Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 15, No. (4) : 2013 : 839-848

ISSN-0972-3005

PRODUCTION OF CELLULASE FROM *BACILLUS* SPECIES USING CELLULOSE FROM BREWERS SPENT GRAIN (BSG) AS SOLE CARBON SOURCE

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***Key word** : Cellulase, Brewers Spent Grains (BSG), Fermentation and waste utilization*

Abstract–Cellulose from Brewers Spent Grain (BSG) and crystalline cellulose were utilized as sole carbon source for the submerged production of Cellulase. A total of 21 bacterial isolates were obtained from Brewers Spent Grain undergoing deterioration. Out of the 21 isolates, 7 bacterial isolates showed various degrees of Cellulase production on plate assay (Nutrient Agar +2% (W/V) Cellulose) by their zones of clearance of Cellulose. The Brewers Spent Grain was characterized for its composition, and its moisture content, Crude Fibre, Fat, Protein, Ash and Total carbohydrate were 17.62%, 13.94%, 8.08%, 25%, 3.68% and 31.68% respectively. Cellulase production screening of bacterial isolates showed that *Bacillus* species strain B223 had the highest zone of clearance (2.8cm ± 0.02), and was used for the submerged Cellulase production. Microbial population dynamics in the enzyme production medium in the 250ml capacity Erlenmeyer flasks containing BSG cellulose increased progressively from zero hour to the 120 hour. pH of the BSG cellulose medium increased from 5.28 to 6.89, while the set-up containing crystalline cellulose had its pH increased from 5.28 to 7.46. Crude cellulase activity of the cellulase in Filter paper Units (FPU) for the cellulase produced from BSG Cellullose ranged from 328 FPU, 2749PU, 32166FPU, 31910 FPU, and 33130 FPU at zero hour, 24hour, 48hour, 72 hour, 96 hour and 120 hour of fermentation respectively. The optimum pH for the production of Cellulase was 6.89. Specific Cellulase activities of Cellulase produced using BSG cellulose and Crystalline cellulose were between 12.45-4319.4 (Unit/Mg) and 19.6 -5711 (Unit/Mg) respectively. Cellulases are industrial enzymes with broad applications, and production using raw materials like cellulose from Brewers Spent Grain makes the enzyme cost effective, and converts the waste (BSG) to wealth. Further research actions are on-going to purify the crude Cellulase.

INTRODUCTION

Cellulase refers to a group of enzymes produced mainly by fungi, bacteria, and protozoans that catalyze cellulolysis (i.e. the hydrolysis of cellulose). Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. Cellulase is used in the fermentation of biomass into biofuels (Watanabe *et al.*, 1998). Nowadays, enormous amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Celluloses are regarded as the most important renewable resource for bioconversion. Many Cellulosic substances were hydrolyzed to simple sugars for making Single Cell Protein, sweeteners etc. It has been become the economic interest to develop an effective method to hydrolyze the cellulosic biomass. Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters as secondary metabolites (Lee and Koo, 2001). Increasing knowledge of mode of action of Cellulase; they were used in enzymatic hydrolysis of cellulosic substances (Kubicek *et al.*, 1993). Cellulase production from bacteria can be an advantage as the

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enzyme production rate is normally higher due to the higher bacterial growth rate as compared to fungi. Screening of bacteria, optimization of fermentation conditions and selection of substrates are important for the successful production of cellulase (Ariffin *et al.,* 2006). The cellulases have attracted considerable attention in recent years due to their great industrial potentials. The enzymatic hydrolysis requires Synergistic action of cellobiohydrolas or exoglucanase glycoside hydrolases (E.C.3.2.1.91), endoglucanase or (E.C. 3.2.1.4) and cellobiase or β-glucosidase (E.C.3.2.1.21). The latter feature provides them capabilities to produce animal feed, formulation of detergents, juice clarification, paper industry and wine production. Cellulases contribute to 8% of the worldwide industrial enzyme applications (Sadhu *et al.,* 2013). Cellulases have a wide range of applications, and the main potential applications are in food, animal feed, detergent and cosmetics industries, textile, fuel, and chemical industries. Other areas of application include the paper and pulp industry waste management, pharmaceutical industry, protoplast production, genetic engineering, and pollution treatment (Coughlan, 1985a; Coughlan, 1985b). The promising cellulolytic microorganisms can be employed for the production of cellulolytic enzymes such glucopyranoside as CMCase, FPase, and β-glucosidase by using different agro-residues as the carbon source during submerged fermentation. The agro-based materials which have previously served as carbon source for the production of Cellulases include Rice husk, Filter paper and cellulose powder (Kumar *et al.,* 2009). Others include Saw dust or wood shavings. However the use of carboxymethylcellulose has continued to make cellulase production very costly, and consumers and industrialists pay heavily for extra cost of production. Several attempts have been made to cost-effectively produce Cellulase from cheap, affordable and grossly available raw materials. Brewers' spent grain (BSG) is available at low or no cost throughout the year, and is generated in large quantities (20kg/ 100 L of beer produced) not only by large, but also small breweries. BSG is of high nutritive value (Tang *et al.,* 2009), and contain abundant cellulose, hemicelluloses, lignin and high protein content. In View of its high cellulose content, it has become pertinent to use the BSG as source of carbon for submerged production of Cellulase enzyme. Enzymes are highly priced items for industrial applications in Nigeria, and most enzymes used in Nigeria are imported from Netherlands, Denmark, Belgium, and United Kingdom etc. The costs of importation also make finished goods and products very expensive in Nigeria. If the Nigerian industries are able to access and use industrial enzymes produced in Nigeria, it will save a lot of foreign exchange earnings. The objectives of this study were as follows; to study the composition of the Nigerian Brewers Spent Grain, to Isolate and screen for Cellulase hyper-producing bacteria, characterize, identify the Cellulase hyper producing bacterial strains, and produce Cellulase using BSG as sole carbon source through the submerged fermentation method.

MATERIALS AND METHOD

Isolation of *Bacillus* species

The strain of choice was isolated from Brewers Spent Grain undergoing bio-deterioration in the open environment. One (1) gram of the deteriorating Brewers Spent Grain was weighed and aseptically transferred into the first test tube of 1 in 10 dilutions containing sterile distilled water as diluent. The serial dilution was done to the tenth tube. A known volume (1ml) was pipetted with a Micropipette and poured on sterile disposable petri dishes. About 12mls of a freshly prepared Nutrient Agar which has not solidified but at a temperature of 45^0C were dispensed into the petri dishes containing the diluted deteriorated Brewers Spent Grain. The Nutrient Agar plates were allowed to gel in the inoculation chamber. Incubation was done at 37^0C at for 24 hours and colonies were counted using standard microbiological method and formula. Pure cultures were obtained by sub-culturing isolates from the primary plates to obtain discrete colonies

Biochemical characterization and identification

The bacterial strains with high cellulase producing ability were picked for identification and further investigation. Preliminary identification by morphological analysis was conducted by using light microscope after gram staining, and spore staining. Biochemical characterization was carried out on the isolates using Analytical Profile Index (API) kits for *Bacillus*

Spore Staining

A smear of the pure bacterial colonies was made from the secondary plates was prepared, air-dried

and heat-fixed. A beaker of water was put on the hot plate and boiled until steam was coming up from the water. Then the hot plate was put down so that the water is barely boiling. The wire stain rack was placed over the beaker which now has steam coming up from the boiled water. This was done for 5minutes. The heat fixed smear was flooded with the primary dye, malachite green, and left for 5 minutes. The smear was placed in slowly running tap for 1 minute and then flooded with the counterstain dye, safranin, and left for 1 minute. The slide was Washed with water, blot dried with tissue paper. The slide was viewed under x 40 and x100 objective lenses. Spores were light green whereas vegetative cells retained the colour of the counterstain safranin. This procedure has been previously reported by Kanu and Okereke, 2004.

Proximate analysis on the Brewers Spent Grain (BSG)

Determination of moisture content

Moisture content was determined at the world Bank Step B laboratory of Analytical Department, Federal Institute of Industrial Research, Oshodi using the Rapid Moisture Analyzer (A and D Compound LTD; Serial Number- P1024360), Japan. The analyser was put on, and temperature of the halogen and target mass set at 130°C and 5grams respectively. The apparatus was started, and heating of the halogen did not tamper with the volatiles. The analyzer stopped automatically as soon as the sample is dried and percentage moisture content read off on the Visual Display Unit.

Determination of Fat

Fat was determined with the Soxtec fat determination Equipment (Tecator™) at the World Bank Step B laboratory, Federal Institute of Industrial Research, Oshodi, Lagos. The method was previously described by Inuwa *et al.*, (2011). The lipid content of each sample was extracted by soxhlet with the use of an extraction apparatus mentioned above (Tecator™). Each thimble used for the extraction was weighed, labeled before 10 g of the powdered sample was transferred into the thimbles. The weights of the thimble plus the sample then were recorded, and the apparatus filled with 250 cm^3 petroleum ether. The top of the condenser were then plugged with cotton wool and cold water from the tap was circulated through the condenser. This apparatus were then heated gently and the extraction allowed to continue until the solvent around each thimble became colourless after 4 h indicating the end of the extraction. The thimbles were then removed from the apparatus and the cotton wool removed before they were dried to constant weight in an oven set at 70^0C, the thimbles were cooled and then re -weighed. This was repeated for each of the sample. The lipid content of each content was calculated as follows using the formula:

Crude fat (%) = W1 - W2 / W3 x 100

Where; W1 = Weight of the thimble plus dried sample before extraction

W2 = Weight of thimble plus residue after extraction

W3 = Weight of dried sample used in grams

Determination of crude fibre

Crude Fibre in the Spent Brewer's Spent Grain was determined by using standard procedures with the aid of Fibretec machine (Fibertec™ 2010 model). Crude Fibre represents the organic residue left behind after the material has been treated under standardized condition with petroleum ether boiling dilute sulfuric acid, boiling dilute sodium hydroxide solution and dilute hydrofluoric acid. A soxhlet apparatus was used to extract fat from 10 g of grounded sample; 100cm^3 of boiled 1.3% $H_2SO_4$was poured into the extracted sample and allowed to boil for 30 min. It was filtered; the excess acid was washed down from the sample with warm water, and transferred into a beaker. 100 cm^3 boiled 2.5% NaOH was added and allowed to boil for 30 min. It was filtered, excess NaOH removed from the residue by washing down with warm water. The filter paper containing the residue was folded and placed in a crucible of known weight, and placed in an oven, dried at 105°C for 3 hours; the weight was recorded, and dried further for 15 min to constant weight. The crucible was transferred to a furnace and burnt at 500°C to complete ash

Crude fibre (%) = W1 – W2/ W3 × 100

Where;

W1 = Weight of Residue + Crucible before extraction

W2= Weight of thimble plus residue after extraction

W3= Weight of dried sample used in grams

Ash was determined using the method previously reported by Inuwa *et al.*, (2011) with little modification based on the availability of materials. Ten (10) g of the Brewers Spent Grains were dried

and finely ground and then carefully mixed to facilitate the choice of a representative sample in crucibles (duplicate analyses). Dry ashing procedures were used. The samples in crucibles duly labelled were loaded in a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600°C. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO_2, H_2O and N_2. The muffling was done for 3hours until the BSG is ashed. Percentage ash content was calculated as follows; Percentage Ash Content = Mass Ash/ Mass Dry X 100. Mass of the dry BSG was obtained while determining the Moisture content.

Determination of carbohydrate

One gram (1g) of macerated sample was placed in 25mL bottle; 10mL of distilled water was then added and shaken vigorously followed by addition of 15mL of 52% perchloric acid. This was stirred continuously for 30minutes and the mixture was later filtered using Whatman no1 filter paper. One milliliter (1mL) of the filtrate was mixed with $4cm^3$ of Anthrone reagent in a test tube and the absorbance of the mixture was measured spectrophotometer at a wavelength of 620nm. The total soluble carbohydrate was then estimated using the standard curve of Glucose (Pearson *et al.,* 1976).

Determination of protein content in Brewers Spent Grains

Crude Protein was determined by the Kjeldahl method. Dried (5 g) and pulverized Brewers Spent Grain was digested in 2ml concentrated H_2SO_4 in the presence of selenium catalyst in a fume cupboard until a clear digest was obtained (Dubbey, 2008). The Nitrogen content was of the diluted digest was obtained colourimetrically at 630nm according to the procedures previously reported by Charlot (1964). Protein content of Brewers Spent Grain was calculated as Nitrogen x 6.25 as previously reported by Oboh and Masodje (2009).

Screening for hyper producers of cellulase

A total of 21 strains of bacteria strains were obtained from deteriorating Brewers Spent Grain. As they were isolated from rotten lignocellulose BSG, there is high chance that the strains are Cellulase producers. Therefore, a preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC agar which was prepared by adding 5% Caborxymethyl cellulose (CMC) into Nutrient Agar (Oxoid). The CMC agar plates were incubated at 37°C for 3 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured using a meter rule in order to select for the highest Cellulase activity producer. These procedures were adopted from previous work of Bai *et al.,* (2012) with modifications. The largest zone of Clearance was assumed to contain the highest activity.

Submerged production of Cellulase

The submerged production of Cellulase was carried out using mineral salt medium developed using the following materials (g/100mL); Peptone 1, Yeast extract 0.5, $MgSO_4$. $7H_20$ 0.2, $CaCl_2$. $2H_2O$, NA_2CO_3 1.0, $CoCl_2.H_20$ 0.1, and Brewers Spent Grain (BSG) (1% w/v) as source of cellulose. The formulation followed previous studies but with modification (Kyanmi-Horani, 1996; Bai *et al.,* 2012). Five (5) conical flasks each containing the 100 mls of the above compounds were prepared and another five series conical flask were filled with 10omls of the above salts but supplemented with 1% Carborxymethyl cellulose as control. Each of the flask was inoculated with 10mls of young Bacillus culture and the zero hour Parameters such as pH, Total culturable heterotrophic bacterial counts, Crude Cellulase activity, Specific activity, Total protein were determined using standard procedures. The mineral salt medium flask which is inoculated with *Bacillus* isolates were loaded for Fermentation in a rotary incubator at the temperature of 37°C and constant impeller speed of 450 rpm. The interval of analyses was 24hrs. At the point of analyses, a conical flask is withdrawn from the shaker incubator for all Cellulase and microbial assay.

Analyses carried out during submerged production of cellulase

Determination of total culturable bacterial count

This was done using the serial dilution method using the method previously reported by Kanu and

Okereke (2004). One (1) mL of the enzyme medium was pipetted with the aid of a micropipette and microtip and delivered into the first tube of 9 tubes containing 9mls of sterile distilled water, and the transfer of 1mL to the subsequent tube continued to the 9th tube. One mL of appropriate dilution was transferred into a sterile petri dish and about 12mL of cooling nutrient Agar nutrient Agar was poured into the plate containing the diluted sample. Incubation was done at 37°C for 18 hours. Colonies were counted and the microbial load at each interval was quantified using the formula;

$$\frac{\text{No of Colonies}}{\text{Volume used}} \times \text{Dilution factor}$$

Determination of pH

The pH was obtained by dipping the pH meter electrode which was calibrated with a buffer of known pH immediately into the crude enzyme production medium and stable pH reading taken as soon as it appeared on the pH meter.

Determination of protein content of the crude cellulase

Protein content of the enzyme extracts were determined by following the method of Lowry *et al*., 1951 with Bovine serum albumin as standard. Protein extract, 0.2mL was measured into tubes and 0.8ml distilled water was added to it. Distilled water was used as blank while BSA standard curve was equally set up, (10 mg/mL), 1-10mg/mL, 5.0mL of alkaline solution was added to all the tubes, mixed thoroughly and allowed to stand for 10 mins, 0.5mL of Folin- C solution was added to all the test tubes and left for 30mins after which the optical density was read at 600 nm wavelength in a spectrophotometer (T70 PG Instrument UV model). The protein concentration was estimated using values extrapolated from the standard graph of protein.

$$\text{Protein concentration (mg/ml)} = \frac{\text{Absorbance value}}{\text{Gradient}}$$

Determination of crude cellulase activity

The activity of crude cellulase in the centrifugal supernatant was determined according to the method of Mandels *et al*., (1976) which had also been previously reported by Kumar *et al*., (2009). Aliquots of appropriately diluted centrifugal supernatant as enzyme source was added to 50 mg of Whatman No. 1 filter paper strip immersed in 1 mL of 0.05 M sodium citrate buffer, pH 5.0. After incubation at 50°C for 30 min, the reducing sugar released was estimated by the Dinitro Salicyclic Acid (DNSA) method (Miller, 1948; Akinola *et al*., 2004). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar.

Determination of specific activity

The Specific activity of an enzyme gives the measurement of the activity of the enzyme. This is the activity of an enzyme per milligram of total protein (expressed in units/mg). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of protein. Specific activity of the cellulase was determined using the formula below.

$$\text{Specific activity} = \frac{\text{Enzyme activity (units/ml)}}{\text{Protein concentration (mg/ml)}}$$

RESULTS AND DISCUSSION

A total of sixteen bacterial isolates were screened, and only Seven (7) showed the cellulase production properties at different degrees (Table 2). The bacterial isolates showed hyper producing properties of cellulase. Gram positive rods labeled strains B220, B221, B222, B223, B224, B225, and B226 had clearance zones of 1.6cm, 1.7cm, 2.1cm, 2.8cm, 2.4cm, 2.0cm, and 1.4cm respectively. The bacterial strain B223 showed the highest clearance zone of 2.8cm (Table 2, and Figure 1), and was selected as the highest hyper-producer for the submerged production of Cellulase.

The proximate analysis done on the Brewers Spent Grain showed that the BSG contains 13.62%, 8.08%, 25%, 3.68% and 31.68% of Crude Fibre, Fat, Protein, Ash, and Total Carbohydrate respectively

Table 1. The composite studies on Fresh Brewers Spent Grains

Parameter	Values % ± S.D
Moisture Content	17.62 ± 0.48
Crude Fibre	13.94 ± 1.43
Fat	8.08 ± 0.21
Protein	25 ± 0.16
Ash	3.68 ± 0.10
Total Carbohydrate	31.68 ± 0.00

The mean of duplicate readings served as the composite value. Values are in percentages, SD; Standard deviation

Table 2. The zone of clearance of Bacterial isolates during agar plate Cellulase screening

Strain No.	Gram Reaction and Microscopic morphology	Zone of Clearance (Cm) ± S.D.
B220	Gram Positive Rod	1.6 ± 0.04
B221	Gram Positive Rod	1.7 ± 0.03
B222	Gram Positive Rod	2.1 ± 0.02
B223	Gram Positive Rod	2.8 ± 0.02
B224	Gram Positive Rod	2.2 ± 0.43
B225	Gram Positive Rod	2.0 ± 0.37
B226	Gram Positive Rod	1.4 ± 0.41

(Table 5). The moisture content was 17.62%, as such there is need for proper drying under asceptic conditions before storage for future use. However where technology is high and power is stable, the BSG could be dried with Freeze drier or perhaps using a refrigerator. The 31.68% Total carbohydrate observed in the BSG is quite low when compared with the amount in unused barley or sorghum. However this is not surprising because the fermentation process has reduced the amount of carbohydrate and fermentable sugars. Adeniran *et al.,* (2008) reported 79.9% carbohydrate in Brewers Spent Grain. Protein level is similar to the previous reports of Kanauchi *et al*., (2001) which documented 24% proteins in BSG in Japan. The ash content of any biological material is the organic residue that remains thereafter burning the material. Ash content of the BSG under study was 3.68%, and the Crude Fibre was 13.94%. The crude fibre is relatively high showing that the BSG as an organic material is still in its raw or crude form. The high Fibre content makes the BSG a good raw material to beef-up fibre content in different food products. Kanauchi *et al.,* (2001) and Russ *et al*., (2005) independently reported 4.5% and 2.4% Ash in Brewers Spent Grain, while Adeniran *et al.,* 2008 reported 3.3% Crude Fibre in Brewers Spent Grain.

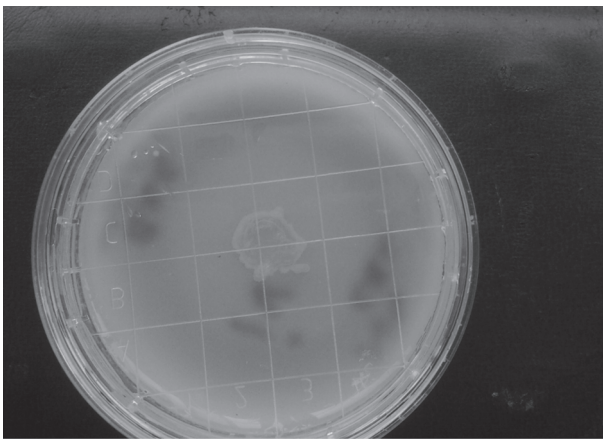

Fig. 1. Zone of clearance of *Bacillus* species (strain B223) used for Cellulase production

There was progressive increase in the total culturable heterotrophic bacterial counts in the enzyme production media containing either Cellulose from Brewers Spent Grain or Crystalline cellulose powder (i.e. refined cellulose powder). At zero hour the Total culturable heterotrophic bacterial count of the inoculated enzyme medium containing Cellulose from Brewers Spent Grain was $6.1x10^1$Cfu/ml, The microbial count progressively increased to 7.28×10^4Cfu/mL, 1.99×10^7Cfu/mL, 2.8×10^8Cfu/mL, 56×10^8Cfu/mL and 18×10^9Cfu/mL on the 24th hour, 48th hour, 72nd hour, 96th hour and 120 hour respectively. The flask containing Cellulose from crystalline cellulose as sole carbon source had a microbial load of 7.6×10^1Cfu/mL. However, during the fermentation period the microbial load increased from 5.10×10^5Cfu/mL, 2.24×10^7Cfu/mL, 6.0×10^8Cfu/mL, 62×10^8Cfu/mL and 16×10^9Cfu/mL at the 24 hour, 48hour, 72 hour, 96 hour and 120 hour respectively (Figure 2).

The pH of the BSG cellulose enzyme production medium at zero hour was 5.29. This acidic pH progressed from 5.80 at the 24th hour of fermentation to 6.89 at the 120hours of fermentation. Crystalline cellulose powder medium began at acidic pH and tilted to an alkaline pH of 7.46 at the 120 hours of fermentation (Table 7). The change in pH is probably attributed to effective utilization of carbon from either Brewers Spent Grain or Crystalline cellulose and the limiting nutrients from yeast extract (Nitrogen) and phosphorus from salts in the medium (Fig. 3).

In addition, Crude Cellulase activity i.e. Filter Paper Unit (FPU) of 328 and 291 Units of Cellulase enzyme was reported for Cellulase produced from the BSG cellulose and crystalline cellulose

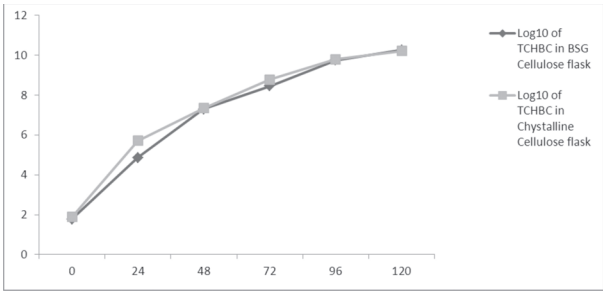

Fig. 2. Changes in total culturable heterotrophic bacterial count during the study period

respectively at the zero hour when the bacterial inoculation into the media was just carried out (Table 8). The Crude enzyme activity was monitored in 24 hours interval. The activity of the Crude Enzyme (Cellulase) produced using cellulose from Brewers Spent grains were observed as 2749.00 FPU, 32166.00FPU, 28880.00FPU, 31910.00FPU and 33130.00FPU respectively at the 24 hour, 48 hour, 72hour, 96 hour and 120 hours of fermentation (Fig. 4.) Similarly, the activities of the crude Cellulase produced using crystalline carboxymethyl cellulose as carbon source were observed as 291.0, 29810.00, 25960.00, 25960.00, 31240.00, and 33110.00 respectively at 24 hour, 48hour, 72hour, 96 hour and 120 hours respectively. Comparatively, on the 96 and 120 hours of Cellulase production, there was no significant difference in the enzyme activity between the cellulase produced from BSG cellulose and refined Crystalline cellulose. However, at the 48hour and 72 hour fermentation study crude Cellulase produced using BSG cellulose as carbon source had significant higher activity than the crude Cellulase produced from Crystalline cellulose powder (Fig. 4). This is quite surprising as the BSG cellulose appears to be theoretically crude, and un-refined as against the refined carboxymethyl cellulose. The fact that the BSG cellulose could be more available to the *Bacillus* producing the Cellulase could justify this observation in crude Cellulase activity variance. It is worthy to note that the BSG cellulose as crude cellulose which has not undergone any purification is good source of carbon during fermentation and with greater chances of enhanced performance after partial purification of the cellulose. With reference to the activity of cellulase produced from BSG Cellulose, this is the best time the Federal and State Governments in Nigeria should allocate meaningful funds for Research and Development in the area of Raw material Development / agro-allied processing which is a major mandate placed on the Federal Institute of Industrial Research, Oshodi, Lagos-Nigeria. The highest Cellulase activity was observed at the 120 hours for both Cellulase produced from Brewers Spent Grain (33130 Units) and Crystalline cellulose (35110.0 Units). The crude enzymes were harvested and packaged at 120hours of fermentation (Fig. 7).

Protein analyses using the Lowry's method was done during the enzyme study period. There was a decrease in the protein level of the enzyme from day 0 to the 5th day. The protein content in the BSG cellulose set-up at time 0 was 10.44Mg/mL and it decreased to 7.67 on the 120 hour/5th day of fermentation. The Crystalline cellulose set-up had a protein content of 11.63 Mg/L on the Zero hour and that decreased to 5.56 on the 120 hours of fermentation. This observation is not surprising since it is a proof that the yeast extracts which supplied nitrogen to the cellulase hyper producing

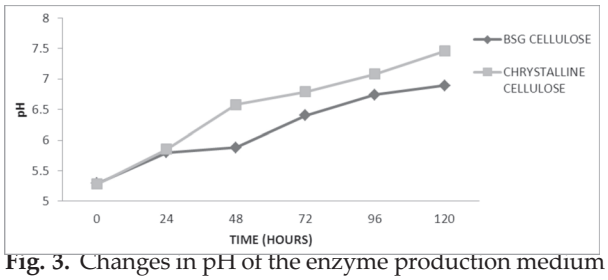

Fig. 3. Changes in pH of the enzyme production medium during the study period

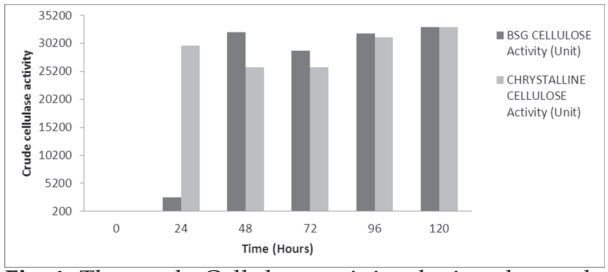

Fig. 4. The crude Cellulase activity during the study period

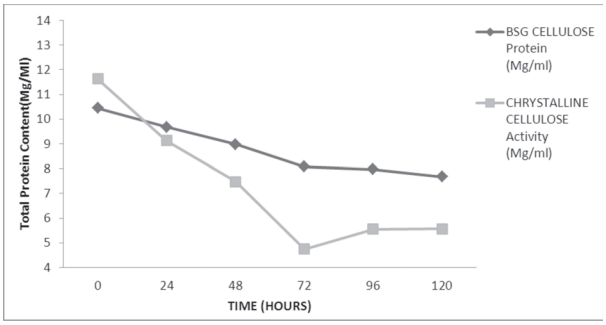

Fig. 5. Total protein content of the Crude Cellulase

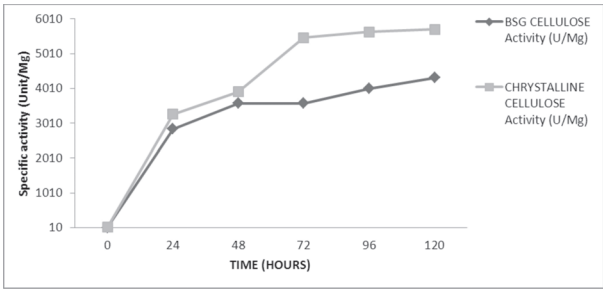

Fig. 6. Specific activity of the Crude Cellulase

Bacillus was metabolized by the organism. The yeast extract which is a protein when utilized by the Cellulase producing bacteria reduces the protein content of the crude enzyme by microbial metabolism (Fig. 5).

Specific Cellulase activity was also determined. It is the mathematical quotient of enzyme activity and protein content of the enzyme. Specific activities of the crude Cellulase were determined and reported in Units/Mg. In both enzymes produced from either BSG cellulose or crystalline cellulose as carbon sources, the specific Cellulase activities increased progressively from day 0 to the 5th day. The specific activity of the produced Cellulase in the BSG cellulose set-up was 12.45Unit/Mg at zero hour. The specific activity also got increased in to 2839 Units, 3518.3 units, 3574.3Units, 4003.8 Unit and 4319 Units in the 24hour, 48hour, 72 hour, 96 hour, and 120 hours respectively. The enzyme production medium with crystalline cellulose as sole carbon source had specific activity of its Cellulase increased from 19.6 Units on zero hour to 3265.10 units, 39056.00, 5465.30, 5628.8, and 5711.00 Units/ Mg at the 24 hour, 48th hour, 72 hour, 96hour and 120 hours of Cellulase production. This is in line with the microbial counts of the enzyme production media which increased in number from day 0 to the 5th day of the study. Optimum specific Cellulase activities were recorded at the 5th day as 4319.4 Units/Mg and 5711.00 Units/Mg for Cellulase produced from BSG Cellulose and Crystalline cellulose respectively (Fig. 6). Ariifin *et al.*, (2006) in a 2L cellulase production medium reported increase in cells (*Bacillus pumulus*) progressively within 54hours, and bacterial growth curve is similar to the bacterial growth curve from this study.

Kumar *et al.*, (2009) reported the production of cellulolytic enzyme in a submerged fermentation method using different using different agro-based substrates including Rice husk and Whatman filter paper as carbon sources.

Okolo (2004) reported the production of cellulolytic enzyme with an edoglucanse and β-glucosidase activities of *448 and 988* units respectively from *Paecilomyces* species using some agro-based wastes such as Corn hob, Rice hull, and wood shavings e.t.c under submerged fermentation method.

Sadhu *et al.*, (2013) reported the production of a thermotolerant endoglucanase from paddy Straw, Whatman filter paper, sugar cane bargasse, banana stem, whatman filter paper, newspaper, avicel. The carboxymethyl Cellulase activities reported while using the aforementioned agro-based waste ranged between 0.07 to 0.073. Bai *et al.*, (2012) observed cellulase production from bacterial isolated from Cow dung, and maximum production of enzyme was obtained at pH 7.0. Comparatively with our study on submerged production of cellulase using Cellulose from Brewers Spent Grain, the optimum pH for cellulase production was 6.8(near neutral pH). In addition the cellulase produced from crystalline Cellulose showed optimum cellulase activity at 7.46, which is slightly alkaline and near neutral pH. Thus our observation from this study remains much similar with the reports of Bai *et al.*, (2012) with respect to pH.

Otajevwo and Aluyi, (2010) reported optimization of cellulase production by *Bacillus subtilis, Clostridium cellobioparum* and *Pseudomonas* in a broth fermentation method. Total activity of the produced cellulase ranged between 1.64 – 5.32IU/mL. Comparatively the *Bacillus* strain B223 Cellulase had high filter paper unit and its specific activities

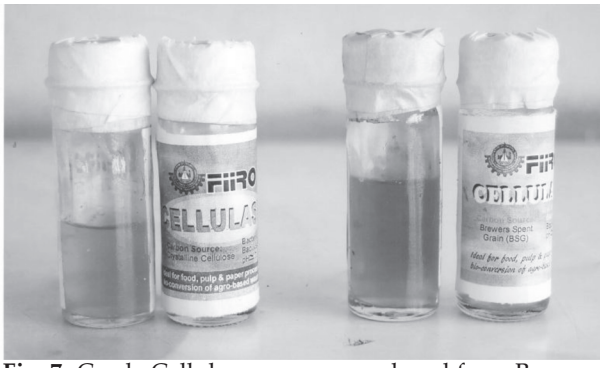

Fig. 7. Crude Cellulase enzyme produced from Brewers Spent Grain (BSG) and crystalline cellulose by *Bacillus* species (Strain B223) in a submerged fermentation method.

are relatively high.

Bai *et al.*, (2012) reported the production of Cellulase using yeast extract as source of Nitrogen in a submerged fermentation method. Awojobi *et al.*, (2012) observed and reported that the best source of nitrogen for Cellulase production was Potassium nitrate (KNO_3) by *Trichoderma* in a solid state fermentation.

CONCLUSION

Cellulase enzyme production accounts for 40% of cost in bioethanol production, pulp and paper, detergent production, e.t.c., to reduce the cost

of production; lignocellulose substrate is used instead of synthetic cellulose due to their reasonable cost, high enzyme production capacity etc. The reduction in cost of production of Cellulase using cheap and available Brewers Spent Grains paves an economically easy way for different industrial processes. In addition, it is worthy to categorically state that this waste utilization programme of utilizing BSG for Cellulase production removes the chance of littering the BSG waste in the environment. This makes the environment clean and free of such waste. In addition, it impacts much economic values on the BSG waste. The microorganism *Bacillus* sp B223 used in this study was able to grow and to produce Cellulase using BSG waste as the sole source of carbon. The results obtained herein make this strain and these low cost substrate worthy of advanced investigation (Cellulase purification), and potentially feasible for biotechnological applications in different areas of Pulp and Paper, Wine clarification, Vegetable juices extraction, Industrial oil extraction from local seeds, application in Animal feed to aid digestion, and detergent production etc.

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