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PRODUCTION OF CELLULASE BY SOLID STATE FERMENTATION OF BREWERY SPENT GRAINS USING *ASPERGILLUS NIGER* FGSC A733

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ABSTRACT

Brewery spent grains (BSG) is a waste product of the brewing process. BSG has been used as animal feed, production of value added products, substrate for growth of microorganisms and for extraction of proteins and acids. BSG is high in lignocellulosic matter and may be a candidate substrate for the production of lignocellulolytic enzymes (cellulases and xylanases). In this study, BSG was evaluated for use as a cheap and readily available substrate for the production of extracellular cellulase by *Aspergillus niger* FGSC A733 during solid state fermentation. The brewery spent grains were subjected to fermentation by *Aspergillus niger* FGSC A733 at different temperatures ranging from 30 - 40 °C and initial pH ranging from 4.0 - 6.0 for process optimization. Time course analysis was done to determine the optimum incubation period for maximum cellulase production. The effect of alkaline pre-treatment of the substrate on the production of cellulase was analyzed. Cellulase activity was determined by estimating the amount of reducing sugars produced by the enzymes using 3.5 dinitrosalycyclic acid (DNS). Maximum cellulase production was at 35 °C, pH 5.0 and an optimum incubation period of 72 hours. Under optimized conditions, a yield of 73.33 U of cellulase/gram of substrate was obtained. Pre-treatment of the spent grains resulted in a three-fold increase in the production of cellulase. BSG was found to be a suitable substrate for production of cellulase.

Keywords: brewery spent grains, substrate, cellulases, solid state fermentation, *Aspergillus niger.*

1. INTRODUCTION

Cellulases are hydrolytic enzymes which work synergistically to break down crystalline cellulose to glucose and dextrins. Cellulase exists as a multi-complex enzyme system consisting of three major components which are; endo-β (1, 4)-glucanase, β-glucosidase and exo-β (1, 4)-glucanase (Kumar et al 2009; Sukuraman et al 2005). The economic significance of cellulases lies in their extensive use in the food, wine and brewery, animal feed, agriculture, textile and laundry, paper and pulp industries as well as in research purposes (Kuhad et al 2011).

Current methods and substrates used for the production of cellulases are a major contributor to the overall high cost of cellulases. Industrial demands for cellulases have mainly been satisfied by the use of submerged fermentations using genetically modified *Trichoderma* species (Avaridan et al 2007). Limited genera that have also been used in the production of cellulases include; *Aspergillus* sp., *Penicillium* sp., *Kluveromyces* sp., and *Bacillus* sp. (Dashtban et al 2009). The use of pure cellulose as a substrate in the commercial enzyme production has also been another major contributor to increased cost of cellulases (Sweeney and Xu 2012).

Strategies towards overcoming challenges of cellulase production have mainly focused on the hyper-producing microbial strains and the use of cheaper substrates during solid state fermentation. The use of waste products from industry and agricultural processes provides cheap, cost effective substrates for solid state fermentation (SSF). The waste substances reduce the overall enzyme production costs as refined and costly materials are replaced (Bulut et al 2004).

Most developing countries such as Zimbabwe continuously produce abundant agro-industrial residues like brewery spent grains, which are under-exploited and can be used as substrates in solid state fermentation (Aliyu and Bala 2011). The SSF process has been shown to achieve higher enzyme yields and productivities than submerged fermentations (Pandey 2003; Singhanai et al 2009; Singhanai et al 2010). The process is particularly advantageous for enzyme production by filamentous fungi, since it simulates the natural habitat of the microorganisms (Holker and Lenz 2005). However, the pre-treatment of the lignocellulosic material before fermentation may be important to improve the accessibility of the substrate to the microbial cellulases (Taherzadeh and Karimi 2008).

Several studies have shown that *Aspergillus niger* can be a potential candidate for enzyme production because the fungus is easy to manipulate and produces copious amounts of the enzyme extracellularly (de Vries and Viser 2002). *A. niger* grows readily on a lot of agro-industrial substrates and is generally regarded as safe (GRAS) (Oyeleke et al 2012). Agro wastes such as corn-cobs, carrot peelings, potato peelings, rice husks, sugar-cane bagasse, saw dust, wheat bran, wheat straw and *Jatropha curcas* seed cake have been shown to produce significant amounts of cellulases. The highest ranked substrates in the production of cellulases are wheat bran and *Jatropha curcas* seed (Bansal et al, 2012, Ncube et al 2012). This study therefore aims at producing cellulases from brewery spent grains (which is a by-product of the brewing process) using *Aspergillus niger* FSC A733 as the fermenting organism.

2. MATERIALS AND METHODS

2.1 Substrate and chemicals

The brewery spent grains were obtained from Delta Beverages (Pvt) Limited, Bulawayo, Zimbabwe. All chemicals used in this research were analytical grade.

2.2 Pre-treatment of the brewery spent grains

Alkaline pre-treatment was performed on dried brewery spent grains by adding 0.1 g of calcium hydroxide per gram of substrate. A volume of 10 ml of distilled water was added per gram of the calcium hydroxide used. The slurry was placed in an incubator at 50 °C for 24 hours. After the incubation, the spent grain slurry was washed three times with distilled water and dried at 105 °C until a constant mass was obtained (Saha et al, 2005). The dried pre-treated material was stored at room temperature in dry, clean polyethylene bags.

2.3 Initial moisture adjustment on the brewery spent grains

The initial moisture content of the spent grains was determined by drying the material to a constant weight using an oven at 105 °C. Moisture adjustment trials were done by adding different volumes of distilled water to 5 g of the substrate to attain a moisture content of 70 %.

2.4 Inoculum preparation

A. niger cultures were maintained on malt extract agar slants at 4 °C and sub-cultured when needed. Before each experiment, the organism was sub-cultured on fresh malt extract agar. Spores were obtained from the *A. niger* cultures by addition of 10 ml of sterile distilled water, followed by gently dislodging the spores on slants with a sterile swab. The spore suspension was collected into a sterile bottle. A spore count was done using the improved

Neubauer bright lined counting chamber. The inoculum was prepared by adjusting the spore suspension to a concentration of 1 x 10^6 spores/ml.

2.5 Solid state fermentation for cellulase production

A volume of 1 ml of the spore suspension was inoculated onto 5 g of autoclaved brewery spent grains in 250 ml Erlenmeyer flasks to achieve a 20 % (v/w) value of the inoculum-substrate ratio. The moisture contents of the inoculated spent grains were then adjusted to 70 % using 10.2 ml of 50 mM acetate buffer, pH 5.0. The content of the flasks were mixed by hand-shaking. Inoculated flasks were incubated at 30 °C, 35 °C and 40 °C in triplicates for up to 96 hours. At 24 hour intervals, samples were taken from the incubated flasks for enzyme extraction.

2.6 Extraction of cellulases from the fermented substrate

The crude enzyme was extracted by adding 50 mM acetate buffer, pH 5.0, at 5 ml of buffer/g of fermented substrate. The mixture was then shaken at 150 rpm on an orbital shaker (Precision Scientific 360P) for 2 hours at room temperature after which the enzyme extracts were centrifuged at 1000 *g* for 20 minutes to obtain a clear supernatant. The crude cellulase was stored at -20 °C in sterile Epperndoff tubes until use.

2.7 Assay for cellulase activity

Cellulase activity was determined by mixing 0.9 ml of 1 % carboxymethyl cellulose (CMC) prepared in 50 mM acetate buffer, pH 5.0, with 0.1 ml appropriately diluted enzyme. The enzyme-substrate mixture was incubated at 50 °C for 5 minutes. The determination of the released sugars was done by the use of 3.5-dinitrosalicyclic acid (DNS) with glucose used as the standard for reducing sugars as described by Bailey et al (1992). One unit of cellulase is defined as the amount of enzyme that liberated 1 µmol of glucose equivalents per minute.

2.8 Effect of initial pH on cellulase production

A standardized suspension of *A. niger* was inoculated on 5 g brewery spent grains moistened with 10.2 ml of the following buffers: 50 mM acetate buffer (pH 4.0), 50 mM acetate buffer (pH 5.0) and 50 mM sodium phosphate buffer (pH 6.0). Nutrient supplements were added to a final concentration (m/v) of 0.02 % KH_2PO_4, 0.05 % NaCl and 0.01 % $MgSO_4$. Inoculated flasks were incubated at 35 °C in triplicates for up to 96 hours. Samples were taken at 24 hour intervals for enzyme extraction and cellulase activity assay.

2.9 Data analysis

Data analysis was done using GraphPad Prism, version 6.02. A one way ANOVA was used to analyze the effect of temperature and initial pH on the mean cellulase activity. The two sample *t*-test was used to analyze for any significant differences in the enzyme activity of the pre-treated and untreated substrate. In all cases a significance level of 0.05 was used.

3 RESULTS

3.1 Time course analysis for cellulase production

The time course analysis of cellulase production (Figure 3.1) showed a gradual increase in enzyme activity with time with a maximum production at 72 hours showing an activity of 12.9 U/g of substrate used. A decline enzyme production was observed at 96 hours of incubation.

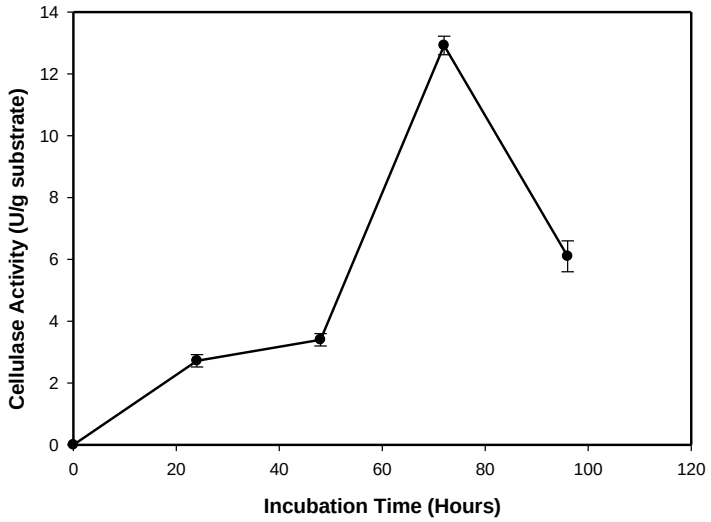

Figure 3.1: Time course analysis for the production of cellulase by *A. niger* on solid state fermentation of brewery spent grains at 30 °C and pH 5.0. Results are expressed as a mean of three determinations

3.2 Effect of substrate pre-treatment on the production of cellulase

The pre-treatment of the substrate had a significant effect on extracellular enzyme secretion ($P < 0.05$). The pre-treated substrate produced a three-fold increase in cellulase activity when compared to the non-treated substrate at 72 hours of incubation (Figure 3.2).

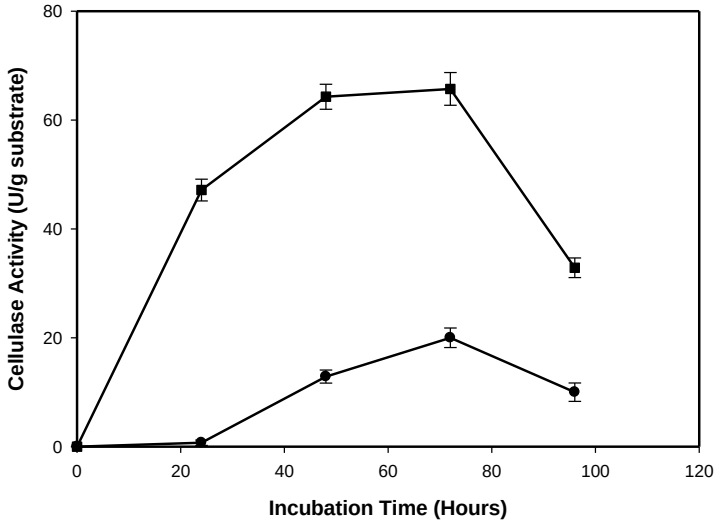

Figure 3.2: Cellulase production by solid state fermentation of pre-treated (■) and non-treated (•) brewery spent grains using *A. niger* at 30 °C and pH 5.0. Results are expressed as a mean of three determinations.

3.3 Effect of temperature on cellulase production

The maximum cellulase activity was obtained at a temperature of 35 °C after 72 hours of incubation. The cellulase activity at 35° C was significantly different from the other temperatures employed ($P < 0.05$).

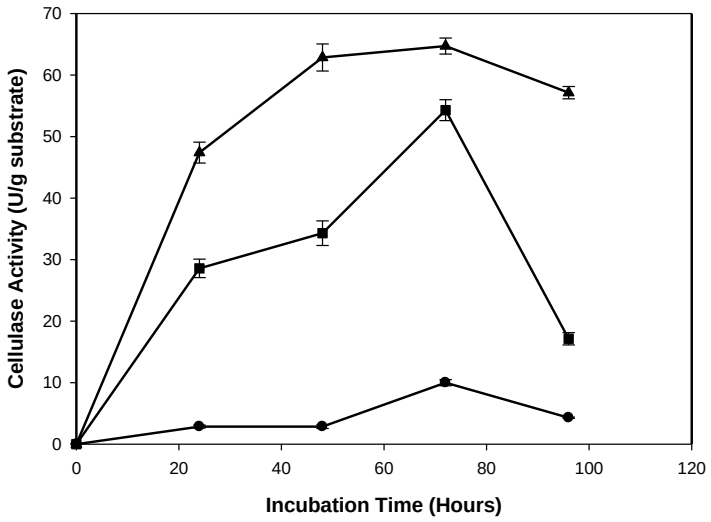

Figure 3.3: Optimum temperature for cellulase production by solid state fermentation of brewery spent grains using *A. niger* at 30 °C (●), 35 °C (▲) and 40°C (■) for up to 96 hours. Results are expressed as a mean of three determinations.

3.4 Effect of initial pH on cellulase production

The optimum pH for production of cellulase at 35 °C was pH 5.0 at 72 hours of incubation. Cellulase activity at pH 6.0 was significantly different from that at pH 4.0 and pH 5.0 ($P < 0.05$). The post-hoc analysis showed no significant difference ($P > 0.05$) in the mean cellulase activity at pH 4.0 and pH 5.0.

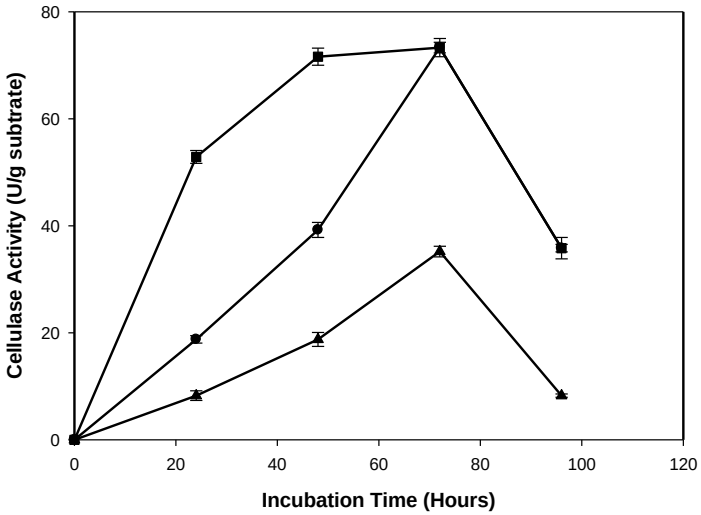

Figure 3.4: Effect of initial pH on cellulase production by solid state fermentation of brewery spent grains using *A. niger* at pH values of 4.0 (•), 5.0 (■) and 6.0 (▲) at 35 °C for up to 96 hours. Results are expressed as a mean of three determinations.

3.5 Optimization of incubation time

The ideal incubation conditions for the maximum production of cellulase by *A. niger* on brewery spent grains was 35 °C and pH 5.0 at 72 hours of incubation.

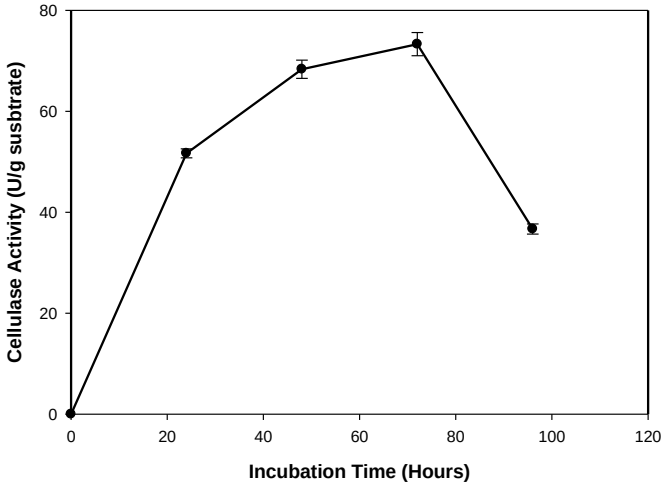

Figure 3.5: Optimum incubation time for cellulose production by solid state fermentation of brewery spent grains using *A. niger* at 35 °C. Results are expressed as a mean of three determinations.

4 DISCUSSION

4.1 Effect of pre-treatment of brewery spent grains

The pre-treatment process increased the cellulase activity three-fold compared to the non-treated spent grains (Figure 3.2). Alkaline pre-treatment modifies the lignocellulosic substrate by increasing pore size, solubilizing lignin and hemicelluloses, and increasing surface area for cellulose degradation. The process also has an effect of reducing the

crystallinity of the complex lignocellulosic substrate. In a similar study, Suresh and Chandrasekaran, (2009) reported cellulase production by *A. niger* to be 1.51 times higher for the pre-treated substrate compared to the non-treated substrate. The pre-treatment process improved substrate utilization by the microbes and thus enhanced enzyme yields.

4.2 Optimum temperature for cellulase production

The brewery spent grains used, supported the growth and production of cellulases by *A. niger*. Cellulase activity at 35 °C was significantly higher than the cellulase activity obtained at 30 °C. This temperature may be conducive for the growth of mycelium and cellulase synthesis. The lower cellulase activity at 30 °C may be due to slowing of the growth rate of the fungi and consequently the extent of development of spores to mycelia (Fawole and Odunfa 2003). Lower temperatures are known to hinder uptake of nutrients by the organisms hence there is reduced metabolic activity in the fungi. The suppressed development of the fungi may have resulted in the low cellulase activity at this temperature as the cellulose was converted to sugars for the nutrition of the fungi (Oyeleke et al 2012). The activity of cellulase observed after incubation at 40 °C was less than that observed at 35 °C incubation (Figure 3.3). This may have been a result of the organism using a lot of energy for maintenance purposes such as protection of the cell system against thermal inactivation.

4.3 Effect of initial pH on cellulase production

The optimum pH for the production of cellulase at 35 °C was pH 5.0 (Figure 3.4). There was no significant difference in the cellulase activities obtained at pH 4.0 and pH 5.0. Significantly lower activities of cellulases were produced at pH 6.0. The low cellulase activity at pH 6.0 could be a result of the change in the ionic state of the substrate that interfered with the uptake of the substrate and therefore the direct impact on the growth of the organism. Drastic variations in pH are also harmful to the organisms because of interference with cell membrane transport proteins and inhibition of the activity of enzymes concerned with the growth of the organism. The enzyme-substrate complex is not formed hence the substrate becomes unavailable to the microorganism (Abdul et al 2011). As the organism grows, there is secretion of metabolites which may alter the environment, thus hindering further growth.

4.4 Effect of incubation time on the production of cellulases

Maximum cellulase activity was obtained after incubation of the organisms on brewery spent grains for 72 hours (Figure 3.5) at 35 °C. In a similar study by Bansal et al (2012), the incubation period was found to be directly proportional to the amount of enzymes and other metabolites to a great extent. During the first few hours of incubation, the spores germinated to form mycelia. Proliferation and build-up of biomass of *A. niger* followed as the cellulases were produced to hydrolyze the substrate and provide soluble nutrients for the developing mycelia. As the amount of mycelia increases, there is also an increase in the amount cellulase produced up to a maximum period, after which the substrate may have been consumed for growth purposes hence enzyme synthesis was decreased (Chandra et al 2007).

The decrease in cellulase activity at 96 hours may have been due to the action of proteases on the cellulases produced. Fungi naturally produce significant quantities of extracellular proteases, which potentially degrade the cellulase produced (Devanathan et al 2007, Acharya et al 2008). There may be a decline in the production of primary metabolites after 72 hours due to the fungi reaching the stationary phase. The accumulation of secondary metabolites stimulates programmed cell death which is accompanied by a decrease in cell biomass and enzyme secretion.

5 CONCLUSION

The study demonstrated that brewery spent grains can be a suitable substrate for cellulase production using *Aspergillus niger* FGSC A733 in solid state fermentation. Variations in pH and temperature showed a significant effect on enzyme production. The highest activity was recorded at pH 5.0 and a temperature of 35 ^{o}C. Under optimized pH and temperature conditions, enzyme production showed a gradual increase with incubation time with a maximum enzyme secretion of 73.3 U/g of substrate after 72 hours. The study also demonstrated that cellulase production can significantly be improved by alkaline pre-treatment of the spent grains using calcium hydroxide. Pre-treated spent grains produced an enzyme activity three times higher than the untreated material. The highlight of this study therefore is that brewery spent grains, as a cheap and abundant agro-industrial waste can be a potential substrate for production of cellulases in solid state fermentation.

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