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Screening of Microbial Isolates from Petroleum Effluent Polluted Site and Optimization of Culture Conditions for Cellulase Production

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Abstract

Microbial cellulases have shown potential application in various industries including: paper and pulp industry, textile, laundry, biofuel production among others. Cellulase is predominantly identified among saprophytic wood decaying *Basidiomycetes* sp where they participate in a cascade of processes leading to wood decay. Out of the eight strains of white rot fungi isolated from the polluted site, strains of *Pleurotus* sp identified using cultural methods plausibly plausible showed best potentials upon screening with standard chromogene of p-nitropheny- α-D-glucopyranoside for the production of cellulose; Cultural production parameters optimized to show best suited for the enzyme production from the white rot fungi include: Incubation days, carbon sources, nitrogen and physiological pH. Lignocellulosic sugar rice bran gave highest catabolite induction of cellulase in the fermentation media with peak activity of 105 µmol/min, and ammonium sulphates ($(NH_4)_2SO_4$) as nitrogen source (109.53 µmol/min). Physiological pH of 7.0 was optimal for cellulose production while incubation day7.0 was found most suitable for the extracellular cellulose production. Petroleum effluent polluted soil has potentials for heterotrophic activity for isolates of Basidomycetes and these isolates have also shown much activity for cellulase production.

Keywords: *Basidomycetes* • Cellulase • Decaying woods • *Pleurotus* • Heterotrophic

Introduction

Emerging myriads of biotechnological applications of classes of cellulase have called for increased quest for optimal and large scaling production of the enzyme. Biotechnology conversion of cellulosic biomass is potentially sustainable to develop novel bioprocess and products. Mechanistically, cellulase is a family of three groups of enzyme: Endo (1,4) β-D glucanase (EC 3.2.1.4); exo (1,4) β-D glucanase (EC 3.2.1.91) and β-D glucosidase (EC 3.2.1.21). They catalyze the β-D hydrolysis of cellulose leading to the release of β glucose, cellubiose etc.

Cellulases are inducible enzymes produced by wide range of organisms including both bacteria and fungi in cellulosic and hemicellulosic composite fermentation medium system [1]. These organisms can be aerobic, anaerobic, mesophilic or thermophilic.

At present, lignocellulose and its component lignocellulosic compounds are major raw materials for forestry, pulp and paper industry and for biofuel production. In nature, the *Basidiomycetes* family is able to effectively degrade lignin by employing lignin degrading enzymes [2]. These organisms can be divided into wood-colonizing white-rot fungi and soil litter-decomposing fungi [3]. These fungi are major causative agents of white rot disease of rubber tree (*Hevea brasiliensis muell*. Arg.) as they serve as the source of ligninolytic enzymes (cellulase) [4]. These enzymes act in a complementary way during lignin degradation [5] of which cellulase among all the arrays of the enzymes is thought to play the most crucial role in lignin degradation, as it is found in all lignin degrading fungi [6].

Bio-utilization of celluloses in many vast biological industries has attracted the attention of researchers; Optimization of physiological parameters for abundant and favorable production of the protein is very crucial industrially to meet its demand and utilizations [7].

Materials and Methods

Equipment and reagents

All the equipment and reagents used through-out the present study were well calibrated, good working conditions and of analytical grade. The chemicals were purchased from Sigma Aldrich (Germany), BDH (England).

Sample collection

Samples for microbial isolation were aseptically collected from a petroleum effluent polluted soil located at Onne jetty site, Rivers State, Nigeria. It was transferred to the laboratory in a clean container for isolation of strains of white rot fungi as described by [8].

Isolation and identification of microorganisms

White rot fungi was isolated from the decaying plant wood (basically the stem part) using standard microbiology procedures and as well identified as described in the handbook of microbial ecology by [8]. Basic microbiology and biochemical techniques which involved: slide mounting, morphology examinations, sugar fermentation and specific enzyme test.

Molecular identification of the strains of *Pleorutus sp*.

Gene extraction was done using acuprex DNA extraction kit at the conserved region of the fungi genetic sequences (ITS-2 and 4). The extracted genome was amplified using RT-PCR with designated primers targeted for initiation of the geneomic polymerization. The amplified genetic materials were purified under agarose electrophoretic well.

Screening of isolates for cellulase production

Strains of white rot fungi were screened for the production of cellulose using standard chromogene of DNS in the presence of ditch patches of cellulose cut into discs and infused in a nutrient broth which contained 3.5 ml of 0.1 M phosphate buffer (4.5), peptone broth (1.5 ml) as described by

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[9]. The inoculated prepared fungal broth was incubated at 37°C for 2-3 days.

Enzyme production and optimization of production parameters

Crude extract of cellulase was produced from the white rot fungi using solid state fermentation system as described by [10].

Each fermenter (250 ml erlenmeyer flask) containing 100 ml of solid media immersed on a solid matrix optimized for the cellulase production containing: 1% nitrogen sources, 0.4% K_2HPO_4, 2% carbon sources, tween 80, 0.01%, sodium acetate, 01%, di-ammonium citrate, 0.05%, $MgSO_4.7H_2O$ 0.2%, $MnSO_4.4H_2O$, 0.02% at specific physiologic conditions.

Physicochemical components such as incubation time, pH, carbon and nitrogen were optimized during the production process

Effect of carbon substrates

Four (4) different rich sources of carbon substrates (both lignocellulosic and non-lignocellulosic) used include: Rice bran, glucose, wheat bran and combination of glucose and wheat bran for the production of cellulase in the semi-liquid medium at 2% w/v concentrations. Fermentation was carried out to the optimal day of enzyme production at the best physiological condition.

Effect of nitrogen sources

Four (4) different nitrogen sources used ncluded: Ammonium sulphate, beef extract, peptone and combination of ammonium sulphate and beef extracts for the production of cellulase in the semi liquid medium at 1% and 0.5% w/v concentrations respectively. Fermentation was carried out to the optimal day of enzyme production at the best physiological condition.

Effect of incubation period

The effect of incubation period was determined by incubating production medium at different time intervals for 14 days at optimum production conditions. The samples were drawn out every 24 hours and further processed for enzyme activity.

Effect of physiological pH

pH of 4.0-8.5 in the range of 0.5 units was adjusted and varied in each liquid medium using 1% HCl v/v and NaOH w/v. They were incubated at the best optimal conditions deduced for cellulase production.

Assay protocol for the enzyme activity

Cellulase activity was evaluated by assaying for β-glucohydrolase (GH) activity of the enzyme. This was achieved by measuring the release of β anomeric glucose from the cellulosic material using a modification of the 3, 5-dinitrosalicylic acid (DNS) reagent assay method described by Miller (1959).

Protein determination

The total protein content of the enzyme was estimated as described by [11] using bovine serum albumin (BSA) as the standard protein. Absorbance was taken at wavelength of 750 nm (Figures 1-3).

Figure 1. Pure colonies of strains of *P.djamor.*on PDA medium.

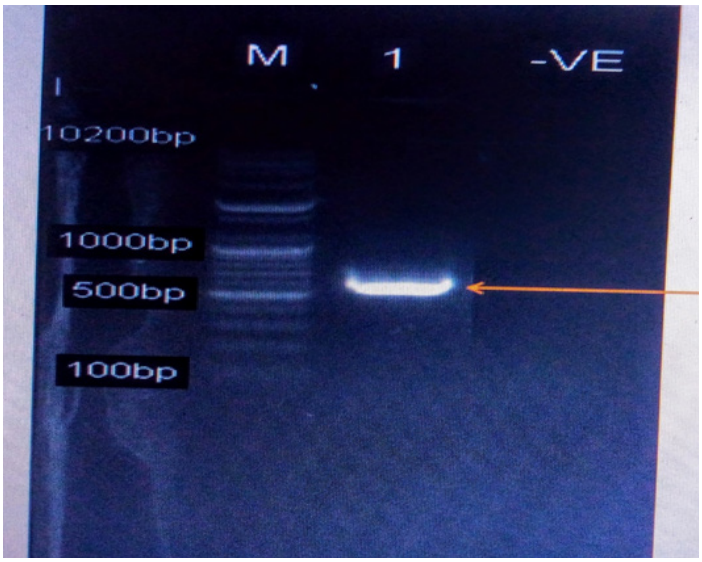

Figure 2. Agarose gel electrophoretic view of the amplified fungal genome from the internal transcribed spacer region 1 and 4.

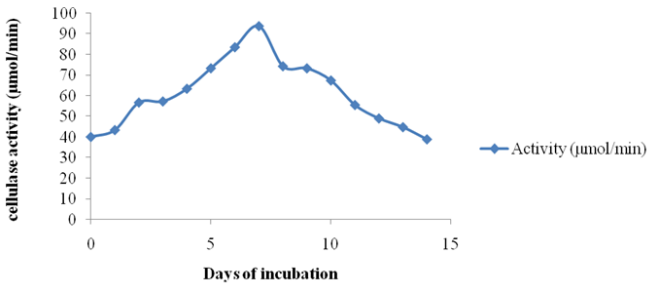

Figure 3. Effect of incubation days on cellulose production by White rot fungi in a solid state medium using glucose as the only carbon source.

Results and Discussion

Basic microbiology and biochemical tools were used for general identification of the white rot fungi isolated. Molecular screening depicted the identified organism as *Pleorotus* after molecular amplification using the RT-PCR. Strains of white rot fungi isolated from the polluted soil showed potential of production of cellulase as screened using the chromogenic compound, p-NPG , in the presence of cut ditch patches of cellulosic material infused in the nutrient broth as shown (Figure 1). The yellow coloration of the screening broth after 96 hours of incubation at 37°C showed a positive test [12-16] reported similar observation in their different study on white rot fungi lignolytic enzymes from decaying rubber plant using glucoronic acid compounds as their standard screening substrate.

The effect of incubation days on the production of cellulase from white rot fungi in a solid state fermentation reveal that the enzyme showed the highest activity on day the 7th day following 14 days of incubation period. Extracellular protein production on these designated days respectively is evident of catabolite inducement of the substrate present in the fermentation media to the organisms.

pH 7.0 showed the optimal range for production of the extracellular protein (cellulase) from the strains of white-rot fungi with peak activity of 102.34 and 102.34 micromole per minute; this shows the adaptability of the fungi at lower pH conditions. As reported in proceeding of mycology handbook, strains of *Basidomycetes* are favourable inhabitants of high hydrogen ions constituted environments [17] reported a pH of 5.5 for the production of cellulase from decaying woods of mahogany (Figures 4-6).

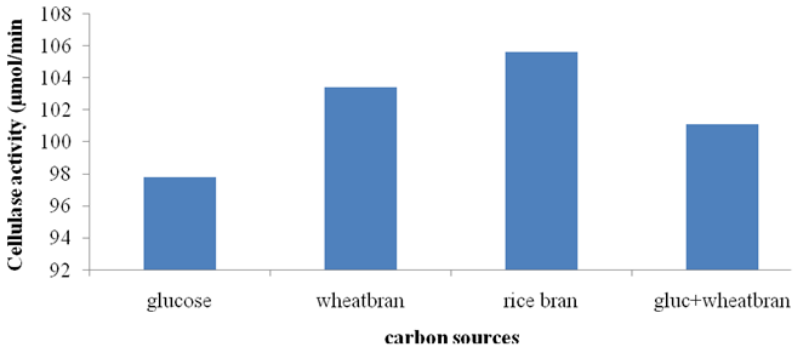

Figure 4. Effect of carbon sources on cellulase production by White rot fungi in solid state medium using varieties of carbon sources.

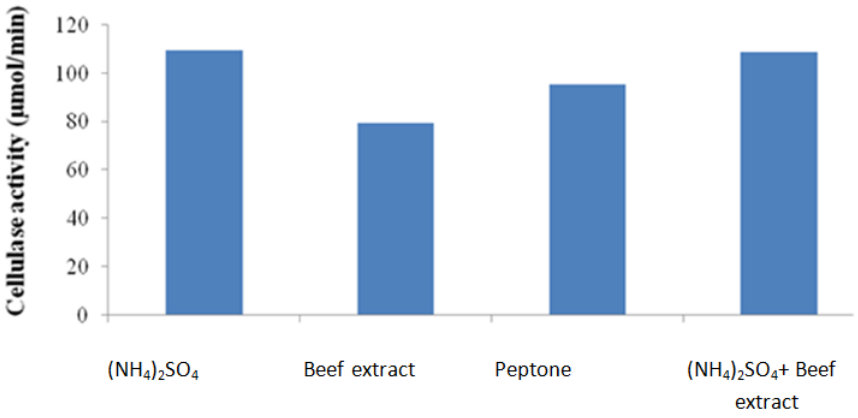

Figure 5. Effect of Nitrogen sources on cellulase production by White rot fungi in a solid state medium using ricebran as the only carbon source.

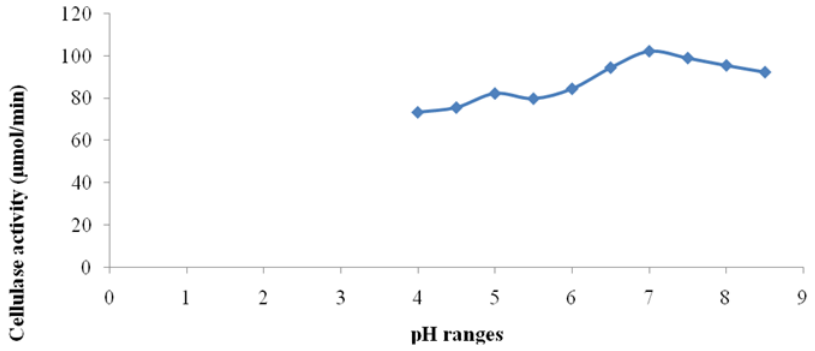

Figure 6. Effect of different pH on cellulose production by White rot fungi in a solid state medium using rice bran as substrate

Fermentation nutrients optimized during the production process showed variant inducible strengths of both lignocellulosic and non-lignocellulosic materials for enzyme production; rice bran gave better catabolite induction of cellulase in the fermentation media with peak activity of 105 µmol/min, wheat bran showed similar effect on the production of cellulase while glucose was least as a catabolite for cellulase production. Among the nitrogen sources optimized for the enzyme production, ammonium sulphate $(NH_4)_2SO_4$ showed greater induction on organism for cellulase production with activity of 109.53 µmol/min [18-24]. Differential preference of the organisms to the available substrates for protein production can be attributed to the geneomic composition as well as physical surrounding of the strains of the white rot fungi as reported by [4].

Conclusion

Cellulases among other members of lignolytic enzymes are integral part of the evolving biotechnology industry. Strains of white rot fungi from petroleum effluent polluted soil showed great potentials for cellulase production. The utilization of economic sustainable materials by the organism in pruducing the enzyme paves way for increased production of the enzyme, to meet its current demand industrially.

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Author's Contributions

Ani Adline Eberechukwu: Conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Chinakwe E C: Supervised the research, analyzed the data, interpreted the data and revised the manuscript.

Nwogwugwu U. N.: Guided the experimental design, performed the experiment and processed the data.

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