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# Properties of Bacterial Cellulases and Secondary Metabolites of Cellulolytic Bacterial Fermentation of Rice Husks

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors FOO, FAO, REU, EOU designed the study. Author FAO performed the statistical analysis, wrote the protocol and author FOO wrote the first draft of the manuscript. Authors REU and EOU managed the analyses of the study. Author FOO managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

The unchecked accumulation of rice husk which is one of the agro-industrial wastes on land serves as source of environmental pollution. This present study showed that submerged fermentation of rice husk for cellulase production and extraction of secondary metabolites was successful using bacterial isolates. The identities of the cellulase hyper-producing strains were *Bacillus subtilis* strain SDDlas, *Bacillus amyloliquefaciens* strain CF11, *Bacillus cereus* strain AT and *Bacillus amyloliquefaciens*. The partial purification of cellulase was achieved by Protein precipitation using ammonium sulphate fractionation, and membrane dialyses. The metallic salts $CaCl_2$, $MgSO_4$ and $FeSO_4$ activated cellulase activity whereas CuSO4 inhibited it. The compounds common to the four Bacillus strains at zero hour are: 9,17-Octadecadienal and propanal while secondary metabolites

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found after seven days fermentation are cis-13-Octadecanoic acid, pentadecanoic and di-isoctyl phthalate. These organic acids are of industrial importance in pharmaceutical and cosmetic industries.

*Keywords: Fermentation; secondary metabolites; cellulase and fermentation.*

## 1. INTRODUCTION

Rice husk as one of the most widely available agricultural wastes in many rice-producing countries all over the world is generated in very large amount, and approximately 600 million tonnes of rice paddy is produced per annum. The rice husk generated in different countries of the world through processing of rice generally constitutes nuisance as waste in the environment. The constituents of rice husks are not limited to 75-90% organic matter such as cellulose, lignin, minerals such as silica, alkali, alkaline earth metals and trace elements (Madhumita et al. [1]). In addition, rice husk is composed of crude protein (2.9-3.6%), oil (8-12%), crude fibre (39-42%) and ash (15-22%). The content of each constituent depends on rice variety, Soil chemistry, climatic conditions, and even the geographic location (Oyenuga [2]; Madhumita et al. [1]).

Fermentation technology which is as old as the early man is highly interested in the anaerobic use of microorganisms for the conversion of solid or liquid substrate into various products. Substrates used vary widely; and equally, any material that supports microbial growth is a potential medium (Chisti and Moo-Young [3]). Under anaerobic conditions, in the dark and in the absence of electron acceptors, organic compounds are catabolized by strictly anaerobic or facultative anaerobic bacteria by internally balanced oxidation–reduction reactions, a process called fermentation. In fermentation, the organic compound serves as both electron donor and acceptor, and adenosine triphosphate is synthesized by substrate level phosphorylation (Konings et al. [4]; Deep et al. [5]).

Microbial degradation of lignocellulosic waste and the downstream products resulting from it is accomplished by a concerted action of several enzymes, the most prominent of which are the cellulases. Cellulases hydrolyze cellulose (β-1, 4-D-glucan linkages) and produce as primary products glucose, cellobiose and cello-oligosaccharides. There are three major types of cellulase enzymes-Cellobiohydrolase (CBH or 1, 4- β-D-glucancellobiohydrolase, EC 3.2.1.91), Endo-β -1, 4-glucanase (EG or endo-1, 4-β-D-glucan 4-glucanohydrolase, EC 3.2.14) and β-glucosidase (BG-EC 3.2.1.21) (Demain [6]; Abhishek and Virendra [7]).

The process of fermentation equally can be used to generate secondary metabolites, which are polymeric substances produced during the stationary phase of microbial growth. Unlike primary metabolism, the pathways of secondary metabolism are still not understood to a great degree and thus provide opportunities for basic investigations of enzymology, control and differentiation (Demain [6]).

The aim of this study was to ferment rice husk using cellulolytic bacteria for production of Cellulase and secondary metabolites of industrial importance.

## 2. MATERIALS AND METHODS

### 2.1 Cellulase Production by Submerged Fermentation Using Rice Husk

The isolation of cellulase producing bacteria isolation, screening, and production were carried out at the Biotechnology Laboratory, Federal Institute of Industrial Research, Oshodi, Lagos State-Nigeria. The isolated bacterial strains were used for cellulase enzyme production in submerged fermentation process were obtained through serial dilution and pour plating onto nutrient agar (NA) and cellulolytic agar (NA and 2% cellulose). Incubation was done at 37°C for 18 h and cellulolytic bacteria were sub-cultured. Hyper-producing strains of *Bacillus* were identified using battery of biochemical tests and Polymerase Chain Reaction (PCR). Fermentation medium consists of (g/l) glutamic acid (0.3 g), $(NH_4)_2SO_4$ (1.4 g), $K_2HPO_4$ (2.0 g), $CaCl_2$ (2.0 g), $MgSO_4.7H_2O$ (0.3 g), peptone (7.5 g), $FeSO_4$ (5.0 g), $MnSO_4$ (1.6 g), $ZnSO_4$ (1.4 g), tween 80 (20% w/v), rice husk (30 g) and 1000 ml distilled water, homogenized on hot plate magnetic stirrer. Thereafter, the pH was adjusted to pH 5.5 using pH meter (T70 PG Instrument UV model). The medium (400 ml each) was dispensed into 500ml Erlenmeyer flasks. The flasks and the content were autoclaved at 121°C for 15 mins. The liquid medium was allowed to

cool at 25°C. Thereafter, bacterial suspension from young cultures (18-24 h), were added to 9.0 ml sterile water. From this inoculum suspension, 1.0 ml aliquot of each inoculum was inoculated into the fermentation medium. Incubation was done at 30°C for 7 days with agitation rate of 200 rpm in a gyratory shaker. Thereafter 7 days fermentation period, the crude enzyme was harvested by centrifugation of the broth fermented broth at 10,000 x g for 10 min at 4°C using a centrifuge.

## 2.2 Cellulase Purification

### 2.2.1 Ammonium sulfate fractionation

The cellulase purification studies were carried out at the Biotechnology Laboratory, Federal Institute of Industrial Research, Oshodi, Lagos State-Nigeria. The crude enzyme was initially saturated up to 70% with solid ammonium sulphate and then centrifuged at 10000 rpm at 4°C for 10 min. The supernatant obtained was further concentrated to 40%, 60%, 70% and 80% respectively using ammonium sulphate, stirred for 1h and then centrifuged. The supernatant was discarded and the pellets or precipitate collected and re-suspended in the minimal volume of 0.1 M phosphate buffer; pH 5.0 (Abirami et al. [8]).

## 2.3 Dialysis Membrane

The treatment of the dialyses tube was achieved by boiling in 2% sodium bicarbonate for 10 min to remove sulphides. It was rinsed once in distilled water and again boiled in 10mM EDTA solution for 10 min to remove heavy metals and followed by rinsing in water. The membrane was then placed in 1% sucrose solution for 30min. The cell-free crude extract was re-suspended in 5ml of 0.1M phosphate buffer (pH 5.0) solution and introduced into a dialysis bag, partially filled. The solution was dialysed against 500 ml of the same buffer for 12 h at 4°C in a refrigerator to remove the excess salt with one change after 4 h thus; obtained enzyme preparation was concentrated against sucrose. This was followed by centrifugation of the resulting solution at 10000 rpm at 4°C and the supernatant was tested for cellulase activity and protein concentration (Palanivel et al. [9]).

## 2.4 Characterization of Enzyme

The purified fraction showing highest specific activity was characterized by varying the parameters that influence enzyme activity.

### 2.4.1 Effect of pH on activity of cellulase

This was determined with CMC 1% (w/v) as substrate suspended in various buffer systems: 0.1M sodium acetate buffer (pH 4.0-5.0): 0.1 M sodium phosphate buffer (6.0-7.0); tris-HCl buffer (8.0) and a glycerine- NaOH buffer (pH 9.0-10.0). The enzyme and substrates were incun=bated at varied pH values from 4.0-10.0 at 50°C for 2 h and subsequent analysis was determination of reducing sugars using DNSA method (Sharma et al. [10]).

### 2.4.2 Effect of temperature on activity and stability of cellulase

The effect of temperature on cellulase activity was determined by estimating the cellulase activity at pH 5.0 within a temperature range of (30°C-100°C) for 2 h using CMC as substrate. The residual cellulase activity was determined by assay of reducing sugar using the conventional DNSA methods (Sharma et al. [10]).

### 2.4.3 Effect of metal ions on enzyme activity

The reaction of the enzyme and filter paper substrate was allowed to proceed at 50°C with duplicate test tubes containing 5 mM$CaCl_2$ ($Ca^{2+}$), $MgSO_4$ ($Mg^{2+}$), $MnSO_4$ ($Mn^{2+}$), $FeSO_4$ ($Fe^{3+}$), $CuSO_4$ ($Cu^{2+}$) and $ZnSO_4$ ($Zn^{2+}$). Test tubes were labeled according to the source of carbon (filter paper, CMC), and above mentioned salts. The cellulase extract was diluted accordingly, and 0.l ml of the diluted enzyme was pipetted into the labelled test tube already loaded with 0.5 gram of shredded filter paper. Thereafter, 0.5 ml of 5 mM solution of each of the above mentioned salts (one salt to one test tube) was pipetted into a test tube. The incubation of the reaction mixture was done at 50°C and the assay done using previously described DNSA Method (Orji et al. [11]). Tubes containing distilled water and substrates (without the enzyme) but treated in the same way as sample served as the blank.

## 2.5 Determination of Secondary Metabolites

### 2.5.1 Sample preparation

A submerged fermentation medium (100 ml) was prepared using 100 ml volumetric flasks labelled A8, B6, C1, D1. To each of the medium, 3 g of rice husk was introduced. From a day-old culture plate, an aliquot of each isolate: A8, B6, C1, D1 was picked and inoculated into the fermentation medium, homogenised and incubated at 37°C in

a rotary shaker for seven days (7 days). After the fermentation period, filter paper method was used to separate the fermented rice husk from the liquid filtrate using four test tubes each in duplicate. The sediments were weighed respectively with varying values of A8:7.22 g, B6: 5.09 g, C1: 4.87 g and D1:8.33 g before dispensing into a 100 ml volumetric flask.

Then 30 ml of methanol was dispensed into each flask containing the supernatant i.e. fermented rice husk and was vigorously homogenised at intervals of 1 h for 6 h before it was kept aside for 24 h a 25°C. The same process was repeated for three days. Finally, it was filtered and evaporated to dryness using rotary evaporator and was reconstituted with methanol ready for GC-MS (Kalpana et al. [12]).

### 2.5.2 Gas chromatography mass spectrometer (GCMS)

The GC-MS analyses were carried out at the Central Research Laboratory of the University of Lagos-Nigeria. GCMS analysis of extracts of cellulolytic bacteria fermentation of rice husks were performed using Agilent Technologies 7890A GC system coupled with MS 5975C VL MSD and Gas chromatography interfaced to a Mass spectrometer (GC-MS) equipped with a stationary phase HP5MS having length of 30 m internal diameter 0.32 mm and thickness of the column was 0.25 µl. For GC-MS detection, an electron ionization system with ionizing energy of 70eV was used. Helium gas was used as the carrier gas at constant flow 2 ml per min and an injection volume of 1 µl was employed. The oven temperature was programmed from 80°C to hold for 2 min at the rate of 10°C per min, having final temperature of 300°C that held for 6 min. Total GC running time was 36 min. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was NIST mass. Interpretation on mass spectrum GC-MS was conducted using the database of national Institute Standard and technology (NIST) having more than 62,000 patterns. The adopted procedures, which was have been previously used by Kalpana et al. [12].

## 2.6 Molecular Identification of Cellulolytic Bacteria

The strain which showed maximum cellulase activity was further subjected to molecular identification using 16S rRNA (Patagundi et al. [13]).

### 2.6.1 Isolation of genomic DNA

2.6.1.1 DNA extraction

*2.6.1.1.1 Procedure*

DNA Extraction was carried out on the samples using the Jena Bioscience Bacteria DNA Preparation Kit according to the manufacturer's instruction which also conformed to the modified method of Patagundi et al. [13].

Two millilitres (2 ml) of overnight grown nutrient broth culture was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet was re-suspended in 10 min 10 Mm Tris, 100 mM Sodium chloride solution and centrifuged at 10000 rpm 4°C for 10 min. After discarding the supernatant, the pellet was re-suspended in 100 µl of $T_{50}E_{20}$ buffer containing 20 µl of lysozymes (50 mg/ml) and incubated at 37°C for 20 min, in that solution 1 µl of RNase (10 mg/ml) was added and incubated at 25°C for 20 min. To this mixture SDS (2% $T_{50}E_{20}$) was added and incubated at 50°C for 45 min with proper mixing. Two microlitres (2 µl) proteinase K (20 mg/ml) was added and incubated at 55°C for 30 min. The sample was extracted in same volume phenol, chloroform and Iso-amyl alcohol (25:24:1) and DNA was precipitated with one volume of 3M of sodium acetate. The pellet was washed with 70% Ethanol, dried and dissolved in µl of $T_{10}E_1$ buffer and stored at -20°C for further use. The concentration of DNA was determined using UV-1800 Spectrophotometer. The DNA was stored at 20°C for further use (Patagundi et al. [13]).

2.6.1.2 PCR amplification of the 16SrRNA gene (27F and 1492R)

Polymerase chain reaction was carried out to amplify the 16SrRNA gene of the bacteria using the primer pair 27F- 5′-AGAGTTTGATCCTGGCT CAG -3', and 1492R 5'-GGTTACCTTGTTACGACTT -3'. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1x concentration containing 1x Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP)(Solis Biodyne), 25 bpMol of each primer (BIOMERS, Germany), 2 units of

Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.

Thermal cycling was conducted in an EppendorfVapo protect thermal cycler (Nexus Series) for an initial denaturation of 95°C for 15 min followed by 35 amplification cycles of 30 sec at 95°C; 1 min at 61°C and 1 min 30 sec at 72°C. This was followed by a final extension step of 10 min at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for $1^1/_2$h. After electrophoresis, DNA bands were visualized by ethidium bromide staining. A 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker (Khataminezhad et al. [14]).

### 2.6.2 Sequencing

PCR products were purified with Exo sap followed by DNA Sanger sequencing and data was analyzed by ABI Sequencing Analysis software (version 5.2).

## 3. RESULTS AND DISCUSSION

Four bacterial isolates (A8, B6, C1, and D1) that exhibited highest cellulolytic potential on CMC agar plates (i.e with maximum zone of inhibition) were selected for submerged fermentation processes and were further identified to the species level by 16S rRNA gene sequencing. All 16S rDNA sequences obtained matched with sequences deposited for *Bacillus subtilis* strain SDDlas (A8), *Bacillus amyloliquefaciens* strain CF11 (B6), *Bacillus cereus*, strain AT (C1) and *Bacillus amyloliquefaciens* (D1) after BLAST analysis (Table 1).

The partially purified cellulase (70%) of A, B, C, and D had activities of 433 units, 493, 174 and 735 units respectively. In addition, at 80% level of purification the cellulase of A, B, C, D were 477, 522, 358 and 411 units respectively. The partially purified cellulase activity, (462.978, 593.028, 551.412 and 447.372 U/ml) was found to be maximum at 50°C. The result is in accordance with the work of Alagasamy et al. [15] where partially purified enzyme activities were maximum at a temperature range of 50-60. The result shows that high temperature of 50°C. is optimal for cellulase production using these *Bacillus* strains (Table 2). The temperature of 50°C observed in (Fig. 1) to be the optimum for the activities of A8, B6, C1, and D1. In addition, pH of 5.5 recorded the highest activities of 634, 707,671 and 531 units, for cellulase of A8, B6, C1 and D1 respectively (Fig. 2). The activities of cellulases produced by the four *Bacillus* strains at various ranges of temperature were found to have the maximum activity at 50°C.

**Table 1. Molecular identities of cellulolytic and fermentative bacteria**

| Our reference | Homology/ percentage identity (%) | Name/ Identity of isolate | Accession number |
|---|---|---|---|
| A8 | 92 | *Bacillus subtilis*strain SDDlas | - |
| B6 | 97 | *Bacillus amyloliquefaciens* strain CF11 | JX438693.1 |
| C1 | 99 | *Bacillus cereus* strain AT | JQ425477.1 |
| D1 | 98 | *Bacillus amyloliquefaciens* | KP261063.1 |

**Table 2. Effect of purification on specific activities of bacterial cellulases**

| Isolate code/ source of enzyme | Enzyme activity (70% in µ/ml) | Protein content | Specific cellulase activity (µ/mg at 70%) | Enzyme activity (80% in µ/ml) | Protein content (µ/mg) | Specific cellulase activity (µ/mg at 70%) |
|---|---|---|---|---|---|---|
| A8 | $433.5^{c}$ | $1.7425^{b}$ | 248.780 | $477^{b}$ | $1.435^{a}$ | $332.404^{b}$ |
| B6 | $495^{b}$ | $1.0865^{c}$ | 455.591 | $522^{a}$ | $0.82^{b}$ | $636.585^{a}$ |
| C1 | $174^{d}$ | $2.378^{d}$ | 73.171 | $358.5^{c}$ | $1.6605^{a}$ | $215.899^{c}$ |
| D1 | $735^{a}$ | $1.4145^{b}$ | 519.618 | $411^{c}$ | $1.025^{b}$ | $400.976^{d}$ |

*Means followed by the same superscript in the same column are not significantly different ($p<0.05$) (Fisher LSD test)*

**Table 3a. Changes in occurrence of secondary metabolites during fermentation of rice husk by cellulolytic bacterium (Bacillus subtilis strain SDDIas)**

| Sample codes | Time/Hour of extraction during fermentation | Number of compound on the chromatogram | Retention time | Name of compound | Percentage content (%) |
|---|---|---|---|---|---|
| A8 | **Day 1 (0 h)** | **1** | 17.632 | 9,17-Octadecadienal | 16.67 |
| A8 | **Day 1** | **2** | 17.838 | Methyl stearate | 14.34 |
| A8 | Day 1 | 3 | 21.535 | Bis (2-ethylhexyl) phthalate | 5.17 |
| A8 | Day 1 | 4 | 24.567 | 2-Ethylacridine | 9.09 |
| A8 | Day 1 | 5 | 27.085 | 2-Ethylacridine | 51.87 |
| A8 | Day 1 | 6 | 27.434 | 1H-Indole, 5-methyl-2-phenyl | 2.86 |
| A8 | Day 7 (168 h) | 1 | 13.163 | Caryophyllene oxide | 28.58 |
| A8 | Day 7 (168 h) | 2 | 14.016 | Germacrene D | 9.20 |
| A8 | Day 7 (168 h) | 3 | 14.262 | Neointermedeol | 16.30 |
| A8 | Day 7 (168 h) | 4 | 14.525 | Caryophyllene oxide | 7.55 |
| A8 | Day 7 (168 h) | 5 | 14.777 | Bergamotol, Z-.alpha | 10.48 |
| A8 | Day 7 (168 h) | 6 | 19.492 | Phenanthrene | 5.28 |
| A8 | Day 7 (168 h) | 7 | 23.200 | 1-Phenanthrenemethanol | 22.61 |

**Table 3b. Changes in occurrence of secondary metabolites during fermentation of rice husk by cellulolytic bacterium (*Bacillus amyloliquefaciens* Strain CF 11)**

| Sample codes | Time/Hour of extraction during fermentation | Number of compound on the chromatogram | Retention time | Name of compound | Percentage |
|---|---|---|---|---|---|
| B6 | Day 1 | **1** | 5.416 | Imidazole, 2-(beta. Carboxy) propionyl amino propanal | 9.77 |
| B6 | Day 1 | 2 | 5.467 | 2H-Pyran,2-(3-butynyloxy)tetrahydrocyclobutanone | 17.28 |
| B6 | Day 1 | 3 | 5.507 | Benzene, | 22.09 |
| B6 | Day 1 | 4 | 5.667 | 1-Ethanol, 2-(ethylsulfinyl) | 11.51 |
| B6 | Day 1 | 5 | 14.084 | Butane, 2-chloro-2,3-dimethyl Carbonic acid | 15.64 |
| B6 | Day 1 | 6 | 14.268 | Benzoic acid, 3-Pyridyl ester | 23.71 |
| B6 | Day 7 (168 h) | 1 | 17.804 | Pentadecanoic acid, 14-methyl-, methyl ester | 16.62 |
| B6 | Day 7 (168 h) | 2 | 19.881 | cis-13-Octadecenoic acid | 67.24 |
| B6 | Day 7 (168 h) | 3 | 20.173 | Heptadecanoic acid | 11.08 |
| B6 | Day 7 (168 h) | 4 | 25.729 | Bis(2-ethylhexyl) phthalate | 5.06 |

**Table 3c. Changes in occurrence of secondary metabolites during fermentation of rice husk by cellulolytic bacterium (*Bacillus cereus* strain AT)**

| Sample codes | Time/Hour Day of extraction during fermentation | Number of compound on the chromatogram | Retention time | Name of compound | Percentage |
|---|---|---|---|---|---|
| C1 | Day 1 | 1 | 3.991 | Chloroacetic acid | 6.05 |
| C1 | Day 1 | 2 | 4.603 | Propanal, Oxime | 5.30 |
| C1 | Day 1 | 3 | 5.462 | 1,3 - Decadiyne | 57.43 |
| C1 | Day 1 | 4 | 6.715 | Propanal | 9.46 |
| C1 | Day 1 | 5 | 8.054 | Cyclobutanemethanol | 5.95 |
| C1 | Day 1 | 6 | 9.324 | 2-Chloro- 2- methyl butane | 6.74 |
| C1 | Day 1 | 7 | 13.146 | 1,1- Difluoro-2-vinylcyclopropane | 9.07 |
| C1 | Day 7 (168 h) | 1 | 17.810 | Pentadecanoic acid | 7.42 |
| C1 | Day 7 (168 h) | 2 | 19.841 | 9,12-Octadecadienoic acid | 22.98 |
| C1 | Day 7 (168 h) | 3 | 19.887 | 12-Octadecenoic acid, methyl ester | 48.29 |
| C1 | Day 7 (168 h) | 4 | 20.173 | Methyl stearate | 12.06 |
| C1 | Day 7 (168 h) | 5 | 21.775 | 8,8,10,10-nonamethylcyclopentasiloxane | 2.18 |
| C1 | Day 7 (168 h) | 6 | 25.723 | Diisooctyl phthalate | 7.07 |

**Table 3d. Changes in occurrence of secondary metabolites during fermentation of rice husk by cellulolytic bacterium (*Bacillus amyloliquefaciens*)**

| Sample code | Time/Hour Day of extraction during fermentation | Number of compound on the chromatogram | Retention time | Name of compound | Percentage (%) |
|---|---|---|---|---|---|
| D1 | Day 1 | 1 | 17.758 | 9,17- Octadecadienal | 15.43 |
| D1c 2 | Day 1 | 2 | 17.930 | 13,16- Octadecadiynoic acid, Methyl ester | 28.16 |
| D1c 3 | Day 1 | 3 | 25.414 | 2- (n-propyl) Oxybenzylideneacetophenone | 38.83 |
| D1 c 4 | Day 1 | 4 | 26.513 | 2- Myristynoyl-glycinamide | 17.58 |
| D1 | Day 7 (168 h) | 1 | 17.804 | Pentadecanoic acid | 16.62 |
| D1 | Day 7 (168 h) | 2 | 19.881 | cis-13-Octadecenoic acid | 67.24 |
| D1 | Day 7 (168 h) | 3 | 20.173 | Heptadecanoic acid | 11.08 |
| D1 | Day 7 (168 h) | 4 | 25.729 | Bis(2-ethylhexyl) phthalate | 5.06 |

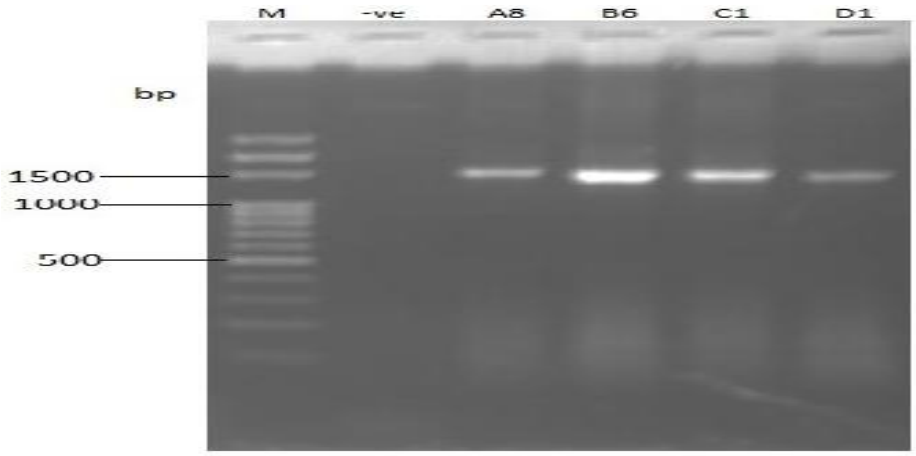


**Plate 1. DNA of Isolates (A-D). Lane M, A8, B6, C1 and D1 Showed Various Lanes from Marker, Control, C1 and D1 (from left to right)**

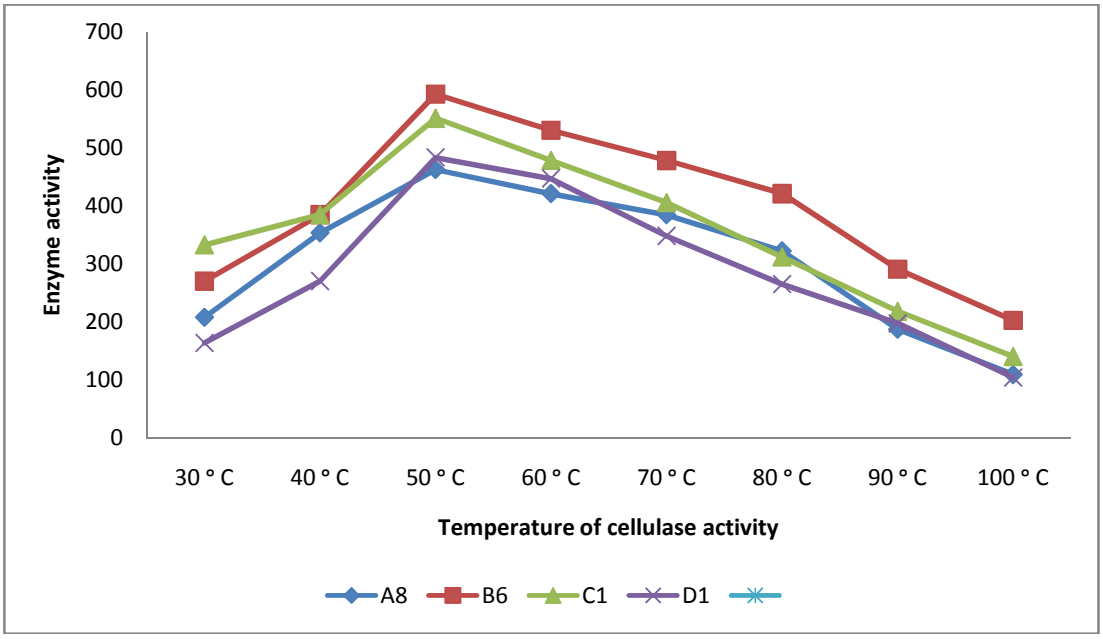


**Fig. 1. Effect of various temperatures on crude cellulase activities**

This work is in accordance with the result of Yin et al. [16where fermentation of corn husk by *Bacillus subtilis Bacillus licheniformis* and *Bacillus circulans* yielded cellulases of different molecular weight. However, the highest cellulase activity of 634.644, 707. 472, 671.058 and 541.008 U/ml was obtained by *Bacillus subtilis*, strain SDDIas (A8), *Bacillus amyloliquefaciens* strain CF11 *(Bacillus cereus* strain AT (C1), *Bacillus amyloliquefaciens* (D1) respectively at pH 5.5. This is slightly different from the work of Amir et al. [17] that obtained highest cellulase activity at pH 5.0 and 5.5 using *Bacillus* strain BOrMGS-3 and fungus respectively. The reason could be that the strains used in this enzyme production possess genes that are more effective at high temperatures.

The effects of monovalent and divalent ionic metals were also considered. The cellulase of A8 tolerated $CaCl_2$, $MgSO_4$, and $FeSO_4$. In addition, the cellulases of A8, B6, C1 and D1 had reduced activities 156, 104, 119, 83 units respectively on exposure to 50 mm concentration of copper ion (Fig. 3). It can be deduced that effect of metallic salts such as $CaCl_2$, $MgSO_4$ and $FeSO_4$

activated the cellulase activities more than other ions whereas $CuSO_4$ inhibited the enzyme activities. This result is in contrast to the work of Nema et al. [18] where increase in cellulase activities was observed in the presence of $Co^{2+}$ and $Mn^{2+}$ whereas $Fe^{2+}$ inhibited activity of cellulase. Increase in the presence of magnesium indicates that magnesium is a probable cofactor.

Forty three compounds were detected using GC-MS analysis after seven days submerged fermentation of rice husk. Methanol was used for the extraction of samples for analysis (sample A-D) (Tables 3a-3d).The samples from each submerged fermentation media A8, B6, C1 and D1,were collected at day one (0 h) that is before fermentation commenced and the last day, day (7168 h) that is, when fermentation has completed, in order to determine the kind of metabolites produced by the four bacterial isolates A8, B6, C1 and D1 in each fermentation medium. Twelve chemical compound revealed from GC-MS analysis that were contained in medium A8, B6 on day one (0 h) before fermentation commenced were: 9,17-Octadecadienal having retention time of 17.632 and percentage content (%); methyl stearate with retention time 17.838 (14.34%); Bis (2-ethylhexyl) phthalate, retention time of 21.535 (5.17); 2-Ethylacridine, 27.085 retention time of 9.09; 2-Ethylacridine, 27.085 retention time of 51.87%;1 H-Indole, 5-methyl-2-phenyl, retention time of 27.434(2.86%) whereas six compounds(secondary metabolites) were generated after fermentation process (i.e.at 168h) and their retention time and percentage content are presented respectively thus: Caryophyllene oxide, 13.163 (28.58%); Germacrene D, 14.016 (9.20); Neo intermedeol, 14.262 (16.30%); Caryophyllene oxide, 14.525 (7.55%); Bergamotol,z-alpha (10.48); Phenanthrene, 19.492 (5.28%) and 1-Phenanthrene methanol, 23.200 (22.61).From fermentation medium containing isolate B6, 10 compounds were recovered from the GC-MS analysis of extracts from fermented rice husk.

It was observed that cis-13- Octdecennoic acid (67.24%) was found to be the major compound followed by Benzoic acid, 3-Pyridyl ester (23.71%),Benzene (22.09%), 2H-Pyran, 2-(3-butynyloxy) tetra hydro cyclobutanone (17.28%), Pentadecanoic acid (16.62%), 1-Ethanol, 2-(ethylsulfinyl) (11.51 Heptadecanoic acid (11.08%), Imidazole (9.77%) and Bis (2-ethylhexyl) Phthalate (5.06%) (Table 3b).

Thirteen compounds were found in submerged fermentation medium C1 (Table 3c). It was observed that 1, 3- Decadiyne was in abundance before commencement of fermentation (0 h). However, 12-Octadecenoic acid and methyl-ester was the major component after 7 days of submerged fermentation (168 h), followed by 9, 12- Octadecadienoic acid (22%) and methyl

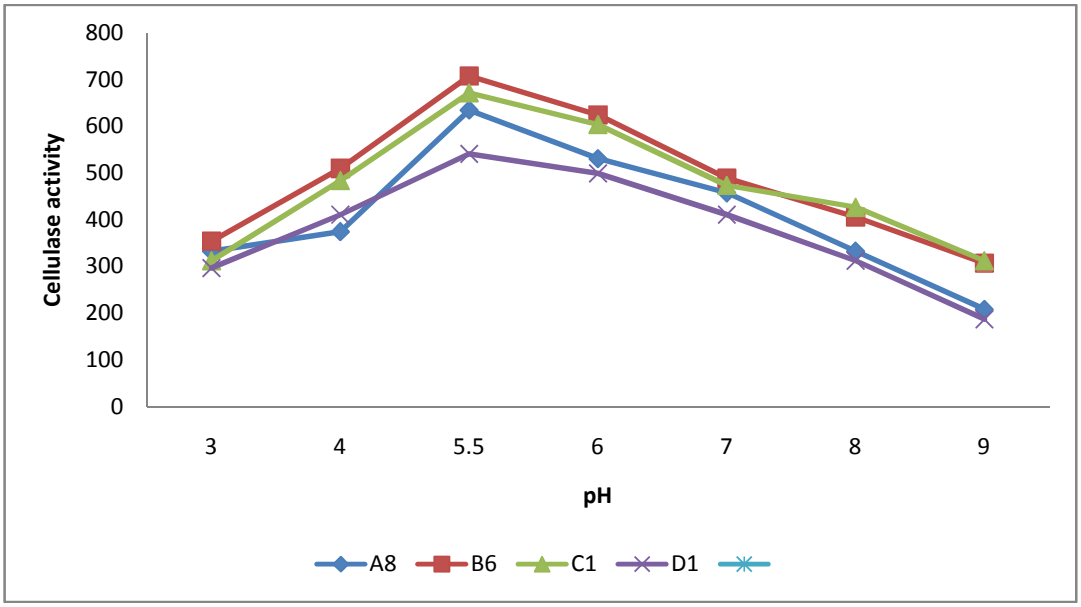


**Fig. 2. Effect of pH on crude cellulase activities**

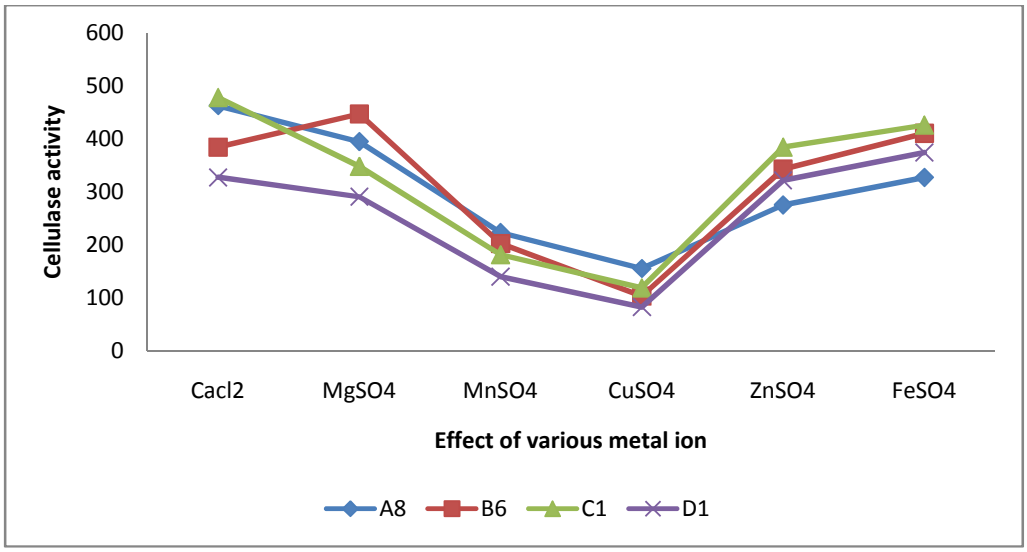


**Fig. 3. Effect of 50 Mm concentration of metal ions on cellulase activities**

stearate (12.06). The percentage of 8, 8, 10, 10-nonamethyl-cyclopentasiloxane after fermentation was very minute (2.18) followed by Diisooctyl phthalate (7.07%) then Pentadecanoic acid (7.42). Minutes quantities of 1, 1- Difluoro-2-vinylcyclopropane (9.07%), 2- Chloro- 2- methyl-butane (6.74%), Cyclobutane-methanol (5.95%), Propanal (5.30-9.46%), Oxime (5.30%) and Chloroacetic acid (6.05%) fractions were found in the submerged fermentation medium at zero hour.

The GC-MS analysis of the D1 submerged fermentation medium at 0 h on day 1 revealed 2-(n-propyl) Oxybenzylidene acetophenone (38.83%) to be the most abundant compound followed by 13, 16- Octadecadiynoic acid, methyl ester (28.16%), 2- myristynoyl-glycinamide (17.58%) and 9, 17- Octadecadienal (15.43%). However, cis- 13- Octadecenoic acid was the major compound in abundance after seven days submerged fermentation (168 h) with 67.24%, followed by pentadecanoic acid (16.62%), then heptdecanoic acid (11.08%) with the least being bis (2- ethylhexyl) phthalate (5.06%) (Table 3D).

The results of this study are in agreement with to the work of Kalpana et al. [12] which reported that GC-MS analysis of extract from *Entadapursaetha* seed using the same method yielded similar compounds of fatty acids. The results reveal possible biotransformation activities of organic compounds during the fermentation process. The *Bacillus* strains transformed Octadecadienal acids which belong to the anal group into pentadecanoic acid. *Bacillus* strains used in this study was able to transform aromatic benzoate, destabilizing the aromatic ring at the initial step and attachment of oxygen atom to the ring prior to ring cleavage. Apparently attachment of oxygen to the ring structure facilitated ring catabolism under anoxic conditions. Similar process was observed in phthalate.

## 4. CONCLUSION

This observation showed that cellulolytic bacteria biodegradation of rice husks could generate organic compounds including organic acids. These organic acids are of industrial importance in pharmaceutical and cosmetic industries.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES


1. Madhumita S, Bhattacharyya S, Behera RC. Rice Effect of temperature on morphology and phase transformations of nanocrystalline silica obtained from rice husk. 2009;82(5):377-386.
2. Oyenuga YA. Nigeria's foods and feeding-stuffs, their chemistry and nutritive values. Ibadan University Press. 1968;9-15.
3. Christi Y, Moo-Young M. Clean-In-Place systems for industrial bioreactors: Design, Validation and Operation. J. Ind. Microbiol. 1994;13:201-207.
4. Konings WN, Lolkema JS, Bolhuis H. The role of transport processes in survival of lactic acid bacteria. Antonie Van Leeuwenhoek. 1997;71:117–128.
5. Deep K, Poddar A, Das SK. Cloning, overexpression, and characterization of halostable, solvent-tolerant novel β-endoglucanase from a marine bacterium Protobacterium Panuliri LBS5$^{T}$ (DSM 27646$^{T}$). Appl. Biochem. and Biotechnol. 2015;178 (4):695-709.
6. Demain AL. Induction of microbial secondary metabolism. Internatl. Microbiol. 1998;1:259-264.
7. Abhishek P, Virendra SB. Production of cellulolytic enzymes. Bioprocessing of renewable resources to Commodity Akihiko, Bioproducts. Virendra, S. B. and K. (Eds.) Published by John Wiley & Sons, Inc. 2014;12-14.
8. Abirami V, Bharathidasan R, Meenakshi K, Kanthymathy R, Pannneerselvam A. Partial purification and characterisation of an extracellular xylanase from *Penicillium janthinellum* and Neurospora crassa. J. Nat. Prod. Plant Resour. 2011;1(4):117-125.
9. Palanivel D, Arjunan T, Venkatachalam B. Cellulosic membrane dialysis tube. Afric. J. Biotechnol. 2013;12:7-14.
10. Sharma K, Pandey A, Shashank K. Chemistry and biochemistry of lipid peroxidation products. Biochem. and Anal. Biochem. 2014;3(2):1-6.
11. Orji FA, Dike EN, Lawal AK, Elemo GN, Olatope SO, et al. Production of cellulase from *Bacillus* spp., using cellulose from Brewers Spent Grain (BSG) as sole carbon source. Asian J. Microbiol. Biotechnol. and Environ. Sci. 2013;15:839-848.
12. Kalpana DV, Shanmugasundaram R, Mohan VR. GC-MS analysis of ethanol extract of Entada Pursaetha seed. Biosci. Disc. 2012;3(1):30-33.
13. Patagundi BI, Shivasharan CT, Kaliwal BB. Isolation and characterization of cellulase producing bacteria from soil. J. Int'l Curr. Microbiol. And Appl. Sci. 2014;3(5): 59-69.
14. Khataminezhad M, Nejadsattari T, Nazemi A, Nuhi A, Mohammad R. Isolation and molecular identification of extracellular lipase-producing *Bacillus* spp. from soil. Annals Biol. Res. 2014;5(1):132-139.
15. Alagasamy S, Ashok P, Chandran C, Carlos R, Paul D. Rice bran as a substrate for proteolytic enzyme production. Brazil. Arch. Biol. And Technol. 2006;49(5):843-851.
16. Yin LJ, Lin HH, Xiao ZR. Purification and characterization of a cellulase from *Bacillus subtilis* YJI. J. Marine Sci. and Technol. 2010;18(3):466-471.
17. Amir I, Aish M, Zahid A, Hussain I, Muhammad I. Optimization of enzyme production from corn cobs using *Alternaria alternata* by solid state fermentation. J. Cell and Molecular Biol. 2011;9(2):51-56.
18. Nema N, Alamir L, Mohammad M. Production of cellulase from *Bacillus* cereus by submerged fermentation using corn husk as substrate. J. Int'l Food Res. 2015;22(5):1831-1836.




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