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Cellulase from Cashew Shell Cake using *Aspergillus niger* Production, Purification and Potential Applications

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Abstract

The Cellulose content in the Cashew Shell Cake was utilized for the production of enzyme Cellulase CEPC-C11; induced by *Aspergillus niger* (MTCC- 1344) using the Solid-State Fermentation (SSF). Optimised conditions by SSF for Cellulase production were at 30ºC, pH 7.0 and 216 hours of incubation. The enzyme activity of Cellulase was found through C_1 and C_x Cellulase Combined Assay. Partially Purified Cellulase was concentrated and powdered by acetone precipitation method. Purified Cellulase showed a maximum specific enzyme activity of 15.322 U/mg of protein at 50ºC, pH 5.0 and on 30 minutes of incubation. Thus cellulase enzyme yield of 20% /Kg of substrate showing Km and Vmax of 1.786 and 19.45 U/mg respectively was obtained. This enzyme which is capable of hydrolyzing native cellulose; that has functional temperature ranging from 25ºC to 70ºC and pH ranging from 3.5 to 8.0 respectively; could find various industrial applications.

Keywords: Cellulase, *Aspergillus niger*, Solid-State Fermentation (SSF), Cashew Shell Cake, Submerged Fermentation (SmF).

Introduction

Cellulose is one of the most common, abundant and renewable organic polymer on earth which is a principal waste from agriculture. Hence any process to utilize this rich biomass is of at most importance. Cellulase enzyme hydrolyzes the β-1,4-glycosidic bonds in the Cellulose to release glucose units. The enzyme is a complex of *Endo-β 1,4 glucanase* (EC 3.2.1.4), *Exo-β 1,4 glucanase* (EC 3.2.1.91) and *β-Glucosidase* (EC 3.2.1.21) (Cellobiase). In order to achieve complete hydrolysis of cellulose to glucose synergetic actions of all three enzymes are required.(Pandey *et al.,* 2005), (Milala *et al.,* 2005).

High performance Cellulase enzymes are in demand in the textile and chemical industry (used in the formulation of washing powders) as well as in the food industry (extraction of fruit and vegetable juices and in starch processing); for cost effective manufacturing of renewable products from biomass. Commercially the cellulose degrading enzymes have enormous potential in industrial applications. Glucose produced from cellulosic substrate could be further used as a substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol, butanol, methane etc., (Walsh *et al.,* 2002).

These enzymes are used to convert non-food biomass to

fermentable sugars, which can ultimately be converted to sustainable products including bio-fuels and household goods. (Bhat *et al.,* 2000).

Fungi and bacteria are the main natural agents of cellulosic degradation. (Lederberg *et al.,* 1992). Cellulase biosynthesis achieved through Submerged Fermentation (SmF), generally uses purified cellulose; while a simpler Solid State Fermentation (SSF) technique, were Cellulosic agricultural waste can be directly used and thereby provides numerous advantages. (Pandey *et al.,* 1994), (Vandevoorde *et al.,* 1987).

In cashew Industry one of the major industrial wastes are the cashew shell cake that is devoid of the cashew nut shell liquid (CNSL). Every year huge tones of cashew shell have been wasted away in India alone. The main purpose of the study was to provide a value addition; to the cashew industry by-product:- cashew shell (cashew shell cake). The Cellulose content (11 to 16%) in the cashew Shell cake was subjected to utilization; for the production of a bio-enzyme Cellulase CEPC-C11 induced by *Aspergillus niger* (MTCC 1344) using the Solid-state fermentation (SSF).The potential application studies for the Cellulase CEPC-C11 enzyme.

Materials and Methods

Isolation and screening of microorganisms

Cellulase producing microorganisms were isolated from soil containing cashew shell cake and CNSL from cashew factories by dilution pour plate technique. The plates were incubated at 37°C for 24 hrs. The individual colonies were transferred to nutrient agar slants. These colonies were then streaked on to CMC agar plates and incubated at 37°C for 24 hrs. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 minutes and washed with 1 M NaCl. To indicate the cellulose activity of organisms, diameters of clear zone around colonies on CMC agar were measured.

Microorganism

Many organisms produce Cellulase to perform Cellulolysis necessary for growth and product formation under appropriate conditions. Microorganisms used in the study were isolated from cashew shell cake and maintained on Potato dextrose agar (PDA) slants at 4°C and sub cultured every 4 weeks. *Aspergillus niger, Aspergillus flavus, Pencillium* species and *Trichoderma ressie* were the organisms isolated and studied of which *Aspergillus niger* (MTCC 1344) was found the most efficient in Cellulase production.

Fermentation

Solid State Fermentation (SSF) was carried out by using Cashew Shell Cake as the solid substrate. For 100gm of substrate; culture mineral media containing $Na_2HPO_4.2H_2O$ (1.1%), $NaH_2PO_4.2H_2O$ (0.61%), KCl (0.3%) and MgSO4.7H2O (0.01%) was added; maintaining a pH of 7 and a final moisture content of the substrate of 60%. The flasks were autoclaved at 121°C at 15 lbs for 20 minutes (3 times). After cooling the substrate were inoculated with the spore suspension of *Aspergillus niger* (MTCC 1344) and incubated at 30°C for 10 days; from which every day sample was analyzed.

Extraction and partial purification of Cellulase

Extraction of Cellulase was done first by transferring the fermented Cashew Shell Cake into a nylon cloth and pressed manually followed by filtering through Glass fibre filter paper and centrifuged at 9000rpm for 20mins at 4°C. The supernatant was used as crude enzyme solution. Partial Purification of the enzyme was done by acetone precipitation.(Ekundayo *et al.,* 2012).

Enzyme Assay

Total Cellulase activity by C_1 and C_x Cellulase Combined Assay (U/ml) was measured by determining the total amount of reducing sugars in the sample as per the modified Dinitrosalicylic method (DNS) of Miller (1972). In this assay the combined action of C_1 Cellulase (Exo-*â*1,4 *glucanase*) and C_x Cellulase (Endo-*â*1,4 *glucanase*) results in the hydrolysis of native cellulose (filter paper); and the specific enzyme activity is expressed as U/mg (U=Enzyme Units). One unit (1U/mg) equals mg of glucose (reducing sugar) released per mg of protein in a minute. (Denison, D. A., and Koehn, R. D. *et al.,* 1977), (Ghose *et al.,* 1987), (Mandels *et al.,* 2009).

The reaction mixture contains 0.1ml of enzyme (containing varying concentrations of enzyme powder) in 1.0 ml of 0.1M Citrate buffer of pH 5.0 along with 50mg of Whatman's No: 1 filter paper as substrate. This mixture was incubated at 50°C for 30 minutes. The reaction mixture was measured at 540nm in a UV-VIS Spectrophotometer. The concentration of glucose released by enzyme was determined by comparing it against a standard curve constructed similarly with known concentrations of glucose.

Estimation of Protein

Total protein of enzyme was determined by Lowry's *et al.,* 1951) by using standard Bovine Serum Albumin and measured at an OD of 660 nm.

Kinetics

The effect of varying temperature (25ºC to 70ºC), pH (3.5 to 8.0), incubation time (5min-2 hours), enzyme concentrations (10% to 150%) and substrate concentrations (1% to 6%) were determined for the purified cellulase enzyme. Based on these kinetic parameters the Km and Vmax was found from the Michaelis-Menten plot using Graph Pad Prism 5 software.

Application of Cellulase in Juice clarification

To 3ml of carrot juice taken in test tubes 1ml of enzyme was added and incubated for 2 hours at room temperature.

Results and Discussions

Understanding the importance of bioconversion of unwanted cellulosic biomass has led to extensive studies on cellulolytic enzymes produced by micro-organisms. But, high cost of Cellulase production hindered use of the enzyme in industries. Hence for the proper utilization of the cellulosic biomass; it is important to enhance cellulase production and to reduce its production cost. (Pandey *et al.,* 2005), (Milala *et al.,* 2005). In the present study the raw cellulosic biomass from the cashew shell cake devoid of CNSL was subjected to study under Solid State Fermentation by various microorganisms.

Production

Aspergillus niger showed the maximum Cellulase production compared to other microorganisms. Cellulase production by SSF on cashew shell cake using *A.niger* was optimum; when incubated for 216 hours at 30ºC and pH 7.0. Addition of Carbon and Nitrogen had negligible effect on the Cellulase production. Cellulase production by Solid State Fermentation (SSF) showed more Total Cellulase specific Activity 2.890U/mg (Table 1); compared to the Submerged Fermentation (SmF) 1.773 U/mg measured by the C_1 and C_x Cellulase Combined Assay. The CEPC-C11 on concentration was powdered by acetone precipitation method; yielded 20% from a Kg of Cashew Shell Cake used as substrate. This yield percentage with increased Purification fold (5.30 %) and Specific enzyme activity (15.322 U/mg) will be significant mainly because of the SSF employed seemed to be highly cost effective in comparison with SmF. The use of purified cellulose as substrate is uneconomical for large scale production. Hence the importance of SSF; were raw cellulosic biomass could be utilized. (Wiseman *et al.,* 1995).

Kinetics

The kinetic studies of the partially purified powdered Cellulase with respect to varying temperature (25ºC to 70ºC), pH (pH 3 to 8), incubation time (5 to 60 minutes), enzyme concentrations (25%-150%) and substrate concentrations (1%-6%) revealed the optimum at which the enzyme functions. The optimum Total Cellulase specific activity of the CEPC-C11 was 15.322 U/mg at temperature 50ºC, pH 5.0 and at an Incubation time of 30 minutes (Table 1). The Km and Vmax of CEPC-C11 was 1.786 and 19.45 U/mg (Figure 3) respectively.

Cellulase enzyme does posses a lot of applications in various industries; of which its used predominantly in animal feed, food, textiles, detergents and in paper industry. Since lignocellulose accounting for one of the most wasted biomass, the importance of cellulase in conversion of this waste into Biofuel is deemed paramount.

CEPC-C11 that is capable of hydrolyzing cellulose even is acidic conditions (pH 3-5.5) (Figure 1); makes it ideal for biostoning of denim jeans gives the jeans the softness by its action on cellulose fibre and the faded look by the removal of the indigo dye. (textile industry). (Cortez *et al.,* 2001). Since this enzyme is capable of being active in mild elevated temperatures (70ºC) (Figure 2) and slight alkaline pH (pH 8) (Figure 1); it could find its use in detergents too. (Uhlig *et al.,* 1998).

Table 1: The yield and purification fold of CEPC-C11

SI No:	Cellulase	Protein(mg/ml)	Total Specific Enzyme Activity (U/mg)	Purification fold (%)	Yield (%)/Kg of substrate)
1.	SSF-crude	18.24 ± 0.62 mg/ml	02.890 ± 0.21 U/mg	1.00%	100%
2.	CEPC-C11	02.27 ± 0.22 mg/ml	15.322 ± 0.72 U/mg	5.30 %	20%

Values are average ±SD of five replicas

CEPC-C11 proved extremely effective in clarifying the waste carrot juice after 2 hours of incubation (Figure 4). This shows the potential of the enzyme in the food industry especially in the extraction of carotenoids and clarification of vegetable and fruit juices. (Galante *et al.*, 1998).

It has been reported that Glucanases are added to improve the malting in beer production (Barbesgaard *et al.*, 1984)[1]; while CEPC-C11 could also find its applications in wine industry, were exogenous Glucanases are used for better maceration and color maceration. (Galante *et al.*, 1998).

In the paper industry the CEPC-C11 enzyme could be used in the removal of inks from paper and toners; especially since the enzyme is capable of acting in native cellulose. (Franks *et al.*, 1996). Hence the enzyme can be applied in the manufacture of soft papers like paper towels and sanitary paper too. (Hsu *et al.*, 2002).

Conclusion

Cellulase (CEPC-C11) enzyme was produced using SSF; from cashew shell cake using highly potent *Aspergillus niger* strain. The SSF showed more enzyme activity compared to Smf; proving that for utilization of cellulose content in cashew shell cake; SSF was the better method. SSF and the partial purification of enzyme by acetone precipitation (5.3% and 20% purification fold and yield respectively- Table 1); seemed to be highly cost effective. CEPC-C11 enzyme obtained was capable of degrading native cellulose like cotton and filter paper; which proves its potential in applications for textile and paper industry. This will definitely add value to the existing cashew industry. With a Km of 1.786 and Vmax of 19.45 U/mg (Figure 3); the CEPC-C11 proved extremely effective in clarifying the carrot juice after 2 hours of incubation which shows the enzymes potential in juice clarification.

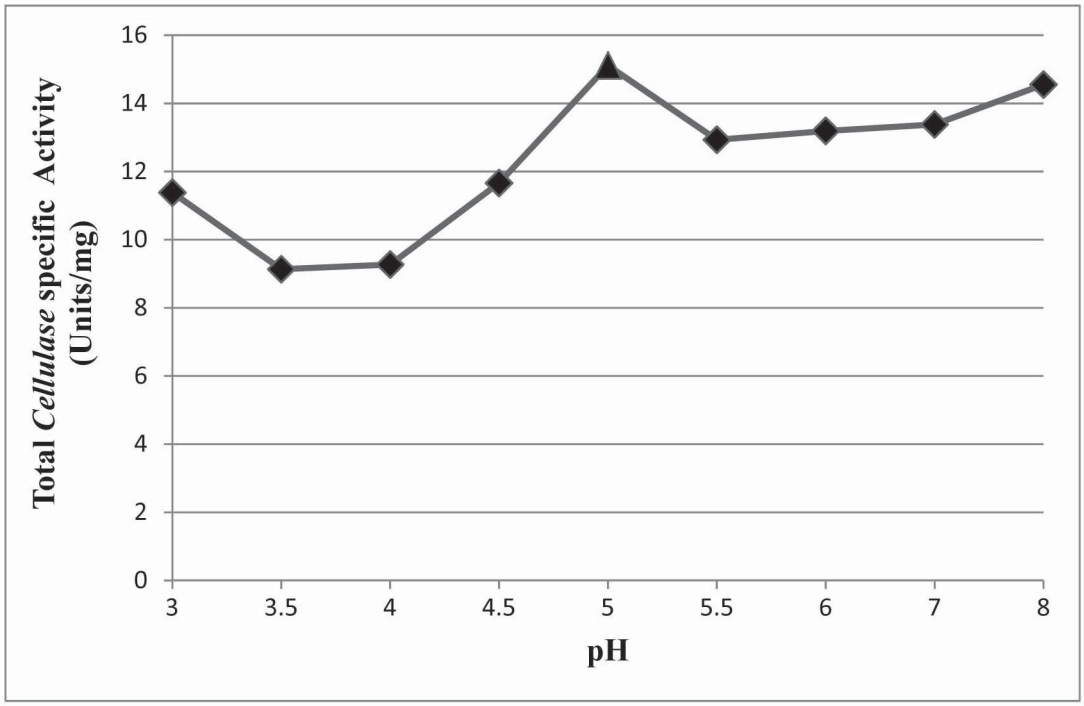

Figure 1: The functional pH for the Cellulase powder **CEPC-C11** ranged from 3.5 pH to 8.0 pH; signifying the enzymes capability to hydrolyze cellulose even in acidic as well in alkaline conditions .

Since this enzyme is capable of being active in mild elevated temperatures (70ºC) (Figure 2) and slight alkaline pH (pH 8) (Figure 1); it could find its use in detergents too. (Uhlig *et al.*, 1998) [17].

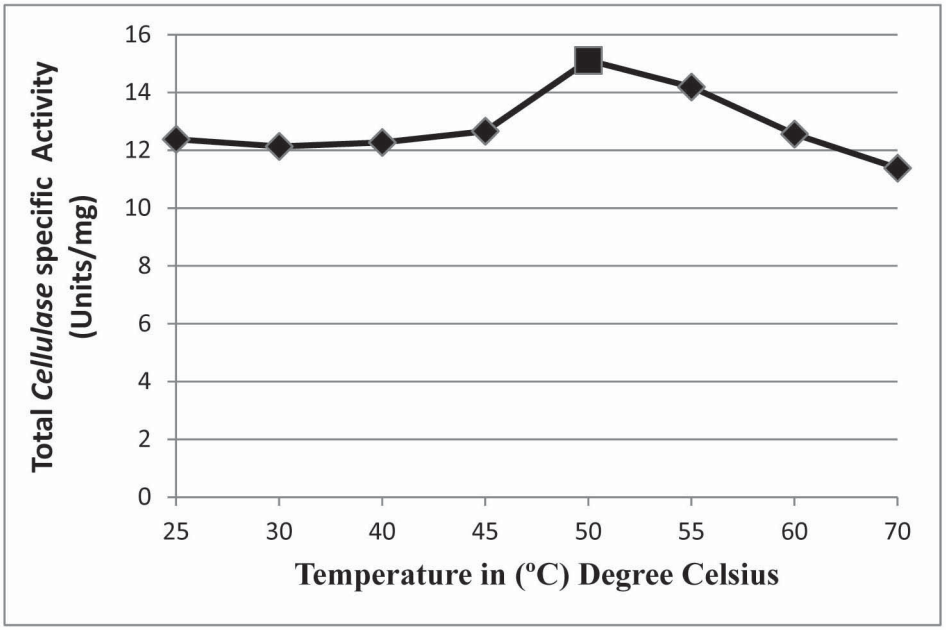

Figure 2: The functional temperature for the Cellulase powder **CEPC-C11** ranged from 25ºC to 70ºC; that shows the enzymes ability to be active even in mild elevated temperatures of 70ºC.

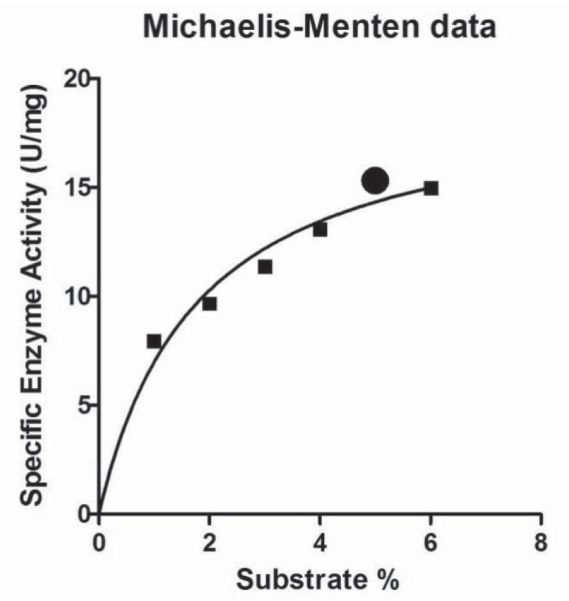

Figure 3: The Km 1.786 and Vmax 19.45 U/mg of the enzyme calculated with respect to the varying concentration (percentage) of substrate and to the Specific Enzyme activity (U/mg)

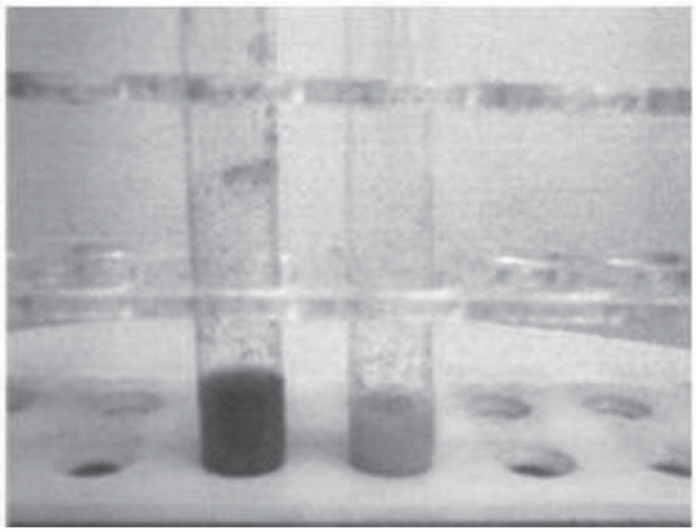

Figure 4: Left – Waste Carrot juice before Cellulase treatment, Right –Clarified Waste Carrot juice after Cellulase treatment

Future Directions

The cellulose content in cashew shell cake gets converted to glucose by the action of CEPC-C11 enzyme. This hydrolyzed cellulosic end product of the cashew shell cake could find its use in the production of fermentable products like bioethanol; by the application of a fermentative microorganism.

CMCase and *Cellobiase* activity studies could bring more understanding of the enzymatic complexes mechanism. Further protein purification studies by Gel Filtration Chromatography using Sephadex G 200 would bring more specificity to the enzyme activity.

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