology is not an accurate way to separate colonies, the bacterial isolates were selected and further purified based on the morphological characteristics on the agar plate. In this study, a total of 58 bacterial isolates were obtained from wood shavings and nine isolates with marked distinct colony characteristics were screened for cellulose degradation. Seven of these isolates were positive when cellulose was used as a sole carbon source, whereas three isolates were able to degrade xylan as the sole source of carbon.

Cellulase and xylanase activities were indicated by the halo zones around the colonies. The diameters of halo zones were measured and the results are depicted in Table 1. Of all positive isolates, PLY1, MLY10, and TLY3 showed both xylanase and cellulase activities; however, PLY1 was chosen for further work based on its highest cumulative activity (114 mm; Table 1).

Table 1. Summary of the relative potentials of the bacterial isolates for cellulase and xylanase production.

Isolate Code	Halo Zone Diameter (mm ± SD) on CMC	Halo Zone Diameter (mm ± SD) on Xylan	Halo Zone Diameter of the Two Enzymes (mm ± SD)
PLY1	**71 ± 7.5**	**43 ± 3.58**	**114**
BLY2	11.6 ± 2.7	-	11.6
BLY10	11.4 ± 1.8	-	11.4
BLC6	22.2 ± 2.4	-	22.2
MLY10	72.7 ± 6.4	40.7 ± 4.7	113.4
TLW8	-	-	0
TLY1	12.5 ± 4.4	-	12.5
TLY3	32.7 ± 4.7	58.7 ± 3.2	91.4

3.2. Identification of the Bacterial Isolates by 16S rRNA Gene Nucleotide Sequencing

Basic Local Alignment Search Tool (BLAST) results of the 16S rRNA gene nucleotide sequence of PLY1 revealed it to have 98% similarity to *Micrococcus luteus* strain Fse9 and the sequence was deposited in GenBank as *Micrococcus luteus* strain SAMRC-UFH3 with accession number KU171371.

3.3. Optimization of Culture Conditions for Enzymes Production by Micrococcus sp. SAMRC-UFH3

3.3.1. Effect of Temperature on Cellulase and Xylanase Production

The effect of temperature on cellulase and xylanase production by the test bacteria was investigated and the results are shown in Figure 1a,b. It was observed that the optimal temperature for cellulase activity was 25 °C with enzyme activity of 198 U/mL (Figure 1) and cellulase activity decreased with increasing temperature until 40 °C after which a gradual increase was observed up to 50 °C. On the other hand, optimal temperature for xylanase production was achieved at 25 °C with enzyme activity of 1007 U/mL (Figure 2). As temperature increased from 25 °C, xylanase activity declined to about 650 U/mL at 45 °C and then increased to about 765 U/mL at 50 °C (Figure 2).

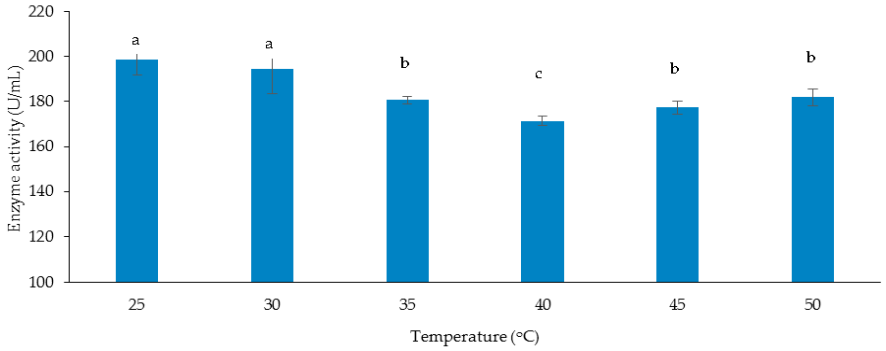

Figure 1. Effect of temperature on cellulase production by *Micrococcus* sp. SAMRC-UFH3. Data are presented as mean ± SD of three replicates. Bar graphs with different letters shows significant difference ($p < 0.05$).

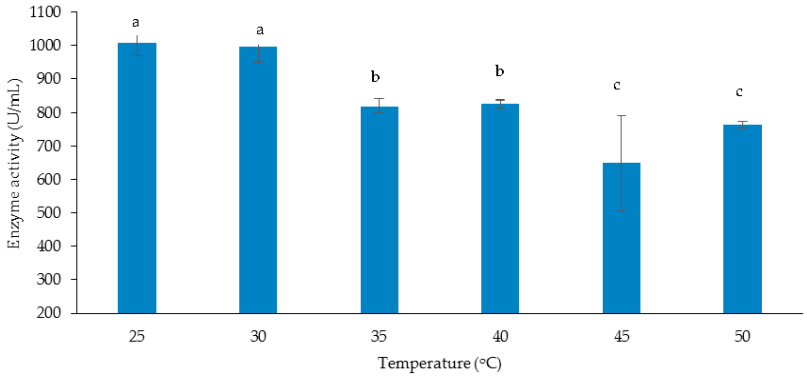

Figure 2. Effect of temperature on xylanase production by *Micrococcus* sp. SAMRC-UFH3. Data are presented as mean ± SD of three replicates. Bar graphs with different letters show significant difference ($p < 0.05$).

3.3.2. Effect of Initial pH of Growth Medium on Cellulase and Xylanase Production

The effect of initial pH of growth medium on cellulase and xylanase production by *Micrococcus* sp. SAMRC-UFH3 was assessed over an extensive pH range of 3–11. The optimum pH for cellulase production was pH 5 with enzyme activity of 173 U/mL (Figure 3), while for xylanase production, the optimum pH was observed to be pH 10 with enzyme activity of 2487 U/mL (Figure 4).

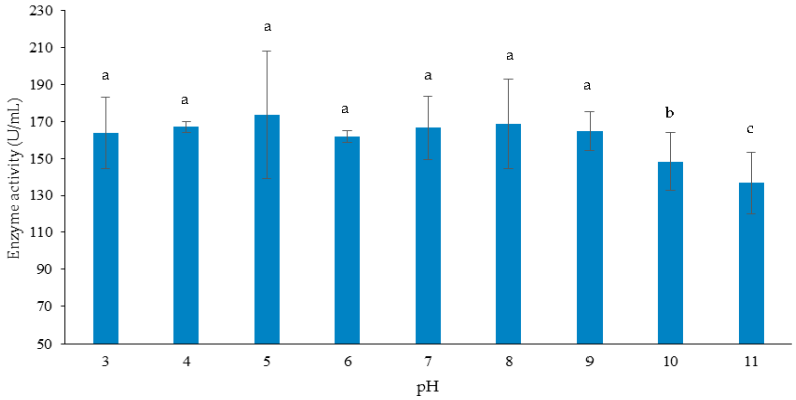

Figure 3. Effect of initial pH of growth medium on cellulase production by *Micrococcus* sp. SAMRC-UFH3. Data are presented as mean ± SD of three replicates. Bar graphs with different letters show significant difference ($p < 0.05$).

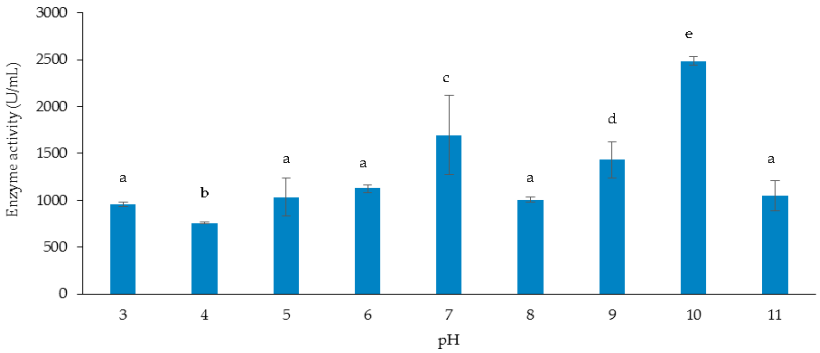

Figure 4. Effect of initial pH of growth medium on xylanase production by *Micrococcus* sp. SAMRC-UFH3. Data are presented as mean ± SD of three replicates. Bar graphs with different letters show significant difference ($p < 0.05$).

3.3.3. Effect of Agitation Speed on Cellulase and Xylanase Production

With respect to the effect of agitation rate on the enzyme production by *Micrococcus* sp. SAMRC-UFH3, no appreciable difference between static and 50 rpm for cellulase production was observed, although the highest activity was obtained at 50 rpm with activity of 173 U/mL (Figure 5). Further increase in agitation speed resulted in a decrease of activity with the lowest activity recorded at 200 rpm. For xylanase production, activity (1814 U/mL) was optimal at 200 (Figure 6).

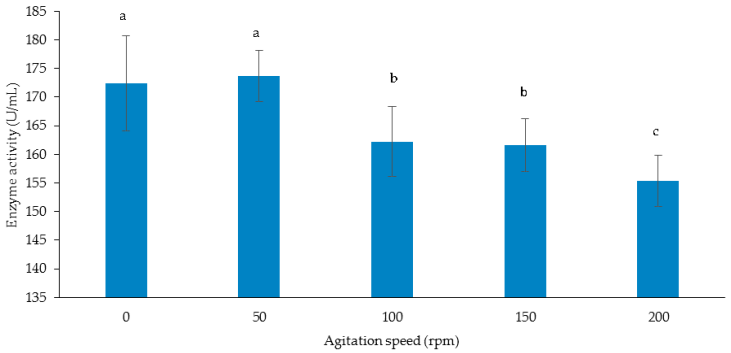

Figure 5. Effect of agitation speed on cellulase production by *Micrococcus* sp. SAMRC-UFH3. Data are presented as mean ± SD of three replicates. Bar graphs with different letters show significant difference ($p < 0.05$).

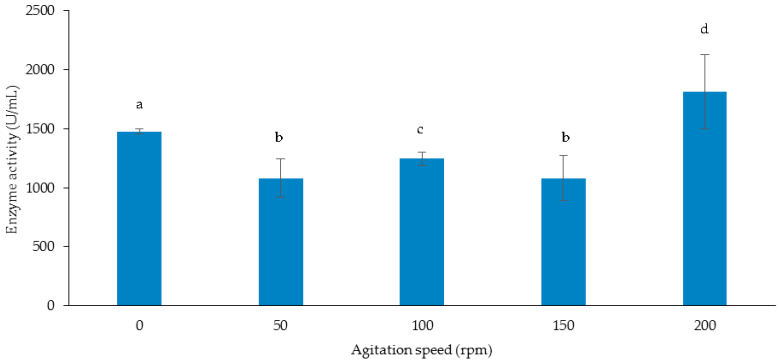

Figure 6. Effect of agitation speed on xylanase production by *Micrococcus* sp. SAMRC-UFH3. Data are presented as mean ± SD of three replicates. Bar graphs with different letters show significant differences ($p < 0.05$).

3.4. Time Course of Cellulase and Xylanase Production

Optimum growth conditions for *Micrococcus* sp. SAMRC-UFH3 were used to carry out a time course assay of cellulase and xylanase production over an incubation period of 108 h. Results presented in Figure 7 show that cellulase production progressively increased for the first 14–16 h, and remained relatively constant thereafter for the next 60 h, attaining maximum activity of 102 U/mL at 96 h before declining at 108 h. Xylanase production increased progressively and peaked at 84 h attaining maximum enzyme activity of 1296 U/mL followed by a rapid decline over the next 24 h (Figure 8).

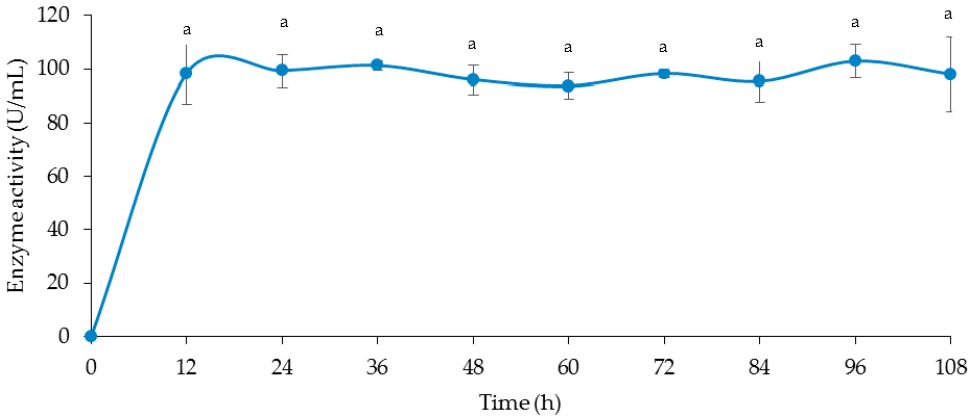

Figure 7. Time course assay of cellulase production by *Micrococcus* sp. SAMRC-UFH3 using CMC as the substrate. Data are presented as mean ± SD of three replicates. The same letter shows that there is no significant difference for all the treatment cases ($p > 0.05$).

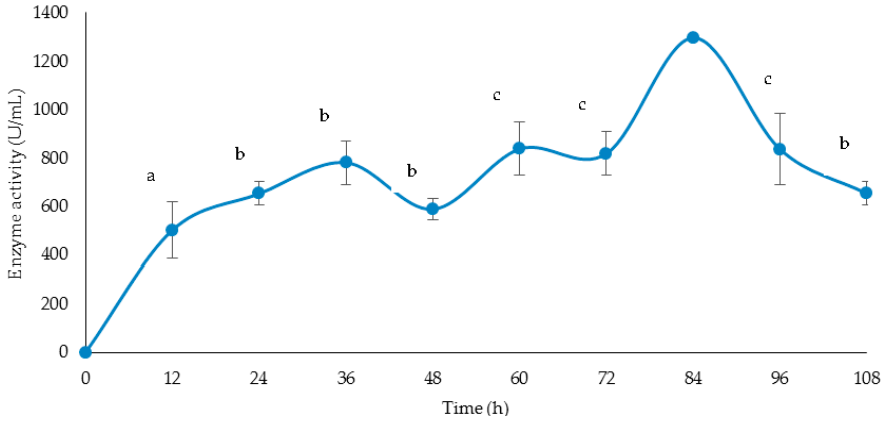

Figure 8. Time course assay for xylanase production by *Micrococcus* sp. SAMRC-UFH3 using xylan as the substrate. Data are presented as mean ± SD of three replicates. Graphs with different letters show significant difference ($p < 0.05$).

4. Discussion

Lignocellulosic biomass is an alternative and cost effective resource for the production of biofuels due to its abundance and renewability [32]. Sources for lignocellulosic biomass include, among others, leaves, stems, and stalks from sources such as corn fiber, corn stover, sugarcane bagasse, rice hulls, woody crops, and forest residues. In the technologies for effectively converting cellulosic biomass to fermentable sugars, cellulases are regarded as one of the key elements.

Cellulases have a wide range of applications, such as in food, brewery, wine, pulp and paper, textile, detergent, feed, and agriculture. Cellulases are also used in the textile industry for bio-polishing of fabrics and making the stone-washed look of denims and in household laundry detergents for

improving fabric softness and brightness. Cellulases are used in cotton preparations, wool and dyeing treatments, in effluent treatment, and also in pharmaceutical industries [17].

Potential applications of xylanases include the bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in the consistency of beer, and the digestibility of animal feed stock [4]. The application of xylanase in the saccharification of xylan in agro-wastes and agro-foods intensifies the need for exploiting the potential role of them in biotechnology.

Cellulolytic and xylanolytic enzymes are synthesized by a number of microorganisms. Among the microbes, bacteria are more reliable due to their adaptability to different environmental conditions, relatively fast growth than any other microbes and extremely have the capacity to produce highly stable enzymes complement which serves as highly potent sources of individually important enzymes [33,34]. The study focused on the isolation and screening of bacteria from sawdust samples collected from Melani Village (wood factory) with promising cellulose and xylan-degrading potentials for modification and decomposition of lignocellulosic biomass for industrial application.

Micrococcus sp. SAMRC-UFH3 was found to degrade both cellulose and xylan (Table 1) to varying degrees as shown by different halo zone diameter measurements after staining with Gram's iodine solution. Cellulose was the preferred substrate with 71 mm halo zone diameter on CMC supplemented agar medium compared to xylan that had a smaller halo zone diameter of 43 mm on birch wood xylan agar. However, despite the larger halo zone in cellulose-supplemented medium compared to xylan-supplemented medium, the hydrolytic action of these enzymes is not a reflection of the size of the halo zone but depends on the environment from which they are isolated [35]. This is an indication that the test bacteria have the possibility to secrete a wide variety of enzymes, which may assist natural selection of the microorganisms to survive in a competitive environment [23]. The relative hydrolytic capability of the enzymes produced by the bacterial isolates went from weak; moderate to strong depending upon the diameter of the clear zones of hydrolysis enclosed surrounding the colonies in the screening process [36].

Degradation of cellulosic materials is a multifaceted process and several microbial cellulolytic enzymes are required [37]. Cellulose and hemicellulose can be degraded by chemical hydrolysis (using acids or alkali) or enzymatic hydrolysis. Several methods of acid hydrolysis using sulphuric or hydrochloric acid at varying concentrations, temperatures, and pressures have been used in the industry for lignocellulose degradation [38,39]. The degradation of lignocellulosic materials involves a complex depolymerisation process of the polysaccharide components into sugar monomers that requires several different types of enzymes [40]. Enzymes known as cellulases are responsible for the breakdown of cellulose into glucose monomers.

The microbial production of enzymes depends on the genetic nature of the organism, the physio-chemical parameters, the fermentation medium components and their concentrations. Thus, optimization of the culture conditions is important to achieve maximum yields and to produce a compelling bioprocess framework for industrial applications. Therefore, a judicious selection of these parameters can dramatically improve the enzyme yield. Several authors have reported increased enzyme yield upon optimization of bioprocess conditions [22,41–43].

Cellulase systems consist of endoglucanases, exoglucanase, and β-glucosidase; the synergy of all these enzymes enables the hydrolysis of cellulose to glucose to be made possible [44]. Nutrient sources were found to be the important factor for the cellulase production. Since carbon is considered as the primary nutrient for the bacteria [45], carboxymethyl cellulose (CMC), and xylan were utilized for the cellulase and xylanase production, respectively, in this study.

It has been reported that temperature has an effect on the activity of both cellulase and xylanase produced by *Micrococcus* sp. SAMRC-UFH3 [46]. In our current study, maximum production of both cellulase (198 U/mL) and xylanase (1007 U/mL) were observed at 25 °C by the test bacteria (Figures 1 and 2). The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane. On the contrary, the optimum temperature

for maximum growth of *Bacillus subtilis* 115 and *Bacillus subtilis* for cellulase production was reported at 40 °C [47]. According to the report documented by Ray et al. [10], the minimum cellulase yield was observed when fermentation was carried out at 45 °C, while maximum yield was obtained at 40 °C by both *Bacillus subtilis* and *Bacillus circulans*. Nandimath et al. [32] reported that *Pseudomonas* sp. produced maximum cellulase production at 30 °C. These findings do not differ significantly from those documented by Bakare et al. [48] who found that the cellulase enzyme produced by *Pseudomonas fluorescens* was activated at 30 to 35 °C and peaked at 35 °C. *Geobacillus* sp. T1 could not grow at temperatures less than 40 °C, but at temperatures ranging from 40–60 °C, this bacterium grew well, showing increased cellulase activity. *Geobacillus* sp. T1 on the other hand, produced cellulase optimally at 50 °C [49]. Several reported studies indicate varying temperature optimal for the production of both cellulase and xylanase by different microorganisms. Abdel-Fattah et al. [50] recorded maximum cellulase activity at 55 °C for a thermophilic *Geobacillus* strain isolated from soil, whereas, Monisha et al. [51] and Sepahy et al. [52] reported optimum temperature of 37 °C for xylanase production by both *B. pumilus* and *B. mojavensis* AG137, respectively, in submerged fermentation. On the hand other, different strains of *Bacillus* sp. gave a maximum yield of xylanase production at incubation temperatures of 45 °C and 55 °C [53].

The pH of the fermentation medium is reported to impact the growth of any microbial strain and consequent metabolic product formation [54]. In addition, many enzymatic processes and the transport of various components across the cell membrane are strongly affected by the pH of the medium [55]. A optimum pH is required to maintain the three-dimensional shape of the active site of enzymes and the change in pH results in a loss of functional shape of the enzyme due to alterations in the ionic bonding of the enzyme. In our study, cellulase and xylanase production by *Micrococcus* sp. SAMRC-UFH3 under submerged fermentation conditions was monitored over pH ranging from 3–11. The results presented in Figures 3 and 4 suggest that the pH of the fermentation medium influences enzyme production. Hence, the maximal production for cellulase was observed at pH 5 (173 U/L). Xylanase production was optimum at pH 10 with enzyme activity of 2487 U/mL. In another study reported by Nandimath et al. [32], cellulase was found to be produced effectively within 60 min and at pH 5. Liang et al. [56] further noted that growth medium pH strongly influences many enzymatic reactions by affecting the transport of a number of chemical products and enzymes across the cell membrane. For example, for the production of cellulase by *Bacillus subtilis* and *Bacillus circulans*, initial pH of growth medium in the range of 7–7.5 was found to be optimum [6]. The optimum pH for the maximum cellulase production by both *Bacillus* sp. 8 and *Bacillus* sp. 17 was found to be 7 [57]. The maximum CMCase activity of 0.29 IU/mL was reported in a study on cellulase production by *Bacillus* sp. at pH 7 [58]. From previous studies, only few xylanases have been reported to be active and stable at alkaline pH and high temperatures [59,60]. Increase or decrease in the pH of the medium resulted in declined enzyme production. Pereira et al. [61] and Park et al. [62] suggested that the alteration in pH tolerance observed during xylanase production might be due to different enzyme mixtures secreted and/or the post-translational alterations in the xylanase secretion procedure; for example, glycosylation enhancing reliability in extreme pH and temperature conditions. Xylanase can also be further categorized as facultative alkaliphiles. At pH 6, cellulase gradually decreased while xylanase at pH 11 significantly lost activity with only 34% retained [63]. In a published report of Sepahy et al. [52], an optimum pH of 8 was observed for xylanase production by *B. mojavensis* AG137 in submerged fermentation while Simphiwe et al. [53] reported eight different strains of *Bacillus* sp. showing maximum xylanase production at pH 8 whereas, *Bacillus pumilus* showed maximum xylanase production at pH 7 [51].

The result of agitation on cellulase and xylanase production by *Micrococcus* sp. SAMRC-UFH3 was investigated (Figures 5 and 6). Agitation has been reported to affect the level of aeration and mixing of the nutrients in the fermentation medium [64]. Maximum cellulase production was recorded at 50 rpm with enzyme activity of 173 U/mL (Figure 5). The report of EI-Refai et al. [65] concurs with our findings in which the best agitation rate for cellulase was found to be between 0 and 50 rpm. In the

present study, 200 rpm proved to be the optimum agitation rate for xylanase production by the bacteria with activity of 1814 U/mL recorded (Figure 6). Kokare [66] reported that agitation at 150–200 rpm was found to be the most suitable for xylanase production by *Micrococcus* sp.

Time course experimentation was carried out to monitor the rate of cellulase and xylanase production by *Micrococcus* sp. SAMRC-UFH3 under optimum growth conditions. Results presented in Figure 7 indicate that cellulase production increased progressively until 96 h (Figure 8), when maximum enzyme production of (102 U/mL) was recorded. Our observation corroborates the report of Nagendra et al. [67], who noticed that the optimum incubation period for cellulase was also within 96 h in the late stationary phase of growth. In another study carried out on *B. pumilus* EB3 by Ariffin et al. [68], lower CMCase activity (0.079 IU/mL) was recorded after 24 h of incubation. In the case of xylanase production, the activity was found to be maximal (1296 U/mL) at 84 h (Figure 8). According to the findings of Sharma et al. [69], a shorter fermentation period was documented for xylanase production at stationary phase of growth by *G. thermoleovorans*. The xylanase titer did not significantly increase, but a peak in production was attained in 42 h instead of 72 h. The enzyme production pattern was consistent with that shown by *Bacillus* sp. when grown in a fermenter. Similarly, Mrudula and Shyam [70] reported a shorter incubation period of 48 h for maximum production of xylanase from *B. megaterium* MTTC 2444. In another study, some strains of *Bacillus* showed maximum xylanase production after 24 h using digested bran and 48 h of fermentation using sawdust as substrates, respectively [53]. Gupta and Kar [71] undertook studies on 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 substrates, respectively. Sepahy et al. [52] reported a fermentation period of 48 h by *Bacillus mojavensis* AG137 in submerged fermentation using oat bran as a substrate. Murugan et al. [72] reported 96 h as optimum fermentation period for xylanase production by *Arthrobacter* sp. MTCC 6915 in SSF using sawdust as a substrate.

5. Conclusions

Cellulase and xylanase yields appear to depend on a complex relationship involving a variety of factors, like inoculum size, pH value, temperature, and presence of inducers, aeration, growth time, and so forth. Only a few studies have been reported on cellulase and xylanase production by member of the genus of Micrococcus. The optimum conditions for maximum cellulase and xylanase production by *Micrococcus* sp. SAMRC-UFH3 was at 1% of carboxymethyl cellulose (CMC) and birch wood xylan as a sole carbon source, respectively. The test bacteria produced cellulase optimally at pH 5, 25 °C, 50 rpm, and at after 96 h of incubation period, while for xylanase maximum production was optimal at pH 10, 25 °C, 200 rpm, and at after 84 h incubation period. By using these growth conditions, industrial production of cellulase and xylanase is reliable with *Micrococcus* sp. SAMRC-UFH3 in the future with the cheapest input rate. The test bacterial strain is non-pathogenic and easily growing organism under these characteristics it is suitable for large scale and small scale production. Further studies are in progress in our research group for the purification and application of cellulase in different commercial fields.

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