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Purification and Quantification of Cellulase Produced by *Aspergillus Niger* Using Alkali Treated and Untreated Sugarcane Bagasse

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This Study was conducted to purify and quantify the amount of cellulase produced by *Aspergillus niger* isolated from refuse dump soil using alkali treated and untreated sugarcane bagasse. Proximate composition of the treated bagasse revealed (95.75%) dry matter, (8.75%) crude protein, (23.13%) Crude fiber, (79.55%) Nitrogen Free Extract (NFE), and (2.70%) Ash, for the untreated Sugarcane bagasse (90.75%) of dry matter, (5.13%) crude protein, (5.20%) Crude fiber, (76.82%) Nitrogen Free Extract (NFE) and (1.60%) ash. The cellulases produced were partially purified using ammonium sulphate precipitation technique. Higher enzymatic activity was found with the partially purified alkali treated sugarcane bagasse enzyme solution (0.74225U/mL) compared to the alkali treated sugarcane bagasse crude enzyme solution (0.3599U/mL).

Keywords: Purification, Quantification, Sugarcane Bagasse

1. Introduction

Cellulose is commonly degraded by an enzyme called cellulase. Cellulases are consortia of complex hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components like glucose units. This enzyme is produced by several microorganisms, mainly by bacteria and fungi (Immanael *et al*., 2006, Kamble and Jadhav, 2012). Fungi are group of microorganism that can degrade mixtures of heterogeneous substrates such as municipal solid waste, cattle manure; they possess a complex set of extracellular enzymes.

Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi. Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al*., 2002). Numerous microorganisms are capable of decomposing cellulose, although only a few of these microorganisms produce significant quantities of extracellular enzymes to decompose cellulose (Sukumaran *et al*., 2005). Also only few of these organisms produce significant quantities of cell free enzymes capable of completely hydrolyzing crystalline cellulose *in vitro*. Several studies were carried out to produce cellulolytic enzymes from bio-waste degradation process by many microorganisms including fungi such as *Trichoderma* sp, *Penicillium* sp, *Aspergillus* sp (Miller, 1972; Mandels and Reese, 1985; Lakshmikant and Mathur, 1990). Similarly cellulolytic property of bacterial species like *Pseudomonas* sp, *Cellulomonas* sp, *Bacillus* sp, *Micrococcus* sp, *Cellovibrio* sp and *Sporosphytophaga* sp. were also reported (Naveenkumar and Thippesneswany, 2013).

Sugarcane is used worldwide as a feedstock for sugar and ethanol production. The sugarcane plant consists of stem and straw. The sugarcane straw has three components – fresh leaves, dry leaves, and tops. Cane juice is extracted from the sugarcane stem leaving fibrous residues referred to as sugarcane bagasse. Sugarcane bagasse is about 25% of the total weight of the sugar cane plant and contains 60% to 80% of carbohydrates (Betancur and Pereira, 2010). It is an abundant and cheap source of lignocellulosic biomass with high cellulose content. The sugarcane bagasse contains between 34 – 45 % cellulose, 24 – 25 % hemicellulose, and 20 – 25 % lignin (Kim and Day, 2011).

Bioconversion of these cellulosic materials particularly via enzymatic hydrolysis, had assumed great potential from the point of view of waste management and isolation of suitable microbial strains that could become useful as sources of hydrolytic enzymes. The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as a feasible

alternative for the conversion of lignocelluloses into fermentable sugars and biofuel (ethanol) (Oksanen *et al.*, 2000).

Cellulosic residues are renewable resources of which over a billion tons are generated annually in the world (Klemn and schmauder, 2009). The annual production of cellulose is estimated to be 4.0×10^7 tons (O'Sullivan, 1997). The non-use of these materials constitutes a loss of potentially valuable resources (Mussatto and Teixeira, 2010).

Cellulase is expensive and the demand for more thermostable, highly active and specific cellulases is on the increase; and contributes only 50% to the overall cost of hydrolysis due to the low specific activity. This enzyme has enormous potential in industries and is used in food, beverages, textile, laundry, paper and pulp industries etc. (Lekh *et al.,* 2014).

Treatment of cellulose with either cellulolytic enzymes or microorganism could lead to more efficient degradation of this waste material, promote cycling of nutrients in the environment and reduce the impact of waste accumulation on terrestrial and aquatic ecosystem as well as generation of end products of economic benefits like compost and biofuels (Klemn *et al*, 2009).

The aim of the study was to purify and quantify the amount of cellulase produced by *Aspergillus niger* using alkali treated and untreated sugarcane bagasse. Determine proximate composition of the sugarcane bagasse. Produce, purify and quantify cellulase enzyme using sugar cane bagasse as substrate.

2. Material and Methods

Aspergillus niger was isolated from the refuse dump soil (Paul and Daniel., 2007) and identified by the use microscope (Domsch *et al.,* 1980).

2.1 Preparation of Substrate (Sugarcane Bagasse) for Cellulase Production

The sugarcane bagasse was sun-dried for a period of three weeks and then oven-dried at 50^{o}C for two days. The dried substrate were chopped into bits, pulverized into coarse particle sizes and then washed repeatedly in hot water in order to remove the residual sugar as described by Rezende *et al.* (2002). One kilogram (1 Kg) of the dried sugarcane bagasse was sieved through 0.8 mm (850 µ) mesh (Damisa, *et al.*, 2008).

2.2 Substrate Pre-treatment with Alkali

The powdered sugarcane baggase was treated with alkali solution of sodium hydroxide solution (0.25N) and autoclave at 121^{o}C for 15 minutes. The treated sugar cane bagasse was washed several times with distilled water until pH 7.0 attained. The residue was drained through muslin cloth and dried in an oven at 60^o C until constant weight is attained (Vyas *et al*., 2005).

2.3 Cultivation medium and enzyme production

The fungus were cultivated at ambient temperature of 28°C in Mandel's medium (Mandels *et al.,* 1999) cultivation was carried out in 250 ml capacity Erlenmeyer flasks in a rotary shaker at ambient temperature of 28^{o}C and 350 rpm. The samples were withdrawn for analysis after every 24 hours, centrifuged at 350 rpm for 30 minutes in a table top high speed refrigerated centrifuge at 4^{o}C and the supernatant was taken as the crude enzyme suspension (Damisa *et al.*, 2008).

2.4 Partial Purification of Cellulase

Crude extract of enzyme was centrifuge at 10,000 rpm for 15 min at 4°C to increase clearity .Solid crystals of ammonium sulfate were added until it is 50% saturated and kept for 4-6 hrs at 4°C.The resulted precipitate was collected and centrifuged at 10,000 rpm for 15 min at 4°C.The pellet of precipitated protein was discarded and more crystals of ammonium sulfate were added to attain 85% saturation at 0°C and again kept for 4-6hr at 4°C and centrifuged at 10,000 rpm for 15 min at 4°C. After centrifugation, the supernatant were kept separate and the sediments were dissolved in acetate buffer (pH 5.5). The solution were kept in a dialysis bag and after sealing securely, dialyzed against distilled water with 4 regular changes of the water after every 6 h. The partially purified cellulase was used for assay (Lokhande and Pethe, 2016).

2.5 Cellulase Assay

Endoglucanase activity-carboxyl methyl cellulose:

Carboxy methyl cellulase activity was determined following a method described by the International Union of Pure and Applied Chemistry (Ghose, 1987). The reaction mixture comprise of 0.5 ml carboxyl methyl cellulose (0.2w/v) in 0.05 M citrate buffer, pH 4.8 and 0.5ml crude enzyme in test tubes. The mixture was incubated at 50^{0}C for 1 hour. After incubation 1 ml of dinitro-salicylic acid (DNSA) reagent was added to stop the reaction. The mixture was boiled for 5 minutes in a boiling water bath and 10ml water was added and absorbance was read at 540nm using a

spectrophotometer. The spectrophotometer was blanked with a mixture treated in the same way as the reaction mixture but with 0.05 M citrate buffer replacing the crude enzyme. Glucose standards were prepared with varying concentrations of glucose ranging from 0.1 to 2.0 mg ml^{-1} and the absorbance were read at 540 nm. Absorbance of the reaction mixture was plotted against concentrations of glucose to obtain a calibration curve. The quantity of reducing sugar produced by the action of crude enzyme was read off from the curve. One unit of enzyme activity defined the amount of enzyme that released 1µM of reducing sugar per millilter of substrate per minute (Saliu, 2012).

3. Results and Discussion

3.1 Proximate Composition of Sugarcane Bagasse

Table 1 shows the results of proximate composition of sugarcane Bagasse. The results revealed percentages of (95.75%) of dry matter of which is a measure of the quantity of matter in the sample when the moisture is completely removed. The crude protein content varies. The treated Sugarcane Bagasse (8.75%) and untreated Sugarcane bagasse (5.13%). The crude protein showed the potential of the sugarcane bagasse as a nitrogen source required for growth and efficient enzyme expression. Crude fiber content describes the amount of cellulose content i.e cell wall composition. Crude fiber for the treated Sugarcane Bagasse was (23.13%). and untreated Sugarcane bagasse was characterized by a low content of fiber (5.20%). High crude fiber content correlates positively with increase in xylose content (Stombaugh *et al*, 2000). Xylose is a common sugar in hemicelluloses and prevents close packing of fibers seen in cellulose.

The Nitrogen Free Extract (NFE) consists of carbohydrates, sugars, starches and a major portion of materials classified as hemicelluloses. (79.55%) value content in treated Sugarcane bagasse is an indication of high content of fermentable sugars required for growth and enzyme production by the organism. The inorganic constituents also called the mineral content of the biomass referred to as ash, is shown to vary from (1.60%) for untreated Sugarcane bagasse to (2.70%) for the treated Sugarcane bagasse, The high content of ash indicates the richness of the substrate in minerals required for growth of the organism. This might have accounted for the cellulase production (0.7422U/mL) obtained in this study. The result is similar to the findings of Damisa, (2007) on the effects of mutagenic treatment on the rate of cellulase production by *Aspergillus niger* using corn straw, corn cob and sugarcane bagasse as substrates.

Pre-treatment of the sugarcane bagasse proved to be a crucial factor for the induction of the cellulase production in the study. Activity was very significant in the cellulases of *Aspergillus niger* grown in treated Sugarcane bagasse medium compared to the untreated Sugarcane bagasse. This may be due to the fact that pre-treating the substrates with sodium hydroxide resulted in the swelling of the particles causing easy removal of the lignin and cellulose de-polymerization occasioned by the separation of hydrogen bonds of the cellulose. Pre-treatment with alkali is also relatively cheap. This result agrees with that of Jaafaru and Fagade (2007), who reported a high cellulase activity from treated than untreated corn cob. Also the result agrees with that of Damisa *et al*; (2008), who reported that alkali treated residues showed higher cellulase yield than the untreated residues using corn straw, corn cob and sugarcane bagasse. Cold alkaline pre-treatment or in combination with heat treatment by boiling or autoclaving has been reported (Vyas et al, 2005; Jaafaru and Fagade, 2007). This guided the choice of alkali pre-treatment as the pre-treatment method for the sugarcane bagasse.

Fungi are the most common groups of organism that are capable of hydrolyzing cellulose to the monomer units because they are ubiquitous and can be easily isolated from the environment (Saliu, 2012).

3.2 Partial Purification of Cellulase Enzyme

The comparison of the Cellulase Activities of Crude Enzyme and Partially Purified Enzyme Solutions of the Alkali Treated and Untreated Sugarcane bagasse as shown in Table 2. Higher cellulase activity was observed in the partially purified alkali treated sugarcane bagasse enzyme solution (0.7422U/mL) than the alkali treated crude enzyme solution (0.3599U/mL). Partial purification of the crude enzyme by ammonium sulphate precipitation technique significantly enhanced the cellulase activity. Higher enzyme activity was observed with the partially purified sugarcane bagasse enzyme solution (0.7422U/ml) as compared to the crude enzyme solution (0.3599U/ml). This is an agreement with the work of Saliu; (2012) also Charita and Sunil (2012) where they observed higher cellulase activity in the partially purified enzyme solution.

Table 1: Proximate Composition of Alkali Treated and Untreated Sugarcane Bagasse

Parameters	Proximate composition (%)	
	Treated SB	**Untreated SB**
Ash	2.70±0.014	1.60±0.023
Dry Matter	95.75±0.035	90.75±0.017
Crude Protein	8.75±0.053	5.13±0.034
Crude Fibre	23.13±0.025	5.20±0.019
Crude Fat	4.75±0.035	7.20±0.015
Nitrogen Free Extracts	79.55±0.081	76.82±0.056

Key: SB- Sugarcane Bagasse
*Values are means ± Standard Error.

Table 2: Comparison of the Cellulase Activities of Crude Enzyme and Partially Purified Enzyme Solutions of the Alkali Treated and Untreated Sugarcane Bagasse

Enzyme Product	Enzyme Activity (U/mL)
Crude enzyme	
Treated SB	0.3599±0.088
Untreated SB	0.1415±0.011
Partially purified enzyme	
Treated SB	0.7422±0.067
Untreated SB	0.3624±0.042

Key: SB- Sugarcane Bagasse * Values are means ± Standard Error.

4. Conclusion

The study concludes that pre-treatment of the sugarcane bagasse proved to be a crucial factor for the induction of the cellulase production in the study; this activity was very significant in the cellulases of *Aspergillus niger* grown in treated Sugarcane bagasse medium compared to the untreated Sugarcane bagasse. Proximate analyses revealed that High dry matter and nitrogen free extracts correlated with high growth constituent. Since selection of a suitable carbon and energy source was a strong factor in the process of extracellular production of hydrolases (such as cellulase) by filamentous fungi the substrates appear to be inducers of the enzyme. Partial purification of the crude enzyme by ammonium sulphate precipitation technique significantly enhanced the cellulase activity in the study.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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