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THE EFFECT OF MEDIUM COMPOSITION ON β-GLUCOSIDASE PRODUCTION BY *TRICHODERMA VIRIDE* USING COW DUNG

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ABSTRACT
Different medium components were screened for their effect on β-glucosidase production. Plackett-Burman experimental design was used and twelve experimental runs were generated using Design-Expert software to determine the main effect of glucose, sucrose, lactose, carboxymethyl cellulose, peptone, beef extract, NH_4Cl, $NaNO_3$, $MnCl_2$ $CaCl_2$ and K_2HPO_4 on β-glucosidase production. Out of the eleven medium components selected, six components (glucose, lactose, $NaNO_3$, NH_4Cl, $MnCl_2$, K_2HPO_4) were found to contribute positively to the overall β-glucosidase production with a maximum production of 34.40±0.72 U/g. Thus, the use of Plackett-Burman experimental design in this study enabled the rapid identification of important medium components affecting the production of β-glucosidase.

Keywords: β-glucosidase, *Trichoderma viride*, Plackett-Burman Design, Cowdung, Medium Components.

INTRODUCTION

β-glucosidase (E.C 3.2.1.21) is an enzyme that catalyzes the hydrolysis of β-1,4-glycosidic bond and releases D-glucose from non-reducing end of cellobiose and oligosaccharides (Kumar *et al.,* 2017). It is considered to be part of the cellulase system, since it stimulates the rate and extent of cellulose hydrolysis by relieving cellobiose-induced inhibition of endo- and exo- β-glucanases (Saha *et al.,* 1994). β-glucosidases, particularly those derived from microbial sources, have been used in many biotechnological processes such as bioethanol production (Coughlan, 1985), improvement of aroma in wine and fruit juice industry (Gueguen *et al.,*1998) and detoxification of cassava (Obilie *et al.,*2004).

Continuous generation of vast quantities of agro-industrial residues as well as their disposal especially in developing countries is associated with several environmental problems (Singhania *et al.,* 2008). Utilization of the agricultural residues as nutrient sources for microbial β-glucosidase production may reduce the final enzyme production cost, which is one of the major challenges affecting the large-scale production (Pandey *et al.,* 2000). The most common substrates used for β-glucosidase production include sugarcane bagasse, wheat straw, rice straw, saw dust, corncobs, corn stover, banana peels, rice husk and orange peel (Iqbal *et al.,*2011a; Iqbal *et al.,*2011b; Irshad *et al.,*2013). β-glucosidase, exists widely in nature and can be isolated from bacteria, fungi, plants and animals (Joo *et al.,*2010). The ability of fungi to grow on surfaces of various substrates and penetrate into their inter-particle spaces makes them one of the best adapted species in the use of agro-residues (Viniegra-Gonzalez and Favela-Torres, 2006). Additionally, fungi are more efficient in the bioconversion of several renewable substrates due to their tolerance to minimal water condition (Diaz *et al.,* 2006). *T. viride* has been used in the production of microbial enzymes including β-glucosidase.

Cow dung is one of most abundant and unexploited resources; it consists of ash (13.3%), nitrogen (1.4 %), cellulose (35.4 %) and hemicelluloses (32.6 %) (Misra *et al,* 2003). This composition makes it attractive for production of several value added products. In this context, this study was aimed at selecting the significant medium components using cow dung as the main substrate through Plackett-Burman (PB) experimental design for β-glucosidase production by *T. viride*. This design was selected based on its ability to screen and evaluate the relevant medium components that affect the β-glucosidase production, so as to generate reliable and more manageable set of components, as well as indicating how each component affects the overall response (Plackett and Burman, 1946). Although there are many published reports on β-glucosidase production using different agricultural residues; to the best of our knowledge the literature contains no reports on the use of cow dung for extracellular β-glucosidase production by *T. viride.*

MATERIALS AND METHODS

Sample and Microorganism Collection

Cow dung was obtained from Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. It was dried for seven (7) days and finely ground using mortar and pestle to have a

uniform particle size of 1mm. The ground cow dung was stored in air tight containers before further use. *T. viride* was obtained from the culture stock of Department of Microbiology, Ahmadu Bello University, Zaria.

Fungal Strain and Inoculum Preparation.

Seven-day-old potato dextrose agar slant containing *T. viride* was used to prepare the inoculum culture. In this study, the spore suspension was prepared by aseptic addition of 10 ml of sterile distilled water into the culture slant. Conidial clumps were broken using a sterile glass rod. The tube was shaken to make homogenous mixture of the suspension which was then counted using a hemocytometer, and the inoculum concentration of 1 x 10^6 spores per ml was prepared (Irfan *et al.,* 2014).

Screening of different medium components that affect β-glucosidase production using Plackett-Burman (PB) experimental design

The variables chosen for the present study were glucose, sucrose, lactose, carboxymethyl cellulose, peptone, beef extract, NH_4Cl, $NaNO_3$, $MnCl_2$ $CaCl_2$ and K_2HPO_4. Twelve experimental runs were generated using Design-Expert software (version 6.0.8, Stat-Ease Corporation, USA). Each component was tested at two concentration levels, low (−1) and high (+1), and the concentration ranges taken for the components were 0–0.5 % each for glucose, sucrose, lactose, carboxy methyl cellulose (CMC); 0–0.2 % each for peptone, beef extract, $NaNO_3$, NH_4Cl and 0–0.1 % each for $CaCl_2$, $MnCl_2$, and K_2HPO_4. These concentration levels were decided based on several literature reports on β-glucosidase production using different substrates. The experimental values of the medium components used in the screening experiment (Plackett-Burman) are represented in Table 1. All experiments were carried out in triplicates and average value of β-glucosidase activities was taken as the response. The experiments were carried out according to the design matrix where temperature, the initial moisture content and inoculum concentration were maintained at 30^0C, 60% (v/w) and 5% (v/w) respectively.

Main Effect =

$$\frac{\sum \text{Response for high levels of factor } \mathbf{x} - \sum \text{Response for low levels of factor } \mathbf{x}}{\text{Number of Runs/trials}}$$

β-glucosidase Production by Solid State Fermentation

Eight grams (8g) of the substrate (cow dung) was transferred into a 250-mL Erlenmeyer flask and the moisture content was maintained at 60 % (v/w). The content was mixed thoroughly and autoclaved at 121°C for 15 minutes. After cooling the flask to room temperature, it was inoculated with 5% (v/w) of *T.viride* inoculum under aseptic conditions. The culture was then incubated at 30°C for 7 days, and after incubation, 80 ml of distilled water was added to the fermented substrate. This was placed in an orbital shaker at 150 rpm for 30 minutes for enzyme extraction. After this, the mixture was filtered using Muslin cloth and centrifuged at 10,000 x g for 20 minutes. The supernatant obtained was used to assay for the activity of enzyme (Irfan *et al,* 2014).

Determination of β-glucosidase activity

The enzyme activity was assayed using the method of Tomaz and Roche (2002). The reaction mixture consisted of 0.2 ml substrate (1 % Salicin), 0.1 ml enzyme solution, and 0.1 ml citrate buffer (pH 4.8). The tubes containing the mixture were incubated at 50°C for 30 minutes. Thereafter, 3ml of Dinitrosalicylic acid (DNS) was added into each tube and then transferred into a water bath set at 100^0C for 15 minutes. The tubes were allowed to cool and absorbance was taken at 540 nm. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1 µmol of glucose from the substrate per minute under standard assay conditions. The results are expressed in terms of units per gram of cow dung (U/g).

RESULTS AND DISCUSSION

The use of different solid substrates for the production of extracellular β-glucosidase using different fungal species has been reported by several researchers (Bai *et al.,* 2013). This study involves the use of cow dung as the basal substrate and other media components were screened in order to determine their contribution for β-glucosidase production by *T. viride*. The carbon and nitrogen ratio in cow dung makes it a good substrate for microbial enzyme (Adegunloye *et al.,* 2007). The effect of eleven (11) components (glucose, sucrose, lactose, carboxymethyl cellulose, peptone, beef extract, NH_4Cl, $NaNO_3$, $MnCl_2$ $CaCl_2$ and K_2HPO_4) on β-glucosidase production was examined using Plackett Burman design so as to determine the contribution of each component. Based on Table 1, which showed the distribution of components according to the design matrix and the results obtained in the study; highest β-glucosidase production was found to be 34.40±0.72 U/g as observed in run 4 while lowest β-glucosidase production was found to be 1.21±0.03 U/g as observed in run 1, 6 and 10 respectively. Run 6 (only cowdung) which acts as a control with no exogenous addition of any of the 11 media components indicated that cow dung on its own contains some basal nutrients that lead to the observed β-glucosidase production (1.21±0.03 U/g).

Results obtained for the screening experiment in this study for β-glucosidase production is promising when compared with several other literature reports by different researchers.

El-Naggar *et al.* (2015) applied Plackett-Burman statistical experimental design to screen fifteen variables in developing the fermentation medium for β-glucosidase production by *Aspergillus terreus*. K_2HPO_4, $NaNO_3$ and Tween-80 were found to contribute significantly ($P<0.05$) with maximum β-glucosidase production of 4457.16 U/g. Madhu *et al*. (2009) also applied Plackett-Burman statistical experimental design to screen twelve medium components for β-glucosidase production by *Aspergillus sydowii BTMFSS 55*. Moisture

content, inoculum concentration and peptone were found to contribute significantly ($P<0.05$) with maximum β-glucosidase production of 387.3 U/g. Also, medium components for β-glucosidase production by *Aspergillus niger* were screened using Plackett-Burman experimental design. The most contributing factors that led to a maximum β-glucosidase activity of 9.37 U/mL were found to be wheat bran, glycerol, KCl, corn steep liquor (Yang *et al.*, 2014).

The effect of each nutrient component on β-glucosidase production was presented in Figure 1. It can be seen clearly that the six components (glucose, lactose, $NaNO_3$, NH_4Cl, $MnCl_2$, K_2HPO_4) were found to contribute positively to β-glucosidase production while the remaining five components (sucrose, CMC, Peptone, beef extract and $CaCl_2$) have negative influence on the β-glucosidase yield. However, production of β-glucosidase by *Bacillus stratosphericus strain SG9* showed that esculin, K_2HPO_4, Na_2HPO_4, $MgSO_4$, and mannitol were the most significant factors (Kumar *et al.*, 2017).

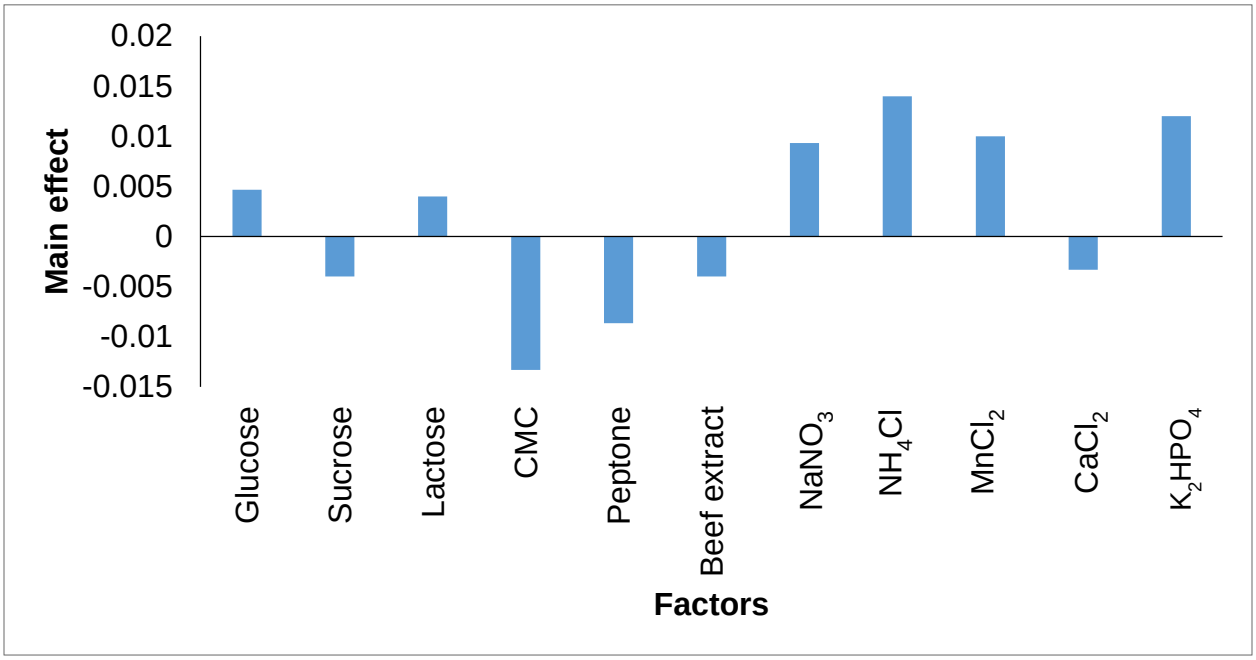

Fig. 1: Main effects of the medium components affecting β-glucosidase production by *T.viride* according to Plackett-Burman experimental results.

Table 1: Plackett-Burman experimental design matrix for screening of various medium components with responses for β-glucosidase production

Run	G (w/v) %	S (w/v) %	L (w/v) %	CMC (w/v) %	P (w/v) %	BE (w/v) %	$NaNO_3$ (w/v) %	NH_4Cl (w/v) %	$MnCl_2$ (w/v) %	$CaCl_2$ (w/v) %	K_2HPO_4 (w/v) %	β-glucosidase activity (U/g)
1	0.50	0.50	0.00	0.50	0.20	0.00	0.20	0.00	0.00	0.00	0.10	1.21±0.028
2	0.00	0.50	0.50	0.00	0.20	0.00	0.00	0.00	0.10	0.10	0.10	7.31±0.063
3	0.50	0.50	0.00	0.50	0.00	0.00	0.00	0.20	0.10	0.10	0.00	6.09±0.45
4	0.50	0.00	0.50	0.00	0.00	0.00	0.20	0.20	0.10	0.00	0.10	34.4±0.72
5	0.00	0.50	0.50	0.50	0.00	0.20	0.20	0.00	0.10	0.00	0.00	2.43±0.20
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.21±0.028
7	0.50	0.50	0.50	0.00	0.20	0.20	0.00	0.20	0.00	0.00	0.00	4.87±0.13
8	0.50	0.00	0.00	0.00	0.20	0.20	0.20	0.00	0.10	0.10	0.00	6.09±0.45
9	0.00	0.50	0.00	0.00	0.00	0.20	0.20	0.20	0.00	0.10	0.10	15.85±0.21
10	0.50	0.00	0.50	0.50	0.00	0.20	0.00	0.00	0.00	0.10	0.10	1.21±0.028
11	0.00	0.00	0.00	0.50	0.20	0.20	0.00	0.20	0.10	0.00	0.10	7.31±0.063
12	0.00	0.00	0.50	0.50	0.20	0.00	0.20	0.20	0.00	0.10	0.00	29.26±0.62

G: glucose; S: sucrose; L:lactose; CMC:carboxy methyl cellulose; P:peptone; BE: beef extract.

Figure 2 showed the percentage contribution of the medium components irrespective of the main effect (i.e positive or negative). The results revealed that NH_4Cl, K_2HPO_4, $MnCl_2$ and $NaNO_3$ were the most contributing media components with 22.80%, 16.80%, 11.60% and 10.10%, respectively. Similarly, CMC had 20.6% contribution, and based on the main effect, a negative influence (inhibitory) was observed.

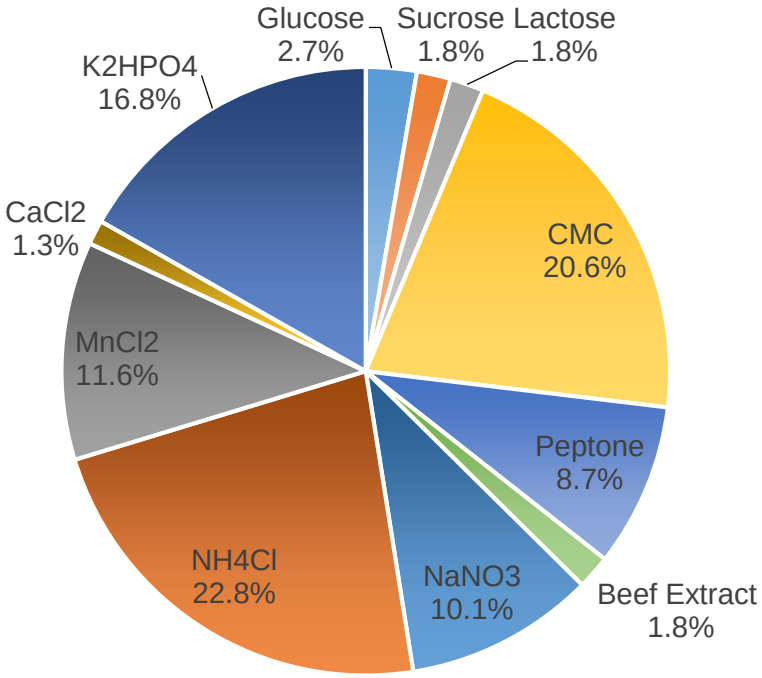

Fig. 2: Pie chart indicating the percentage contribution of the medium components on β-glucosidase production.

The present findings indicate that the supply of nitrogen sources is an absolute requirement for the production of β-glucosidase from *T. viride*. Both inorganic and organic nitrogen sources are important for enzyme synthesis in microorganisms (Tan *et al.*, 2004). The supplementation with nitrogen sources like NH_4Cl and $NaNO_3$ enhanced β-glucosidase production. Inorganic nitrogen sources are used immediately to meet the nitrogen needs, while organic sources act as precursors for many cell growth factors and amino acids necessary for enzyme synthesis and metabolism (Tan *et al.*, 2004). Carbon sources, such as glucose and lactose are required during microbial fermentation because of their contribution in microbial cellular synthesis and energy generation (Stanbury *et al.*, 1997).

Several inorganic minerals are required by different microorganisms for their growth during β-glucosidase production (Huntner, 1972). Optimum β-glucosidase production was obtained with $MnCl_2$ and K_2HPO_4 as mineral salts. Potassium is needed for microbial growth in which it is essential for osmoregulation (Venkateshwar *et al.*, 2010). Manganese is involved in signalling during microbial sporulation and contributes to the stabilization of cell walls (Missiakas and Raina, 1997)

In Agreement to the findings of this study, El-Naggar *et al.* (2015) demonstrated that $NaNO_3$ had maximum effect on β-glucosidase production. Weng *et al.* (2012) also reported that NH_4Cl (2 %) and K_2HPO_4 (1.5 %) were the best nitrogen sources for β-glucosidase production from *A. niger* using wheat bran. *Penicillium purpurogenum* was found to produce high level of intracellular β-glucosidase when grown on medium containing $NaNO_3$ as nitrogen source among the three salts tested i.e. $NaNO_3$, KNO_3 and NH_4NO_3 (Dhake and Patil, 2005). Although the PB experimental design employed in this study does not give information on the exact quantity of components to be used in further experiments, it effectively provides general information on each investigated factor and can serve as an excellent screening tool for development of microbial production system.

CONCLUSION

Determination of the main effect of each medium component on β-glucosidase production using PB experimental design as a preliminary optimization technique clearly showed six components (glucose, lactose, $NaNO_3$, NH_4Cl, $MnCl_2$, K_2HPO_4) contributing positively to β-glucosidase production while the remaining five components (sucrose, CMC, Peptone, beef extract and $CaCl_2$) had a negative influence on the β-glucosidase yield.The findings of this study showed the importance of using PB experimental design as a preliminary

optimization technique, which aided in screening and evaluating the medium components affecting the β-glucosidase production using cow dung by *T. viride*. Among all the tested medium components, NH_4Cl was the most contributing. Based on the results, the substrate (cow dung) and *T. viride* which is among the microorganisms generally recognized as safe (GRAS) contributed in making this process worthy of future investigation. As such, further statistical optimization of process and medium parameters need to be explored using response surface methodology.

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