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OPTIMIZED PRODUCTION OF CELLULASE USING FRUIT WASTE AND ITS APPLICATION IN BIOETHANOL PRODUCTION

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ABSTRACT

Cellulases are a group of hydrolytic enzymes capable of degrading lignocellulosic materials into simpler sugar molecules. These enzymes are mainly produced by the microbial fermentation of bacteria and fungi. Usage of edible crops as substrates for the cellulase production may lead to a food crisis. So the present study mainly focuses on the production of cellulase by using apple waste as substrate with the help of previously isolated fungi *Aspergillus fumigatus* JCF. When apple waste was added with 1%cellulose as inducer a maximum yield of 2.20 IU/ml was obtained. Further, environmental and cultural conditions for the production of cellulase were optimized. A maximum cellulase activity of 2.28IU/ml was obtained at optimized conditions of 20% apple waste, 0.3% ammonium sulphate, 6.2 pH and five days fermentation period. Cellulase thus produced was used for bioethanol production. Biofuel production plays an important role in the current situation of increasing demand of fuel. Saccharified sample when inoculated with baker's yeast yielded a maximum of 11.6gm/l of bioethanol.

KEYWORDS: **Cellulase, *Aspergillus fumigatus* JCF, bioethanol, Baker's yeast**

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INTRODUCTION

Cellulose is one of the most important sources of carbon. Cellulose is a linear polysaccharide polymer with glucose monosaccharide units (300 to over 10, 00units with the formula $C_6H_{10}O_5$). The major source of glucose in the cellulose is photosynthesis reaction. Cellulose degradation and its subsequent usage are important for utilizing it as carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest[1]. But it is very difficult to degrade the ordered structure of cellulose. So much of the cellulose, which is a storehouse of glucose is wasted as such in the environment. One of the important methods to degrade the cellulose is by enzymatic method. A number of organisms, particularly fungi, possessing cellulose-degrading enzymes have been isolated and studied extensively[2]. Cellulase is an enzyme extensively used for the complete hydrolysis of cellulosic substrates into its monomeric glucose component, which is a fermentable sugar. Cellulase is mainly composed of endoglucanase or carboxymethyl cellulase (CMCase) (endo β-1, 4-glucanase), exoglucanase or cellobiohydrolase (exo β-1,4-glucanase), and β-glucosidase (β-D-glucoside glucohydrolase)[3]. Cellulases have a broad variety of applications in food[4], paper pulp, and detergent[5], fuel production[6] as well as waste management and pollution treatment[7]. Cellulase enzyme is mainly produced by microbial fermentation. Submerged and solid state fermentation has been tried for the production of cellulase. The cost of the fermentation media is a major factor that influences the production of cellulase. This expense can be reduced to an extent by the use of agricultural waste as the raw material. Several different types of waste materials like sugar cane bagasse, apple pomace[8], mango peel[9] etc., have already been used as the raw material. But studies on the use of waste fruits as raw material are very little in the literature. Biofuels plays an important role in depleting the energy scarcity that may arise in the near future. The production of biofuels using edible crops can affect the food price and economic balance. Use of lignocellulosic biomass can reduce this cost to an extent[10]. Simultaneous saccharification and fermentation (SSF) is an important process for carrying out hydrolysis and fermentation of waste biomass for the production of bioethanol[11]. This study mainly focuses on the process optimization of cellulase production, enzyme using apple waste and further production of bioethanol by SSF.

MATERIALS AND METHODS

(i)Microorganisms

Cellulase producing fungi which was isolated and characterized as Aspergillus fumigatus JCF in the previous study was used in the current research. Aspergillus fumigatus JCF subcultured in czapex dox agar medium was used for cellulase production.

(ii) Production of cellulase by submerged fermentation

Apple waste was collected from markets in Chennai and the surrounding area. Samples were brought to lab and washed, ground to paste, weighed and was dissolved in water. This was then kept in autoclave for 25 minutes at 121ºC. Autoclaved apple waste was filtered and other required minerals were added to use this as fermentation media for cellulase production. Media for cellulase production was taken in different form like apple waste alone, apple waste along with minerals and apple waste with minerals and cellulose (1% (v/v)). Minerals were, according to Modified Mandels media[12], with a minimal composition containing: KH_2PO_4 0.2%, $CaCl_2$ 0.03%, $MgSO_4.7H_2O$ 0.02%, Ammonium sulphate 0.3%, Peptone 0.25%, Tween 80µl, $FeSO_4.7H_2O$ 5 mg/L, $MnSO_4.H_2O$ 1.6 mg/l, $ZnSO_4.7H_2O$ 1.4 mg/l, $CoCl_2$ 2 mg/l. The pH was adjusted and autoclaved. . Fungal strains maintained on agar slants at 4ºC were used for the preparation of spore suspensions by washing slant cultures with sterilized water with 0.1% Tween 80[13]. This culture was inoculated into the presterilized media. Then media was kept in shaker at 160rpm at 30ºC for five days.

(iii) Experimental design for the optimized production of cellulase

The effect of carbon source (apple waste), nitrogen source (ammonium sulphate), pH and incubation time on the production of cellulase experiments was investigated by using one factor at a time study. The concentration of substrate varied from 5 to 25%, ammonium sulphate was varied from 0.1 to 0.5%, pH of the media was varied from 5.8 to 6. 6 and incubation time varied from 3 to 7 days. The samples were withdrawn at regular intervals of time for estimating enzyme activity.

(iv)Simultaneous saccharification and fermentation for bioethanol production

Apple waste, yeast extract and peptone were mixed in 100 ml citrate buffer. This media was sterilized and was inoculated with 5 ml of crude cellulase (Activity 2.28 IU/ml) and baker's yeast. The fermentation was carried out at 120 rpm and 30ºC. The sample was collected at regular intervals of 12 hr for determining ethanol concentration.

(v) Assay of cellulase enzyme activity

The reducing sugar concentration was determined by the DNS method[14]. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1.5 ml of 1% carboxymethyl cellulose (CMC) in 50 mM sodium citrate acetate buffer (pH 4.8) and 1.5 ml of filtrate. The reaction mixture was incubated at 50 ± 2ºC for 10 min, and the reducing sugar produced was determined by DNS method. To the reaction mixture 3ml of DNS reagent was added and incubated for 15 min at 100ºC. The reaction mixture was cooled and 1 ml Rochelle salt was added and OD was taken at 575 nm. One unit (IU) of cellulase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min.

(vi) Determination of Bioethanol

The amount of ethanol produced in the fermentation media was estimated by using dichromate method. The 1 ml of cell free extract was diluted four times and 1 ml of potassium dichromate was added. After keeping all tubes containing the above mixture in ice water 5 ml of concentrated sulfuric acid was added gently through the walls. Then the optical density was measured on spectrophotometer at 660 nm.

RESULTS AND DISCUSSION

In the previous study several cellulase producing fungi were isolated from different natural wastes. Out of the set of fungi isolated Aspergillus fumigatus JCF was found to be the maximum cellulase producer which was characterized and sequence submitted to NCBI with accession number as KF541346.

1. Effect of apple waste on cellulase production

To check the effect of inducers for cellulase production, cellulase production was carried out in various media with and without inducers. The apple waste extract was prepared by following a modified version of method by Adoki[15]. The result of the study is shown in FIG.1. The media used for the study include apple waste (A1), apple waste and minerals (A2) and media with apple waste, minerals and 1% cellulose (A3). Out of the three media investigated A3 showed maximum cellulase production. This may due to the presence of cellulose as an inducer along with apple waste and minerals. Optimized conditions for cellulase production were established through one factor at a time study. Optimal amount of carbon source was selected by checking cellulase production at varying concentration of apple waste, from 5 to 25% with an interval of 5% (FIG.2). The production of cellulase was increased with increase in apple waste concentration from 5% to 20%. The cellulase production was decreased when the apple waste concentration was increased further from 20% to 25%. Further, increase in the substrate concentration may inhibit the production of enzyme. The maximum cellulase production 2.22 IU/ml was obtained at an apple waste concentration of 20%.

2. Effect of ammonium sulphate on cellulase production

The presence of nitrogenous compounds in the media greatly influences the cellulase production[16]. Nitrogen is one of the major cell proteins and stimulation of cellulase activity by

ammonium sulphate salt might be due to their direct entry in protein synthesis[17]. In the current investigation Ammonium sulphate was selected as the nitrogen source for the study since it is cheap and an effective nitrogen source. Ammonium sulphate concentration was varied from 0.1% to 0.5% with an interval of 0.1% (FIG.3). The production of cellulase was increased with increase in ammonium sulphate concentration from 0.1% to 0.3%. The further increase in the ammonium sulphate concentration 0.3% to 0.5% caused decrease in the cellulase production. The maximum cellulase production of 2.15 IU/ml was obtained 0.3%.

3. Effect of pH on cellulase production

Environmental factors also play an important role in production of cellulase. So conditions were optimized by varying the pH from 5.8 to 6.6 and incubation time from 3 to7 days. The optimum pH for fungal cellulases and crude protein production varies from species to species though in most cases, the optimum pH ranges from pH 3.0–6.0 [18]. The pH of the medium is one of the most critical environmental parameter affecting the mycelial growth, enzyme production and the transport of various components across the cell membrane [19]. In the current study pH was varied from 5.8 to 6.6 with an interval of 0.2.Further increase in pH showed a marked decrease in the cellulase production. Thus optimum pH was found to be at 6.2 for the cellulase production in the current study (FIG.4). The pH 6.2 was also found to be the optimum for maximum cellulase production from Trichoderma lignorum using banana waste[20].

4. Effect of incubation time on cellulase production

The optimization of the incubation time is of prime importance for cellulase production by fungi[5]. So cellulase production was studied at various fermentation periods. Cellulase production was studied at regular intervals of 24hrs from third to seventh day of fermentation. Cellulase concentration was found to increase upto fifth day with a maximum production of 2.27IU/ml. Cellulase production during the course of fermentation period is shown in FIG.5. The optimized conditions may vary depending on the microbial strain and media used for the enzyme production[21].

5. Production of bioethanol by saccharification and fermentation

Bioethanol was produced by saccharification and fermentation of apple waste. The S. cerevisiae was proved to be very effective in converting glucose to ethanol in SSF of lignocellulosic materials[22]. From the results of simultaneous saccharification and fermentation bioethanol production was highest at around 48 hrs of fermentation period. The results are shown in FIG.6. Bioethanol production is an extensively studied subject in biofuel production. Thus by using lignocellulosic wastes as substrates the cost of bioethanol production can reduced extensively. Bioethanol produced by using apple waste was 11.06 g/l at 48 hrs fermentation. The result of the current study was comparable with some of the previous studies. The highest ethanol concentration after hydrolysis by enzyme cellulase-cellubiase in SSF was 6.94 g/l[22].

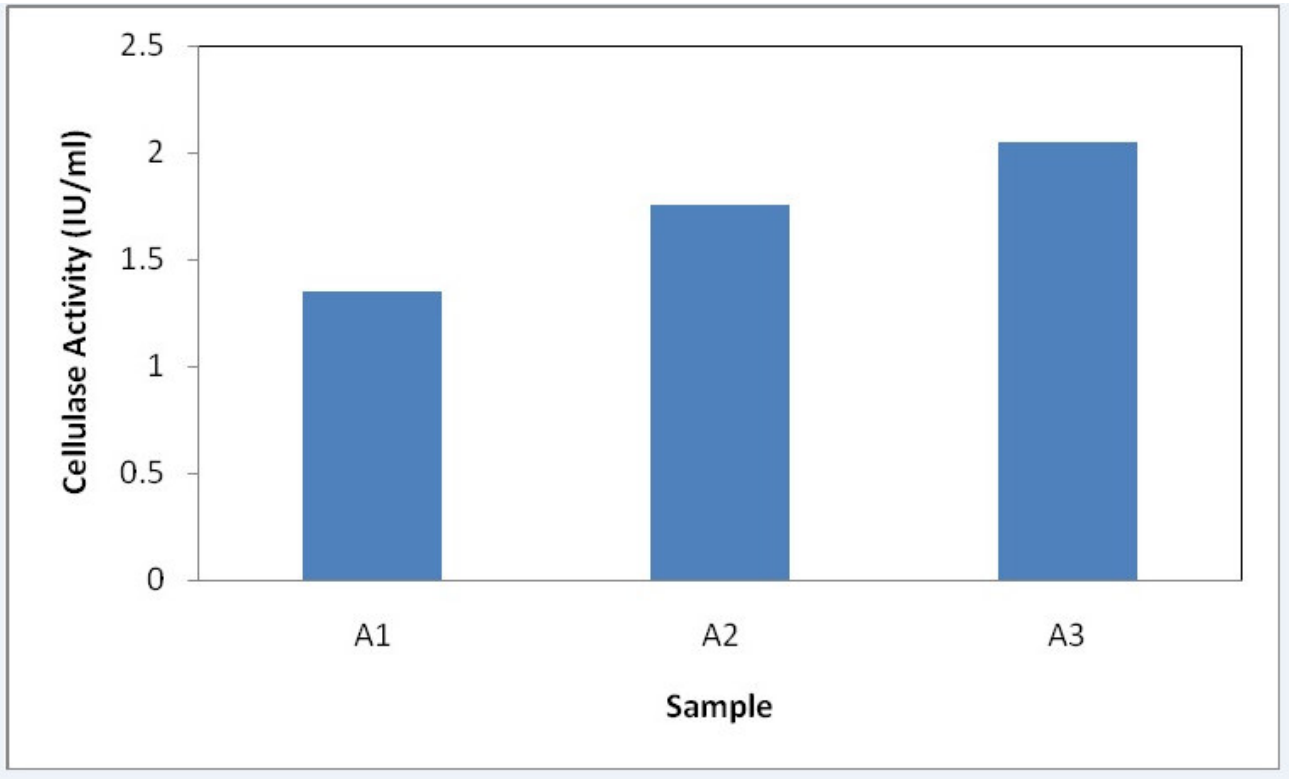

Figure 1
Effect of cellulosic activity at varying media components (A1- Apple waste alone as substrate, A2- Apple waste along with minerals, A3- Apple waste, minerals and 1%cellulose as substrate).

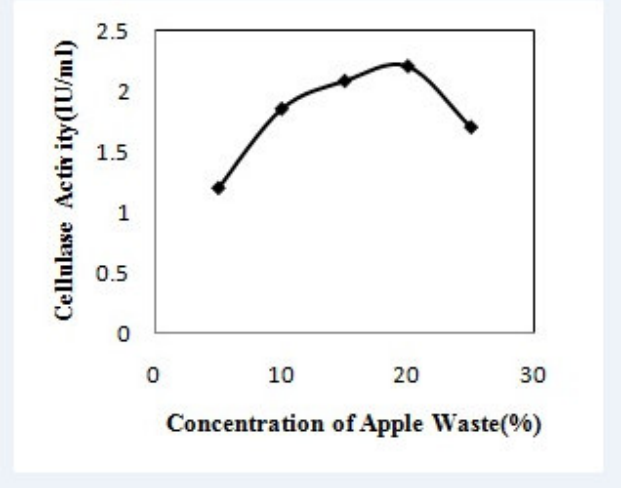

Figure 2
Effect of cellulosic activity at varying concentration of apple waste

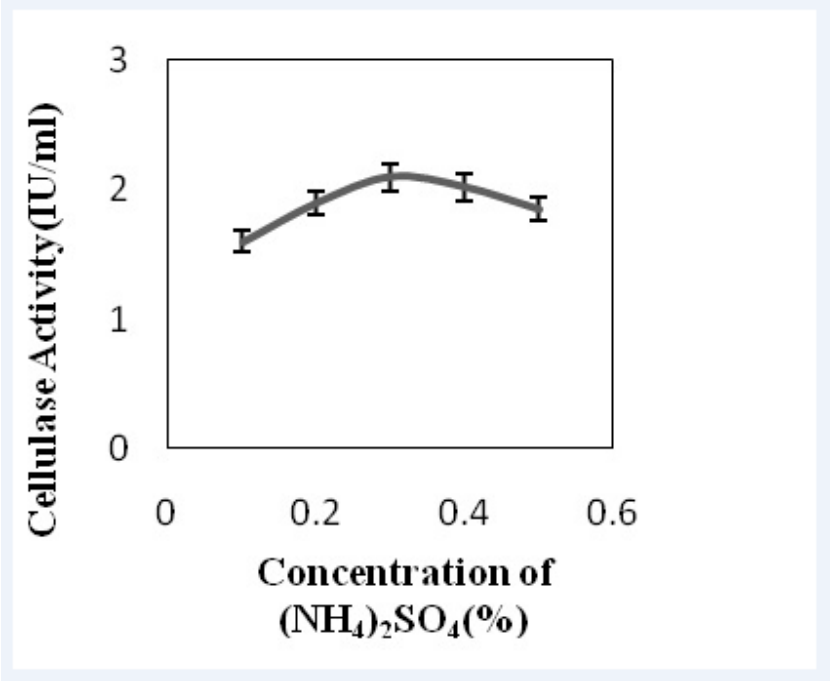

Figure 3
Effect of cellulosic activity at varying concentration of ammonium sulphate

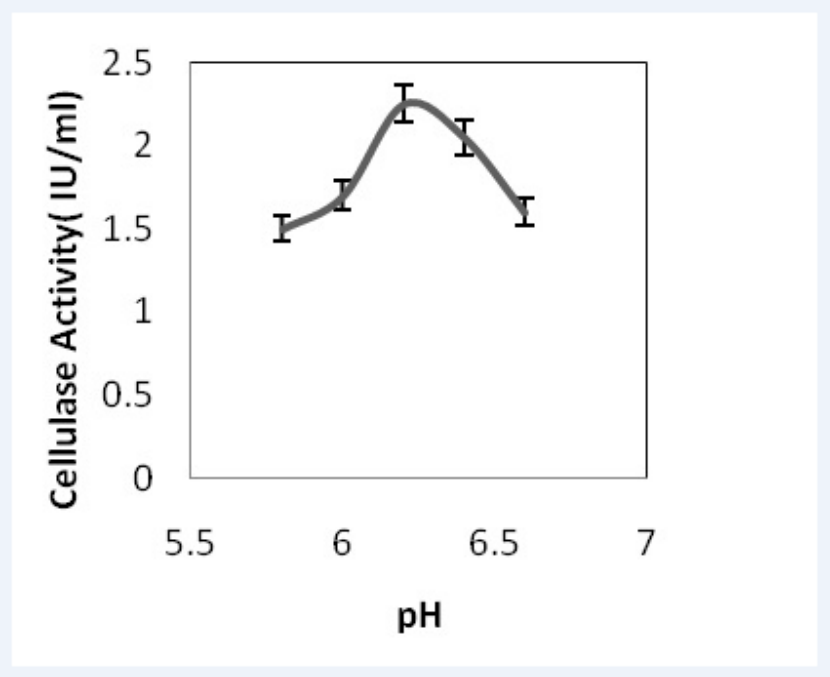

Figure 4
Effect of cellulosic activity at various pH

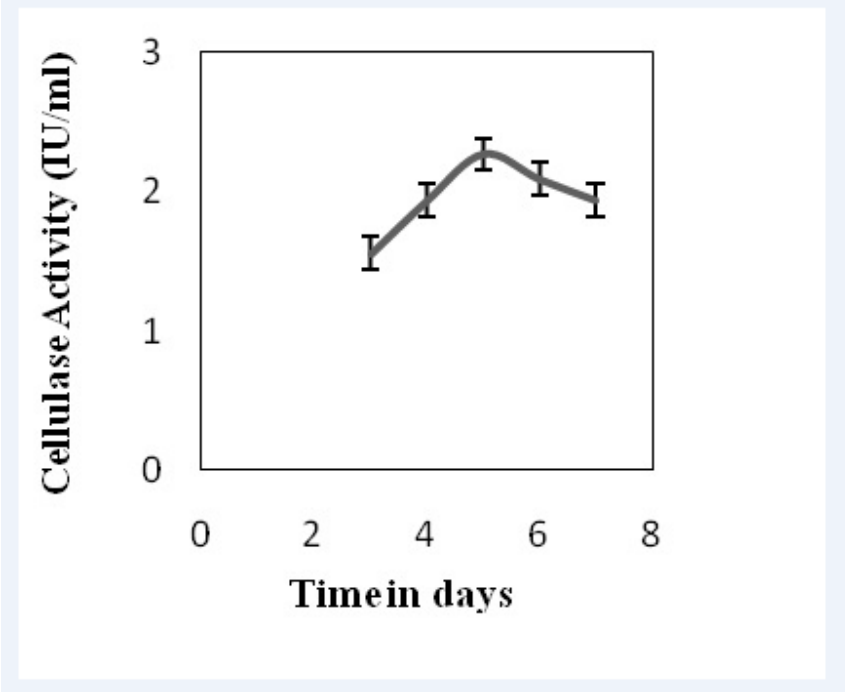

Figure 5
Effect of cellulosic activity at various incubation time

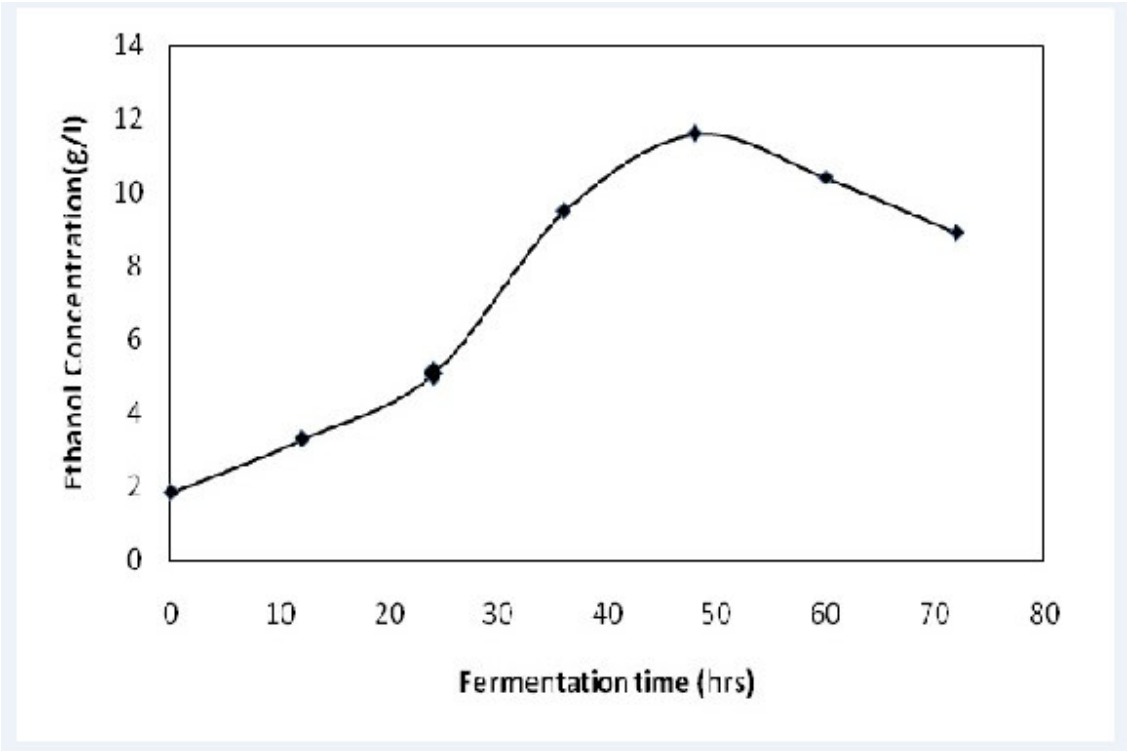

Figure 6
Production of bioethanol by simultaneous saccharification and fermentation

CONCLUSION

Production of cellulase using lignocellulosic biomass is a good method for reducing the cost of production. The current study revealed that apple waste is a good substrate for cellulase production. From the results it has been discovered that Aspergillus fumigatus JCF isolated from rotten jackfruit can be effectively used for the cellulase production. Concentration of apple waste, ammonium sulphate, pH and fermentation period greatly influenced the cellulase production. This experiment was also designed to check the possibility of alcohol production using baker's yeast by simultaneous saccharification and fermentation. A maximum bioethanol concentration of 11.6g/l was obtained in the current study.

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