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Optimization of Process Conditions for the Production of Holocellulase by a *Bacillus* Species Isolated from Nahoon Beach Sediments

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Abstract: Production of holocellulase by a newly isolated marine *Bacillus* species via submerged fermentation technique at varying cultivation conditions was investigated. Enzyme production was optimized by altering one variable per time. Moreover effect of lignocellulosic saccharides in inducing cellulase and xylanase production was also investigated. The 16S ribosomal Deoxyribonucleic Acid (16S rDNA) gene sequence analysis exhibited 98% sequence similarity of the isolate with other *Bacillus* species in the gene bank and was deposited with the accession number KX524510. Optimal xylanase and cellulase production was attained at pH 7, temperature 30°C and agitation speed of 50 and 150 rpm. Furthermore, maximum xylanase and cellulase production were both achieved at 60 h corresponding to the late stationary growth stage, with activity of 16.6 and 0.061 U mL^{-1} respectively. Xylanase production was maximally induced by beechwood xylan, xylose and arabinose with activities of 13.59, 8.78 and 1.90 (U mL^{-1}) respectively; while cellulase production was induced by carboxymethyl cellulose only and no cellulase activity was detected in the culture supernatant of the other carbon sources tested. Optimization increased cellulase and xylanase yields being 0.006-0.061 and 0.23-16.6 (U mL^{-1}) from unoptimized to optimized respectively. The results of the study suggest the bacterial strain to be a proficient producer of cellulase and xylanase with potentials in biotechnological application.

Keywords: Xylanase, Cellulase, Lignocellulose, Optimization, *Bacillus*

Introduction

Holocellulase Enzymes, Cellulases (EC 3.2.1.4) and xylanases (EC 3.2.1.8), have increased relevance in several areas of agro-industrial processes including the improvement of nutritional quality of animal feed, quality of dough for various baked products, for cleaning and anti-re-deposition action in detergent industry, cotton softening and denim finishing in textiles industry (Motta *et al*., 2013). In addition, these enzymes could be employed as environment-friendly means of lignocellulosic waste conversion to various bioproducts (de Souza Vandenberghe *et al.*, 2016).

Presently, commercial production of cellulases and xylanases is mostly from fungi such as *Trichoderma* and *Aspergillus* species, which may have attained maximal yield, having undergone extensive strain improvement over the years (Banerjee *et al*., 2010; Peterson and Nevalainen, 2012). Moreover, these commercial enzymes are still limited by the high cost of production, narrow substrate reaction and instability under industrial process dynamics (Motta *et al*., 2013). Consequently, considerable efforts are being made to increase microbial cellulase and xylanase production by environmental strains (Kim *et al*., 2007). In that context hemi (cellulolytic) bacterial strains are continuously being sourced from diverse environments with a view to finding better alternative producers or other cellulases and xylanases capable of enhancing existing commercial enzyme cocktail preparations (Banerjee *et al*., 2010). Therefore investigation on novel bacterial strains adaptable to industrial process dynamics and capable of

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producing cellulases and xylanases with favourable industrial characteristics is ongoing (Sepahy *et al*., 2011; de Souza Vandenberghe *et al.*, 2016).

Members of the genus *Bacillus* are known to play significant roles in the biodegradation of organic matter during composting and are notable producers of hemi (cellulolytic) enzymes (Amore *et al*., 2013). Several *Bacillus* species includig *B. circulans*, *B. amyloliquefaciens*, *B. halodurans*, *B. licheniformis*, *B. subtilis* and *B. mojavensis* have been reported as producers of cellulase and xylanase (Ray *et al*., 2007; van Dyk *et al*., 2010; Sepahy *et al*., 2011; Acharya and Chaudhary, 2012). However, investigation of hemi (cellulolytic) bacteria from marine environment is on the increase due to their remarkable versatility and adaptation to heterogeneous environmental conditions (Lordan *et al*., 2011). These features potentiate them as producers of enzymes with characteristics adaptable to the dynamics of industrial processes.

Bacterial fermentation profile can be enhanced by optimization of the nutritional and culture conditions including initial pH of media, incubation temperature and agitation speed. Therefore determining the optimal variables is of utmost importance for increased production of the enzyme (Nagar *et al*., 2012). In this study, we report on the optimal conditions for enhanced cellulase and xylanase production by a *Bacillus* strain, isolated from marine beach sediments in South Africa.

Materials and Methods

Source of Bacteria

The bacterial strain was isolated from sediments of Nahoon Beach located in East London, Eastern Cape South Africa, by dilution and heat-shock method, using M1 media composed of (g/L natural seawater); peptone 2, yeast extract 4, starch 10, agar 18 (Mincer *et al*., 2002). The media were autoclaved at 121°C for 15 min and subsequently amended with filter sterilized 50 mg L^{-1} nystatin. Bacterial cultivation was performed at incubation temperature of 30°C for 7 days. Repeated streaking was done to purify the bacterial colonies and the colonies so obtained were screened for lignocellulolytic potential. Screening was carried out to determine qualitative (hemi) cellulolytic enzymes production by formation of halo zones on Carboxymethyl Cellulose (CMC) and beechwood xylan agar for cellulase and xylanase activity respectively, on staining with grams iodine solution (Maki *et al*., 2012).

Identification of the Bacterial Isolate by 16S rDNA

Molecular identification of the isolate was carried out by sequence investigation of the 16S rRNA gene. The bacterial DNA was extracted and amplified by Polymerase Chain Reaction (PCR) following the protocol of Cosa *et al*. (2012). Briefly, DNA template was prepared by suspending a loop-full of bacterial colonies in 70 µL of sterile distilled water, heated in a heating block (Lasec, South Africa) for 10 min at 100°C and cooled for 5 min followed by centrifuging at 3000 rpm for 5 min. The supernatant obtained was placed in a sterile Eppendorf tube and preserved at 4°C, for further analysis. The PCR was run using a universal gene primer with the following forward and reverse primers: F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and R5: 59-ACGGITACCTTGTTACGACTT-39 respectively and a 2 µL template DNA. Nearly full-length of the 16S rDNA nucleotide sequences were amplified. The amplicon size was confirmed by 1% agarose gel electrophoresis (Cosa *et al*., 2012) and sequenced at University of Kwa-Zulu Natal, Durban (South Africa). Verification of the bacterial genus was by Basic Local Alignment Search Tool (BLAST) algorithm system of National Centre for Biotechnology (http://www.ncbi.nlm.nih.gov/Blast.cgi). Sequences from the GenBank database and the bacterial strain were aligned with the MUSCLE programme via the EMBL-EBI portal (www.ebi.ac.uk/Tools/msa/muscle/). The evolutionary analyses and construction of the tree topology of the aligned sequences were conducted in MEGA 6 programme using Maximum Likelihood method based on Jukes-Cantor model (Tamura *et al.*, 2013).

Cultivation Media and Fermentation Conditions

Fermentation and pre-culture medium was composed of (g/L); 0.1% $NaNO_3$, 0.05% $MgSO_4$, 0.1% K_2HPO_4, 0.1% KCl, 0.05% yeast extract, 1% carboxymethyl cellulose (Merck, South Africa) or beechwood xylan (ROTH, South Africa). All fermentations were performed in 250 mL Erlenmeyer flasks. Sterilization of the cultivation media was carried out by autoclaving for 15 min at 121°C, after adjusting the initial pH to 7.0 (Maki *et al.*, 2012). Preparation of the culture inoculum was performed by placing a loopful of bacterial colony into a test tube containing 5 mL of medium and incubated at 30°C for 24 h. Thereafter optical density of the inoculum was adjusted to 0.1 (OD_{600}) using sterile normal saline, 1 mL of which was subsequently transferred into 100 mL of sterile cultivation medium. Fermentation was performed by incubating in a rotary gyrator at 140 rpm and 30°C for 72 h. Fermentation broth was centrifuged at 10, 000 rpm for 15 min under 4°C and the supernatant so obtained served as crude extracellular enzyme (Sharma *et al*., 2013).

Enzyme Assay

Quantitation of cellulase and xylanase production was determined by volumetric enzyme activity following the method of Saratale *et al.* (2012), with slight modifications. Estimation of released reducing sugar from carboxymethyl cellulose and beechwood xylan was in accordance with Miller (1959), using the 3, 5-Dinitrosalicylic Acid (DNS) method.

Cellulase Assay

Cellulase activity was determined by a reaction volume comprising 1 mL of crude extracellular enzyme and 1 mL of 1% (w/v) carboxymethyl cellulose (Merck, Modderfontein, South Africa) dissolved in 50 mM phosphate buffer (pH 7.0), which was incubated in a water bath (Lasec, South Africa) at 50°C for 30 min.

Xylanase Assay

Xylanase activity assay was conducted by adding 1mL suitably diluted enzyme to 1 mL of 1% (w/v) beechwood xylan (ROTH, Cape Town, South Africa), in 50 mM phosphate buffer (pH 7). The enzyme and substrate reaction mixture was incubated for 10 min at 50°C. Sample controls were subjected to the same reaction conditions but were terminated at zero time. Reactions were terminated by adding 2 mL 3, 5-Dinitrosalicylic (DNS) acid reagent, placed in a vigorously boiling water bath for 10 min and cooled in ice water. The absorbance reading was taken at 540 nm and the reducing sugars released were estimated using D-glucose and D-xylose standard line graphs, for cellulase and xylanase activity respectively. One unit of enzyme was defined as the amount of enzyme per millilitre needed to liberate one micromole of reducing sugar from CMC or beechwood xylan per minute under the stated assay conditions.

Determination of Optimal Fermentation Conditions for Holocellulase Production

Submerged fermentation of cellulase and xylanase was carried out at varying cultivation conditions by adjusting one variable while keeping others constant (Battan *et al*., 2007). Optimized conditions were then used in consecutive optimization fermentation (Fatokun *et al*., 2016).

Effect of Initial pH on Holocellulase Production

The effect of initial pH on cellulase and xylanase production was investigated by altering initial pH of the medium with 1N HCl or 1N NaOH. Adjustment of the pH ranged from 3-11 before sterilization and was subsequently incubated under the earlier stated conditions.

Effect of Incubation Temperature on Holocellulase Production

A 100 mL of fermentation medium in 250 mL Erlenmeyer flask was inoculated with a 1 mL inoculum of the bacterial suspension adjusted to optical density of 0.1 (OD_{600}) and incubated at different temperatures ranging from 25-50°C for 72 h. Thereafter the fermentation medium was centrifuged at 10, 000 rpm, 4°C for 15 min and the supernatant was used for assaying activity of the extracellular enzymes.

Optimizing Agitation Speed for Holocellulase Production

Cellulase and xylanase production was carried out under static and different agitation speed. Agitation speed was adjusted from 50-200 rpm, with an interval of 50 rpm and was incubated at pre-optimized conditions for 72 h.

Kinetics of Growth and Production of Holocellulase over Time

A 1 mL of 24 h pre-culture adjusted to 0.1 Optical Density (OD_{600}) was used in seeding 100 mL of fermentation medium and incubated under optimized conditions for 96 h. The medium was withdrawn at 12 h interval to monitor growth and enzyme activity. Growth was recorded as change in optical absorbance at OD_{600}.

Effect of Carbon Source on Holocellulase Production

The basal salts medium was supplemented with different carbon source in order to determine their effect in inducing cellulase and xylanase production by the bacterial strain. Carbon source components of lignocellulose were selected which were; crystalline cellulose in form of Avicel, carboxymethyl cellulose, xylan (beechwood xylan), cellobiose, arabinose, galactose, glucose, mannose and xylose were individually added at a concentration of 1% (w/v) in the fermentation broth. The monosaccharides were sterilized separately prior to mixing with the basal salts medium. Basal salts medium without a carbon supplement served as control fermentation broth. Microbial fermentation was performed for 72 h; after which the broth was centrifuged and assayed for extracellular volumetric enzymes activity.

Statistical Analysis

All the experiments were run in triplicates and data obtained were recorded as mean standard deviation. Comparison of mean was by Fisher's Least Significant Difference following one-way Analyses of Variance (ANOVA), using Statistical Package for Social Sciences (SPSS) run with IBM SPSS Statistics 23 Software (IBM Corp, New York, USA). A $p \leq 0.05$ between variables was considered significant.

Results

According to the BLAST analysis of 16S rDNA sequence, the isolate in this study belongs to the *Bacillus* genus, with a 98% similarity with other Bacillus species in the gene bank including *Bacillus cereus* strainAPT23, *B. thuringiensis* strain KUDC1706 and *B. cereus* strain MSCS33; was consequently named *Bacillus* sp. strain SAMRC-UFH9 and deposited at the gene data base under the accession number KX524510. Phylogenetic tree generated showing the evolutionary relationship of the strain with other *Bacillus* strains in Gen Bank database is shown in Fig. 1.

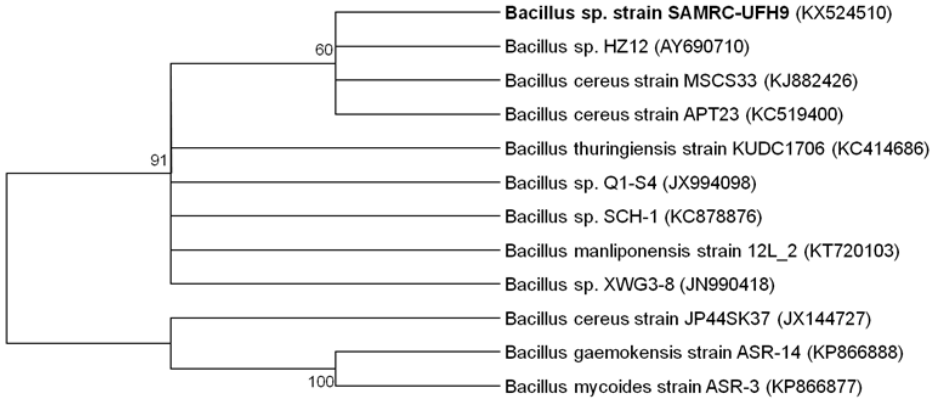

Fig. 1. Phylogenetic tree of *Bacillus* sp. strain SAMRC-UFH9 with other Bacillus strains in the gene bank. The tree topology with higher log likelihood value was selected; and the Bootstrap values (>50%) of 1000 replicates are indicated next to the branches (Tamura *et al*., 2013)

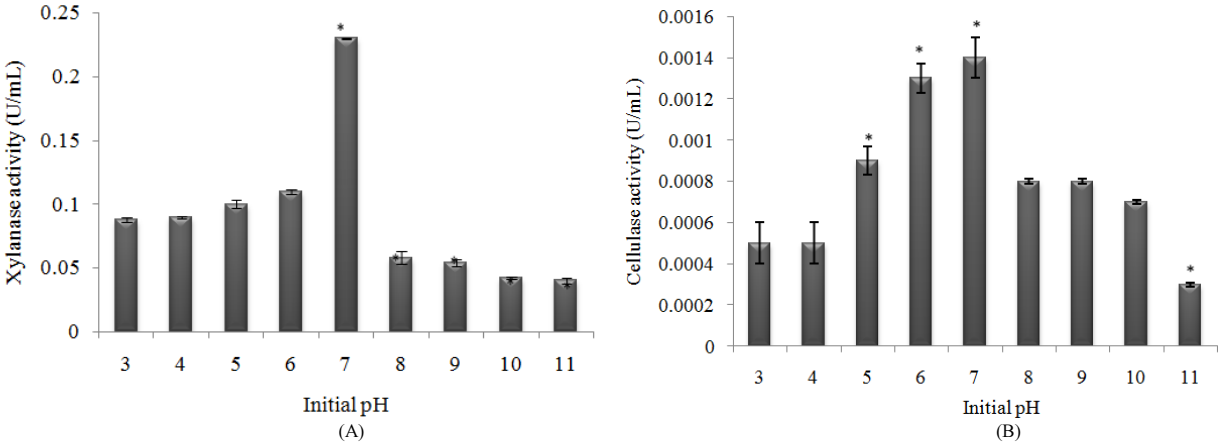

Fig. 2. Effect of initial pH on xylanase (A) and cellulase (B) production. There was statistical significance between pH ranges but cellulase production was at a wider pH range (5-9). Optimal production was at pH 7 for both xylanase and cellulase. Values are mean of triplicate tests ± SD. Asterisk denotes significant difference in xylanase and cellulase production at $p \leq 0.05$

Effect of Initial pH on Holocellulase Production

Effect of initial pH of medium on cellulase and xylanase production was investigated (Fig. 2). Although significant difference was observed between the pH ranges ($p<0.05$), no statistical significance was observed between pH 3 and 4 and pH 5 and 6 for xylanase production. Optimal xylanase production was at initial pH 7 with activity of 0.23±0.001 U mL^{-1}. A decline in production was observed with increase in pH and the least recorded was at pH 11 with activity of 0.04±0.0 U mL^{-1} (Fig. 2A). While there was significant difference ($p<0.05$) among the pH variables for cellulase production, there was no significant difference in its production between pH 3 and 4 and between pH 8, 9 and 10. Furthermore, cellulase production by the *Bacillus* sp. strain SAMRC-UFH9 was at a wide pH range from 5-9 (Fig. 2B) and maximum production was observed between initial pH 6 and 7 with respective activity of 0.0013±0.0001 and 0.0014±0.0001 U mL^{-1} ($p>0.05$), whereas the least cellulase production was at pH 11 with activity of 0.0003±0.0 U mL^{-1}.

Effect of Incubation Temperature

Optimal temperature for xylanase and cellulase production by the *Bacillus* strain was investigated and the result is shown in Fig. 3. Xylanase was produced under all the incubation temperature range tested, nevertheless optimal production was recorded at 30°C (0.24±0.01 U mL^{-1}) and there was no statistical significance between xylanase produced at 35 and 40°C. Furthermore, about 50% of the xylanase activity recorded at the optimal temperature was attained at the temperature range of 45-50°C (Fig. 3A). On the other hand cellulase production was more sensitive to temperature difference, as indicated by a maximum production at 30°C (0.015±0.001 U mL^{-1}) and almost no production being recorded at temperature range of 40-50°C (Fig. 3B). There was no significant difference between temperatures 40, 45 and 50°C, with respective activity of 0.0003±0.0, 0.0001±0.0 and 0.0001±0 (U mL^{-1}).

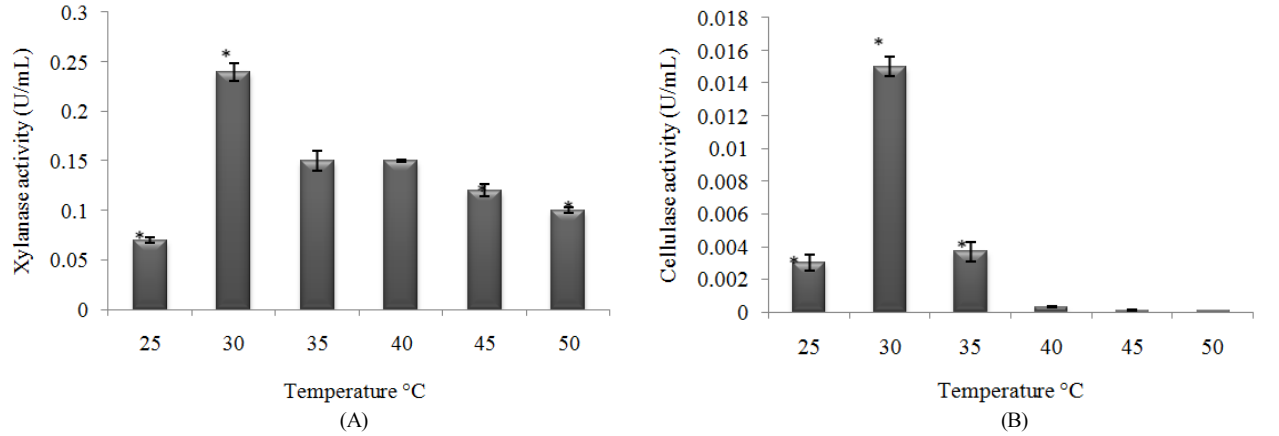

Fig. 3. Effect of incubation temperature on xylanase (A) and cellulase (B) production. Xylanase production was recorded under all the incubation temperature range tested and up to 50% of the activity at optimal incubation temperature was attained at the temperature range of 45-50°C. Cellulase production was more sensitive to temperature difference and nearly no production at 45-50°C. Values are mean of triplicate tests ± SD. Asterisk denotes a significant difference in xylanase and cellulase production at $p \leq 0.05$

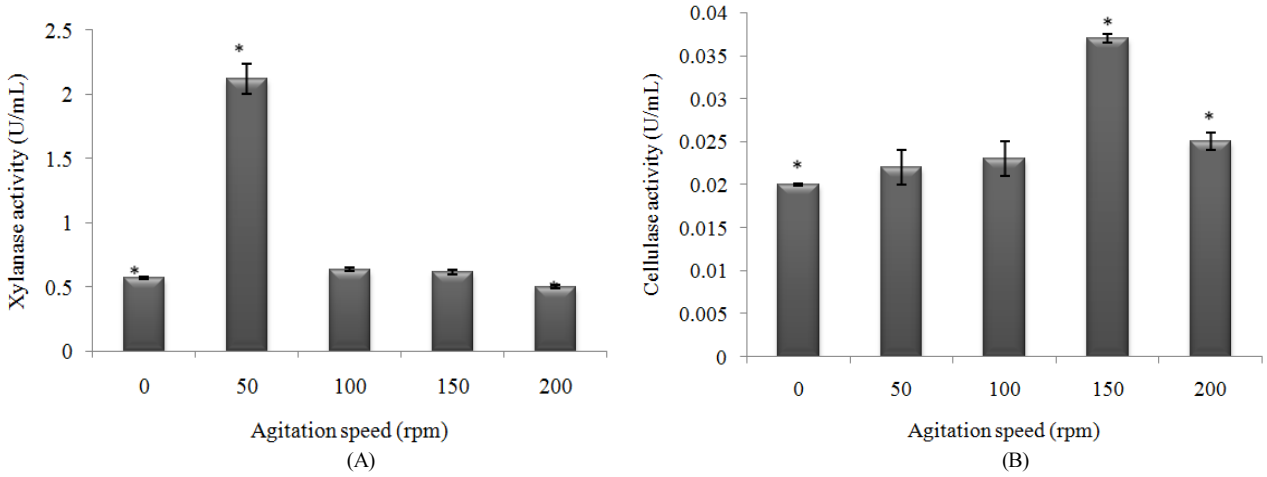

Fig. 4. Effect of static (0) and varying agitation speed for xylanase (A) and cellulase (B) production by *Bacillus* sp. strain SAMRC-UFH9. Xylanase and cellulase production was favoured by low agitation speed (50 rpm), while cellulase production was favoured by higher agitation speed of 150 rpm. Asterisk denotes a significant difference in xylanase and cellulase production at $p \leq 0.05$

Optimizing for Agitation Speed

Production of cellulase and xylanase by *Bacillus* sp. strain SAMRC-UFH9 under static condition and varying agitation speed was investigated and the result is as presented in Fig. 4. Xylanase production was favoured by low speed of 50 rpm with a maximum activity of 2.5±0.13 U mL^{-1} (Fig. 4A). Other recorded xylanase production was at respective agitation speed of 100 and 150 (rpm) with activities of 0.8±0.02 and 0.73±0.02 (U mL^{-1}), although the difference between the two was not statistically significant. The least xylanase production was recorded at 200 rpm, followed by static condition with activities of 0.5±0.01 and 0.7±0.0 (U mL^{-1}) respectively. Conversely, cellulase production was favoured by higher agitation speed of 150 rpm with activity of 0.037±0.001 U mL^{-1}. Other observed cellulase production in order of descent were, 0.025±0.001, 0.023±0.002 and 0.022±0.002 (U mL^{-1}) at agitation speed of 200, 100 and 50 (rpm) respectively.

Growth and Time Course for Xylanase and Cellulase Production

Xylanase and cellulase production was carried out over 96 h period and the microbial growth (OD_{600}) and activity at 12 h interval are as shown in Fig. 5. Xylanase production was initiated after 12 h with gradual increase at 48 h (Fig. 5A), corresponding to the early stationary growth phase. Peak in xylanase production was attained

at 60 h (16.6±0.3 U mL^{-1}), towards the mid stationary growth, which sharply declined at 72 h and finally dropped at 96 h with activity of 4.42±0.2 U mL^{-1}. On the other hand cellulase production was also initiated at 12 h but remained steady till 36 h when there was a gradual increase and attained optimal production at 60 h (0.061±0.002 U mL^{-1}) as well. However, another peak in cellulase production was observed at 96 h (0.06±0.01 U mL^{-1}) after an initial decline at 72 h (0.054±0.0007 U mL^{-1}) corresponding to the late stationary growth phase (Fig. 5B).

Effect of Carbon Source for Holocellulase Production

The production of holocellulase enzymes by *Bacillus* sp. strain SAMRC-UFH9 in response to carbon source was investigated and the result is presented in Fig. 6. Xylanase production was maximally induced by beechwood xylan with an activity of 13.59±0.33 U mL^{-1}, followed by xylose and arabinose with activity of 8.78±0.31 and 1.9±0.04 (U mL^{-1}) respectively. Xylanase production was also induced by other carbon sources but at much reduced activity with the following ascending order of activity: 0.41±0.01, 0.44±0.02, 0.47±0.06, 0.51±0.01, 0.54±0.03 and 0.75±0.02 (U mL^{-1}), for glucose, mannose, cellobiose, microcrystalline cellulose, CMC and galactose respectively. On the contrary cellulase production was induced by CMC only (0.052±0.002 U mL^{-1}); no cellulase activity was detected in culture supernatant of the other carbon sources.

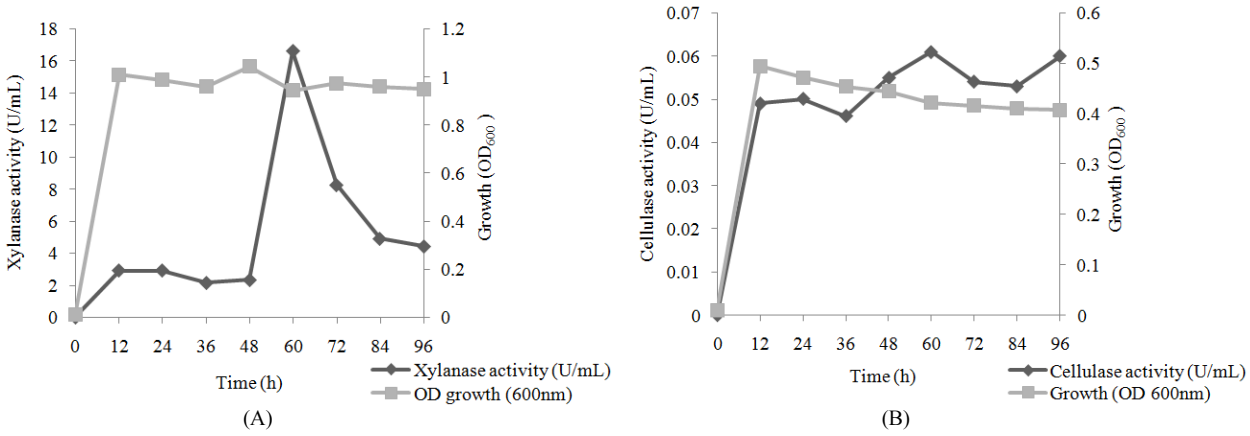

Fig. 5. Growth and time course for xylanase (A) and cellulase (B) production by *Bacillus* sp. strain SAMRC-UFH9. Readings were taken at 12 h interval and values represent triplicate tests. Peak xylanase and cellulase production were both attained at 60 h, at mid stationary growth. Xylanase production sharply declined at 72 h and finally dropped at 96 h while gradual drop in cellulase production occurred between 72-84 h and picked up at 96 h

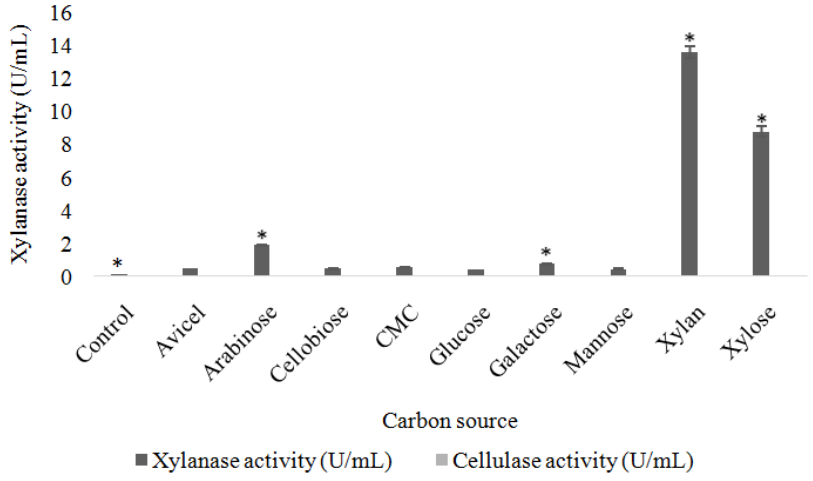

Fig. 6. Carbon inducers of xylanase and cellulase production by *Bacillus* sp. strain SAMRC-UFH9. Xylanase production was induced by all the carbon source tested; cellulase production was induced by CMC only. Xylanase production was maximally induced by Beechwood Xylan; followed by xylose and arabinose. Glucose was the least carbon inducer of xylanase production by the *Bacillus* strain. Asterisk denotes a significant difference in xylanase and cellulase production at $p \leq 0.05$

Discussion

Optimizing various culture conditions is considered as one of the paramount criteria in developing any novel microbial process (Anbu *et al*., 2013). Moreover, response to cultivation conditions are as varied as the microbial population (Table 1), hence optimal conditions for cellulase and xylanase production by any microorganism have to be ascertained in order to maximize product yield (Ray *et al*., 2007).

Effect of Initial pH on Holocellulase Production

Studies have shown that pH of cultivation medium exerts a great influence on microbial growth and metabolic activities (Saini *et al*., 2014). Similar findings with this study for the optimal initial pH 7 for xylanase production have been reported in previous studies on *Bacillus* species, including *B. pumilus* ASH, *Bacillus altitudinis* DHN8, *B. amyloliquefaciens* XR44A and *B. vallismortis* RSPP-15 (Sanghi *et al*., 2008; Adhyaru *et al*., 2014; Amore *et al*., 2013; Gaur *et al*., 2015). Contrary to this result however, optimal initial pH varied for xylanase production by *B. megaterium* (pH 7.5), *B. subtilis* (pH 8) and *B. pumilus* VLK-1 (pH 6), (Tandon and Sharma, 2014; Kumar *et al*., 2014). Nevertheless, the result of this study concurs with previous reports for cellulase production by most *Bacillus* species (Abou-Taleb *et al*., 2009; Bai *et al*., 2012). In contrast, pH 6.5 was optimal for both *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3; while pH 8 was optimum for cellulase production by *Bacillus* sp. 313SI using pretreated rice straw as carbon source (Acharya and Chaudhary, 2012; Goyal *et al*., 2014). Sharma *et al*. (2013) accounted that initial pH 6.5 and 7.5 were optimal for cellulase production and pH 7.5 and 8 for xylanase production by *B. licheniformis*, *Bacillus* sp., *B. megaterium* and *B. subtilis* respectively.

Effect of Incubation Temperature

Metabolic reaction rate is influenced by temperature differential since cellular metabolic activity is slowed down at temperatures below or above the optimal reaction temperature range (Tandon and Sharma, 2014). Therefore optimal temperatures are paramount for efficient microbial growth and enzyme production. Similar finding to this study was made by other authors who reported 30°C as optimal temperature for xylanase and cellulase production by different Bacillus species (Nagar *et al*., 2012; Bai *et al*., 2012; Kumar *et al*., 2014); and very close to the finding of Mukesh Kumar *et al*. (2012) who reported that 32°C was optimal for cellulase production by *B. cereus* MRK1.

Table 1. Optimization conditions, cellulase and xylanase yield for some Bacillus strains

	Cellulase optimization conditions					Xylanase optimization conditions					
Bacillus strain	Substrate	pH	t (°C)	FP (h)	Cellulase yield	Substrate	pH	t (°C)	FP (h)	Xylanase yield	References
B. cereus MRK1	Xylan	8	32	48	-	-	-	-	-	-	Mukesh Kumar *et al.* (2012)
B. subtilis KIBGE-HAS *B. subtilis* CY5	SC/MC	7	40	48	302[a]		-	-	-	-	Bano *et al.* (2013)
B. circulans TP3 *B.licheniformis* MVS1	CMC/MC	7.5	40	96	-	-	-	-	-	-	Ray *et al.* (2007).
Bacillus sp. MVS3	WS/MC	6.5; 7	50	60	-	-	-	-	-	-	Acharya and Chaudhary (2012)
Bacillus sp. JS14	WB	6.5	40	120	2014[b]	-	-	-	-	-	Singh and Kaur (2012)
Bacillus sp. BCCS A3	CMC/MC	9	-	-	49.80[c]	-	-	-	-	-	Kazemi *et al.* (2014)
B. amyloliquefaciens SS_{35}	CMC/MC	6	40	48	0.161[c] -0.693[c] (4-fold)	-	-	-	-	-	Singh *et al.* (2014)
B. subtilis ASH	-	-	-	-	-	WB	7	37	72	-	Sanghi *et al.* (2008)
B. pumilus SV-205	-	-	-	-	-	WB/MC	10	37	36	7382.7[b] (21.63 fold)	Nagar *et al.* (2012)
B. halodurans TSEV1	-	-	-	-	-	WB/MC	-	-	-	23-fold	Kumar and Satyanarayana (2014)
B. vallismortis RSPP 15	-	-	-	-	-	BWX	7	55	48	3768[b]	Gaur *et al.* (2015)
B. pumilus ASH	-	-	-	-	-	WB	8	37	72	5407[b] (13-fold)	Battan *et al.* (2007)
B. halodurans PPKS-2	-	-	-	-	-	OsX/MC	11	37	48	4-fold	Prakash *et al.* (2012)
B. mojavensis AG137	-	-	-	-	-	Ob/MC	8	37	48	194.68[b]-302.466[b]	Sepahy *et al.* (2011)
B. altitudinis DHN8	-	-	-	-	-	SS	7.0	35	42	3.74-fold increase	Adhyaru *et al.* (2014)
B. subtilis 276NS	X/CMC/S	8	35	24	5.7-fold	X/CMC/S	8.0	35	24	1.08-fold	Ali *et al.* (2013)
Bacillus isolates (MAM-29 and MAM-38)	RS	6.4;5.6	50	48	200[c] -311[c]; 203[c]-325[c]	RS	5;4	50	48	162-233[b]; 153-242[b]	Abo-State *et al.* (2013)
Bacillus isolates (R5 and N11; K_{22} and K_{23})	BSM	6.5;5.6	30;35	168;120	169.553% (K_{22}), 181.346% (K_{23})	TGY	7.58.0	45, 40	168	428.997%	Sharma *et al.* (2013). (R_5), 464.512% (N_{11})
Bacillus sp. strain SAMRC-UFH9	CMC	7	30	60	0.0056-0.061[c] (10.89-fold)	BX	7	30	60	0.23-16.6[c] (72.17-fold)	This study

Contrary to the result of this study however, maximum production of xylanase and cellulase by other bacterial species at both lower and higher incubation temperatures have been reported (Sepahy *et al*., 2011; Bibi *et al*., 2014), which is suggestive that optimal incubation temperature for cellulase and xylanase production is dependent on bacterial strain (Gautam *et al*., 2011).

Optimizing for Agitation Speed

Agitation and aeration are crucial factors responsible for oxygen supply and uniform distribution of nutrients under submerged fermentation process, for enhanced microbial growth and enzymes production (Satyanarayana and Adhikari, 2006). Agitation speed for optimal xylanase production recorded in this study (50 rpm), seems contradictory to the findings of Sanghi *et al*. (2009) who reported agitation speed of 200 rpm as optimal for xylanase production by *B. subtilis* ASH and some other authors who reported xylanase production to be optimal under static condition (Saratale *et al*., 2012). Nevertheless, Irfan *et al.* (2012) affirmed that *B. subtilis* had maximal xylanase production at both static and 140 rpm using sugarcane bagasse and wheat bran respectively. However, the optimal agitation speed for cellulase production observed in this study corroborates that of other authors that reported 150 rpm to be optimal for cellulase production by different bacterial strains including *B. alcalophilus* and *B. amyloliquefaciens* with respective activity of 2.32 and 2.97 IU mL^{-1}; *Achromobacter xylosoxidans* BSS4, *Pseudomonas* sp. BSS2 and Bacillus sp. BSS3 with activity 104.68, 91.28 and 68.22 U mL^{-1} respectively (Abou-Taleb *et al*., 2009; Sreedevi *et al*., 2013). Conversely Deka *et al*. (2013) recorded agitation speed of 121 rpm as optimal for CMCase production by *B. subtilis*. Low agitation speed especially in high viscous CMC broth, may result in low level of Dissolved Oxygen (DO), which may be insufficient for microbial growth and metabolic activity (Sarka and Aikat, 2014). Therefore, differences in response to static and varying agitation speed for cellulase and xylanase production seems to be influenced by species and media variations.

Kinetics of Growth and Time Course for Xylanase and Cellulase Production

Literature indicates that fluctuations in culture nutrient at late exponential and stationary growth phase induce the synthesis and release of hydrolytic enzymes including carbohydrases and protease in Bacillus species (Gessesse and Mamo, 1999; Heck *et al.*, 2002). This was observed in this study as the highest production of xylanase and cellulase by the *Bacillus* strain was attained at stationary growth phase. However a sharp decline in xylanase production was recorded thereafter, probably due to proteolysis and enzyme denaturation; while an increase in cellulase activity after an initial decline was observed, which could be attributed to the production of an isoenzyme as have been suggested in literature (Battan *et al*., 2007; Ritter *et al.*, 2013). Differing from this study however, xylanase production by *B. subtilis* ASH was initiated at 24 h and attained maximum yield at 72 h (Sanghi *et al*., 2008). On the other hand, lowest and maximum xylanase production by *Gracilibacillus* sp. TSCPVG was attained at logarithmic and stationary growth phase respectively (Giridhar and Chandra, 2010).

Effect of Carbon Source Inducers for Holocellulase Production

Similar to the carbon source for maximum xylanase production obtained in this investigation, birchwood xylan induced highest xylanase production (3.5 U mL^{-1}) by *Gracibacillus* sp. TSCPVG (Giridhar and Chandra, 2010). Nagar *et al.* (2012) reported wheat bran as the best inducer of xylanase production by *Bacillus pumilus* SV-205. In another study, xylose and arabinose were reported as having the highest inducing influence on xylanase production by *Paenibacillus* sp.N_1, with respective activity of 40.60 and 38.70 IU mL^{-1} (Pathania *et al*., 2012); while Adhyaru *et al.* (2014) reported that xylose, sucrose and starch with respective xylanase activity of 145.13, 115.41 and 110 IU mL^{-1} were the carbon sources that induced appreciable xylanase production by *B. altitudinis*. On the other hand, cellulase production is generally induced by CMC in most bacterial strains (Heck *et al.*, 2002). Documented reports have shown that the spectrum of enzymes produced by bacterial strains in response to different carbon inducers is specie specific, therefore variations among different bacterial strains can usually be observed (Heck *et al.*, 2002).

Conclusion

The upsurge of interest in holocellulolytic enzymes production is attributed to the need to meet the increasing demand for these enzymes. Among other strategies, isolation of novel high-yielding bacterial strain and process enhancement could aid in meeting the demand for these enzymes. The characteristics of rapid growth and metabolic turn over potentiate bacterial strains for efficient holocellulases producers. In this study holocellulolytic enzymes production by *Bacillus* sp. strain SAMRC-UFH9 was improved by several folds; 0.006-0.061 and 0.23-16.6 (U mL^{-1}) for cellulase and xylanase respectively. In addition to producing high activity cellulase-free xylanase, very low activity cellulase even in the presence of cellulose substrate was also recorded. Therefore the bacillus strain could be an important source of xylanase in biotechnological

application, especially in processes requiring low cellulase or cellulase-free xylanase such as the textiles, pulp and paper industries.

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Author's Contributions

Evelyn N. Fatokun: Performed the bench work, data analysis and prepared the manuscript.

Uchechukwu U. Nwodo: Revised and proof read the manuscript from draft to final version.

Ademola O. Olaniran: Performed the gene sequencing of the bacterial isolate and proof read the final version of the manuscript.

Anthony I. Okoh: Supervised the study and proof read the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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