Eggshell Wastes as Potential Protein Supplement in the Production of Cellulase by *Pseudomonas aeruginosa* and *Bacillus cereus*

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Abstract: Eggshells are waste from homes and industries. Its disposal contributes to environmental pollution. Some of the challenges with eggshell wastes management are disposal cost, availability of dumpsite, flies and odour. However, this waste can be converted to useful materials. In this study, different gram of eggshell was substituted for proteins in nutrient broth to grow cellulase-producing bacteria. Crude and pretreated eggshell were added to 100ml of bacteriological media. Filter paper assay was used to determine cellulase activity produced by *Pseudomonas aeruginosa* and *Bacillus cereus*. From the results, highest cellulase activity of 28.80IU was observed from cellulase produced by *Pseudomonas aeruginosa* supplemented with 0.5g of the crude eggshell, assayed at 50^{o}C for 60 minutes while the least activity of 2.50 IU was observed in cellulase produced by *Bacillus cereus* in 2.0g pretreated (15% HCl) eggshell supplemented medium at 40^{o}C for 60 minutes. In the control (Nutrient broth), cellulase produced by *Pseudomonas aeruginosa* showed the highest activity of 42.20 IU at 50^{o}C for 60 minutes while the least activity of 7.50IU was observed in cellulase produced by *Bacillus cereus* at 50^{o}C for 60 minutes. Cellulase activity was low for 120 minutes at 50^{o}C. This research findings show that eggshells contain protein which can be metabolized by *Pseudomonas aeruginosa* and *Bacillus cereus*. The enzyme cellulase acts best at an optimum temperature of 50^{o}C for 60 minutes, it also shows that media supplemented with crude eggshell gave better yield than those of HCl pretreatment. Higher activities were observed in cellulase produced by *Pseudomonas aeruginosa* than those produced by *Bacillus cereus*.

Keywords: Eggshell waste, Waste management, Cellulase, Bacteria and Reaction time,

INTRODUCTION

Eggshells are waste materials generated from homes, hatcheries and fast food processing companies and can therefore be readily collected in larger volume. Improper eggshell waste disposal contributes to pollution in the environment. Eggshell waste disposal is faced with challenges ranging from cost of disposal, availability of a dumpsite, flies and at times odour (Phil and Zhihong, 2009). However, eggshell wastes can be converted to saleable materials such as fertilizer, collagen production and artwork materials. The eggshell wastes can also be used in human and animal nutrition (Amu *et al*., 2005). It has also been used as solid base catalyst in the production of biodiesel (Arabhosseini and Faridi, 2018). A major component of the eggshell is the shell membrane which consists of collagen. When the collagen is extracted, it has diverse uses in pharmaceutical, medicine, food and cosmetics industries.

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Their effect on environmental pollution is great; therefore using them will greatly reduce their polluting effect on the environment. The eggshell and its membrane make up about 10.2% of the whole egg and comprises of calcified shell and shell membranes including inner and outer membranes (MacNeil, 1997). The organic matter of eggshell and shell membrane contains large amount of proteins with little carbohydrate and lipids (Burley and Vadehra, 1989).

Eggshell waste is associated with both domestic and industrial wastes and it is a major contributor to environmental pollution. For sustainable development, wastes should be recycled, reused, and channeled towards the production of value added products. This is to protect the environment and obtain value added products as a zero waste standard is established. In order to achieve sustainable development, waste utilization is a priority (Martin-Luengo *et al.,* 2011).

The most abundant biomass on earth is cellulose (Raju *et al.,* 2013). Plant biomass contains cellulose as the major component. Huge amount of agricultural and industrial cellulosic wastes have been accumulating in the environment.

Cellulose has attracted worldwide attention as a renewable resource that can be converted into biobased products and bioenergy (Li*et al.,* 2009). Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on cellulosic matters (Lee and Koo, 2001). Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid or enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere (Lynd *et al.,* 2002). Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production (Sethi *et al.,* 2013). Therefore in this study, eggshell wastes were used as protein supplement in nutrient broth medium for the growth of *Pseudomonas aeruginosa* and *Bacillus cereus* (cellulase producing bacteria). The activity of cellulase produced by the bacteria were determined using filter paper assay.

MATERIALS AND METHODS

Materials

All glassware were washed with liquid detergent and rinsed with distilled water after which they were dried in a drier before use. All the media used were sterilized using the autoclave at 121^{o}C, 15psi for 15minutes. Eggshells were collected in sterile Aluminum foil and aseptically transferred to the Laboratory

Methods

Collection and Processing of Sample

The eggshell wastes (Plate 1) were collected from fried egg sellers (popularly known as "*mai shai*" in Hausa language) at Gwagwalada in Abuja metropolis in Nigeria. These were taken to the Sheda Science and Technology Complex (SHESTCO) laboratory for further processing. The eggshell wastes were soaked in hot water (100^{o}C) for 10 minutes, after which it was air-dried for 24hours and pulverized into fine powder with the aid of a dried and clean pestle and mortar (Plate 2).

Eggshell pretreatment

Eggshell pretreatment was done by dissolving 20g of the eggshell powder in 100ml of different concentrations of HCl (5%, 10% and 15%). This was then transferred to a shaker for 1hour, and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and residue was washed with deionized water to a neutral pH (7.0). The washed residue was thereafter dried at 45^{o}C. (Stadelman, 2000).

Plate 1: Eggshell wastes collected from Gwagwalada, Abuja

Plate 2: Crude eggshell powder from eggshell

Bacteria used

Pure cultures of cellulase-producing *Pseudomonas aeruginosa* and *Bacillus cereus* were obtained from Biotechnology Advanced Research Centre (Sheda Science and Technology Complex (SHESTCO)), Abuja, Nigeria. The identities of these bacteria were confirmed using standard microbiological techniques such as streak plate techniques, morphological and biochemical characteristics and maintained on Nutrient Agar slant at 4^{o}C.

Preparation of Reagents

The 0.05M sodium citrate buffer used in this study was prepared according to the method of Bailey and Nevalainem (1981). The pH of the 0.05M citrate solution was adjusted to 4.8 and stored in a reagent bottle until further use.

Dinitrosalicylic (DNS) reagent was prepared in a 2L standard volumetric flask, covered with tin foil to avoid light exposure.

Ten grams (10g) of DNS and 16g of NaOH were weighed and added to a beaker containing 600ml distilled water and was transferred onto a magnetic stirrer with continuous stirring, after complete dissolution of the salts 300g of Rochelle salt was added slowly over a period of 30 minutes. The solution was carefully warmed to a maximum temperature of 45°C and later allowed to cool to room temperature (25°C ± 2°C), after which it was transferred to a 1L standard volumetric flask and was made up to the volume. (Bailey and Nevalainem, 1981)

Various concentrations of HCl were prepared by measuring different volumes of concentrated HCl (5ml, 10ml and 15ml) each and carefully adding to 95, 90 and 85ml of distilled water respectively in a standard volumetric flask and stored in reagent bottles.

Preparation of Culture medium

Each nutrient medium (100ml) was prepared using 0.55g Carboxy methyl cellulose (CMC) as a carbon source; 0.5g Sodium Chloride and 0.5g Peptone were added to the medium. However, Yeast and Meat Extracts were excluded from the medium and supplemented with 0.5g, 1.0g, 1.5g and 2.0g each of the crude and pre-treated egg shell; treated with 5%, 10% and 15% HCl which were added separately to the broth as nitrogen supplement in the medium. Nutrient broth was used as control. The media were autoclaved at 121°C for 15 minutes at 15 psi.

Production of the Cellulase Enzyme

The temperature of the sterilized medium was cooled to room temperature under an aseptic condition; inoculum from the stock bacterial cultures was introduced into the Erlenmeyer flasks containing the medium. These were then transferred to an incubator shaker and incubated at 37°C at 140 rpm for 24 hours, after which, the samples were centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatants were carefully transferred into clean sample bottles as crude enzyme source and stored in the refrigerator before use (Nitin *et al.*, 2012).

Filter Paper Assay for Total Cellulase Activity (FPA)

The activity of crude cellulase was determined according to the method reported by Kumar *et al*., (2009). An aliquot of 0.5ml of cell-free culture supernatant was transferred to a clean test tube to which 1ml of 0.05 M Sodium citrate buffer (pH 4.8) and Whatman No.1 filter paper strip 1.0 cm × 6.0 cm (≈ 50mg) were added. These were properly mixed and the tubes were incubated in a water bath at 40°C, 50°C and 60°C for 60 and 120 minutes reaction time for each. The enzymatic reaction was terminated by the addition of 3 ml of 3, 5- dinitrosalicylic (DNS) acid reagent. The tubes were then placed in a boiling water bath at 100°C for 5 minutes and then allowed to cool to room temperature (25°C ± 2°C). The contents of the tubes were mixed by completely inverting the tubes several times and absorbance was taken at 540nm. Cellulase activity was expressed in terms of filter paper unit (FPU) per ml of undiluted culture filtrate, where 1 FPU is defined as the amount of enzyme required to release 1µmol of reducing sugars per ml under standard assay conditions.

Conversion of FPU to IU/ML

0.01g of glucose was weighed and dissolved in 10ml of distilled water to give a concentration of 1mg/ml solution. Serial dilution of the solution was made as shown in the table below.

Glucose (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.08	0.06	0.04	0.02	0.01
Dist.H_20 (ml)	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.92	0.94	0.96	0.98	0.99
DNS (ml)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

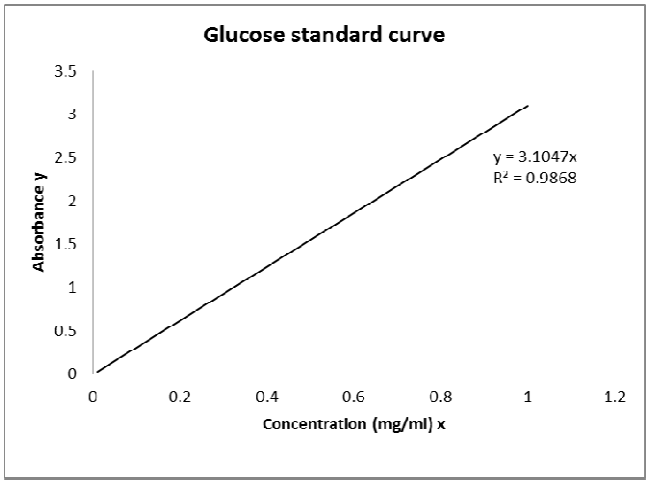
Glucose dilution

The absorbance was taken at 540nm and the results obtained were recorded in the table below

Absorbance reading of different concentration of glucose was taken and recorded.

Glucose Absorbance reading

Concentrations (mg/ml)	Absorbance
1	3.00
0.9	3.00
0.8	2.70
0.7	2.20
0.6	1.581
0.5	1.461
0.4	1.152
0.3	0.850
0.2	0.621
0.1	0.424
0.08	0.170
0.06	0.152
0.04	0.108
0.02	0.082
0.01	0.030



Glucose Standard curve

From the graph, y = 3.1047x

For example, if absorbance is 0.186

From the graph, y = 3.1047x

$$X = \frac{0.186}{3.1047}$$

= 0.0599 mg/ml OR 0.0599g/dm^3

Molar Mass of Glucose = 108g/dm^3

$$\text{Concentration (Mol/dm}^3\text{)} = \frac{\text{Concentration (g/dm}^3\text{)}}{\text{Molar Mass}}$$

$$= \frac{0.0599}{108} = 0.0005546 \text{ mol/dm}^3$$

$$\frac{0.0005546 \text{ mol/dm}^3}{60 \text{ minutes}} = 0.0000092 \mu\text{mol/ml/min}$$

$$= 9.2 \times 10^{-6} \mu$$

It should be noted that IU is the amount of enzyme which releases 1 µmol/ml/min of glucose.

RESULTS

Cellulase Activities Using Crude Eggshell Powder as Protein Supplement

Reaction Time- 60 min.

The results obtained in this study showed highest cellulase activity of 28.80IU/ML (28.8 X 10^{-6}) at 50°C in 60 min assay period. This was recorded in cellulase produced by *Pseudomonas aeruginosa* in 0.5g crude eggshell powder supplemented medium, while the least activity of 5.20 IU/ML (5.20 X 10^{-6}) at 40°C for 60 minutes assay period (Table 1) was observed in cellulase produced by *Bacillus cereus* in 2.0g crude eggshell powder supplemented medium.

Reaction Time- 120 minutes.

The results obtained in this study showed highest cellulase activity of 18.10 IU (18.10 X 10^{-6}) at 50°C in 120 minutes assay period. This was recorded in cellulase produced by *Pseudomonas aeruginosa* in 0.5g crude eggshell powder supplemented medium, while the least activity of 3.30IU (3.30 X 10^{-6}) at 40°C for 120 minutes assay period (Table 2) was observed in cellulase produced by *Pseudomonas aeruginosa* in 2.0g crude eggshell powder supplemented medium.

Table 1: Cellulase Activity in Crude Eggshell Media at Different Temperatures For 60 Minutes Reaction Time (IU/ML= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	**50°C**	**60°C**	**40°C**	**50°C**	**60°C**
0.5	10.00	28.80	19.50	7.20	26.20	18.00
1.0	9.20	27.30	15.70	6.10	25.90	15.10
1.5	7.20	25.40	13.70	5.30	25.40	13.30
2.0	6.40	24.30	10.70	5.20	21.60	10.10
Control	8.0	42.20	26.40	7.50	28.70	22.20

Table 2: Cellulase Activity in Crude Eggshell Media at Different Temperatures for 120 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	**50°C**	**60°C**	**40°C**	**50°C**	**60°C**
0.5	5.30	18.10	11.00	5.50	17.90	10.90
1.0	4.90	15.40	10.90	5.00	15.50	10.00
1.5	3.80	15.00	7.60	4.70	14.60	9.80
2.0	3.30	14.40	7.20	3.60	13.80	7.00
Control	9.10	29.90	25.20	7.60	25.10	24.90

Cellulase Activities in 5% HCl Pretreated Eggshell Powder as Protein Supplement at 60 Minutes Reaction Time.

Cellulase activity of 26.30 IU/ML was obtained at 50°C in 60 minutes assay period when subjected to 5% HCl pretreated powder, in cellulase produced by *Pseudomonas aeruginosa* in 0.5g pretreated eggshell powder supplemented medium, while the least activity of 3.10 IU/ML was recorded at 40°C, in cellulase produced by *Bacillus cereus* in 2.0g pretreated eggshell powder supplemented medium (Table 3).

Cellulase Activities in 5% HCl Pretreated Eggshell Powder as Protein Supplement at 120 Minutes Reaction Time.

Cellulase activity of 17.80 IU/ML was obtained at 50°C in 120 minutes assay period when subjected to 5% HCl pretreated powder, in cellulase produced by *Pseudomonas aeruginosa* in 0.5g pretreated eggshell powder supplemented medium, while the least activity of 3.20 IU/ML was recorded at 40°C, in cellulase produced by *Pseudomonas aeruginosa* in 2.0g pretreated eggshell powder supplemented medium (Table 4.0).

Table 3: Cellulase Activity in 5% HCl Pretreated Eggshell Media at Different Temperatures for 60 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	50°C	60°C	40°C	50°C	60°C
0.5	9.90	26.30	16.10	7.20	25.40	16.60
1.0	9.00	25.50	14.50	6.10	24.50	13.00
1.5	7.00	24.90	13.70	5.30	22.70	12.10
2.0	6.40	23.40	10.70	3.10	20.40	10.10
Control	8.00	42.20	26.40	7.50	28.70	22.20

Table 4: Cellulase Activity in 5% HCl Pretreated Eggshell Media at Different Temperatures for 120 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	50°C	60°C	40°C	50°C	60°C
0.5	5.30	17.80	10.60	5.50	17.30	10.00
1.0	4.90	14.90	10.00	5.00	16.60	9.90
1.5	3.70	14.40	7.50	3.80	14.90	7.50
2.0	3.20	13.20	7.00	3.60	14.40	6.40
Control	9.10	29.90	25.20	7.60	25.10	24.90

Cellulase Activities in 10% HCl Pretreated Eggshell Powder as Protein Supplement at 60 Minutes Reaction Time.

Higher cellulase activity of 24.90 IU/ML was recorded at 50°C in 60 minutes assay period, in cellulase produced by *Pseudomonas aeruginosa* in 0.5g pretreated eggshell powder supplemented medium, while the lowest activity of 2.60 IU/ML at 40°C in 60 minutes assay period was observed in cellulase produced by *Bacillus cereus* in 2.0g pretreated eggshell supplemented medium (Table 5.0).

Cellulase Activities in 10% HCl Pretreated Eggshell Powder as Protein Supplement at 120 Minutes Reaction Time.

Higher cellulase activity of 17.10 IU/ML was recorded at 50°C in 120 minutes assay period, in cellulase produced by *Pseudomonas aeruginosa* in 0.5g pretreated eggshell powder supplemented medium, while the lowest activity of 3.00 IU/ML at 40°C in 120 minutes assay period was observed in cellulase produced by *Bacillus cereus* in 2.0g pretreated eggshell supplemented medium (Table 6.0).

Table 5: Cellulase Activity From 10% HCl Pretreated Eggshell Media at Different Temperature in 60 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	50°C	60°C	40°C	50°C	60°C
0.5	7.40	24.90	15.70	7.10	25.50	13.80
1.0	7.00	24.50	13.70	6.00	24.20	12.90
1.5	6.30	21.50	12.10	5.00	20.80	11.90
2.0	5.80	19.80	11.20	2.60	19.60	11.00
Control	8.00	42.20	26.40	7.50	28.70	22.20

Table 6: Cellulase Activity in 10% HCl Pretreated Eggshell Media at Different Temperature in 120 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	50°C	60°C	40°C	50°C	60°C
0.5	3.90	17.10	10.60	3.70	15.80	8.40
1.0	3.70	14.50	9.60	3.60	15.30	8.10
1.5	3.60	14.30	6.90	3.50	13.10	7.40
2.0	3.10	12.70	7.00	3.00	12.40	6.30
Control	9.10	29.90	25.20	7.60	25.10	24.90

Cellulase Activities in 15% HCl Pretreated Eggshell Powder as Protein Supplement at 60 Minutes Reaction Time.

Higher cellulase activity of 24.90 IU/ML was recorded at 50°C in 60 minutes assay period, in cellulase produced by *Pseudomonas aeruginosa* in 0.5g pretreated eggshell powder supplemented medium, while the least activity of 2.50 IU/ML at 40°C in 60 minutes assay period was observed in cellulase produced by *Bacillus cereus* in 2.0g pretreated eggshell supplemented medium (Table 7).

Cellulase Activities in 15% HCl Pretreated Eggshell Powder as Protein Supplement at 120 Minutes Reaction Time.

Higher cellulase activity of 14.90 IU/ML was recorded at 50°C in 60 minutes assay period, in cellulase produced by *Pseudomonas aeruginosa* in 0.5g pretreated eggshell powder supplemented medium, while the lowest activity of 2.60 IU/ML at 40°C in 60 minutes assay period was observed in cellulase produced by *Bacillus cereus* in 2.0g pretreated eggshell supplemented medium (Table 8).

Table 7: Cellulase Activity From 15% HCl Pretreated Eggshell Media at Different Temperatures in 60 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	50°C	60°C	40°C	50°C	60°C
0.5	8.10	24.90	15.00	7.00	22.90	14.20
1.0	6.90	24.20	12.40	6.00	20.70	14.70
1.5	6.10	22.00	13.40	4.50	19.40	10.80
2.0	5.00	19.70	12.30	2.50	18.00	8.90
Control	8.00	42.20	26.40	7.50	28.70	22.20

Table 8: Cellulase Activity in 15% HCl Pretreated Eggshell Media at Different Temperatures for 120 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)			*Bacillus cereus* (IU)		
	40°C	50°C	60°C	40°C	50°C	60°C
0.5	3.70	14.90	10.40	3.70	12.60	8.40
1.0	3.60	13.50	8.90	3.20	12.50	7.80
1.5	3.60	12.70	6.70	2.80	12.10	7.20
2.0	3.00	12.40	6.60	2.60	12.00	6.20
Control	9.10	29.90	25.20	7.60	25.10	24.90

Effect of Crude and Hydrochloric Acid (5, 10 and 15%) Pretreatment on Cellulase Activities

It could be observed that activities of cellulase enzyme varied with concentrations of acid pretreatment. Highest cellulase activities of 10.00 IU/ML (Table 9), 28.80 IU/ML (Table 10), 19.50 IU/ML (Table 11), 5.70 IU/ML (Table 12), 18.10 IU/ML (Table 13) and 11.00 IU/ML (Table 14) were recorded in crude eggshell supplemented media (Table 9 - 14) while cellulase activities decreased with an increase in the concentration of acid used for pretreatment and *vice versa* (Table 9-14). The least cellulase activity of 2.50 IU/ML (Table 9) was recorded in cellulase produced by *Bacillus cereus* in a medium supplemented with 2.0g of the 15% HCl pretreated eggshell powder at 40°C.

Table 9: Cellulase Activity in Crude and HCl Pretreated Eggshell Media at 40°C for 60 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell(g)	*Pseudomonas aeruginosa*(IU)				*Bacillus cereus* (IU)			
	Crude eggshell	HCl pretreated eggshell			Crude eggshell	HCl pretreated eggshell		
		5%	10%	15%		5%	10%	15%
0.5	10.00	9.90	7.40	8.10	7.20	7.20	7.10	7.00
1.0	9.20	9.00	7.00	6.90	6.60	6.10	6.00	6.00
1.5	7.20	7.00	6.30	6.10	5.30	5.30	5.00	4.50
2.0	6.90	6.40	5.80	5.00	5.20	3.10	2.60	2.50
Control		**8.00**				**7.50**		

Table 10: Cellulase Activity in Crude and HCl Pretreated Eggshell Media at 50°C for 60 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)				*Bacillus cereus* (IU)			
	Crude eggshell	HCl pretreated eggshell			Crude eggshell	HCl pretreated eggshell		
		5%	10%	15%		5%	10%	15%
0.5	28.80	26.30	24.90	24.90	26.20	25.40	25.50	22.90
1.0	27.30	25.50	24.50	24.20	25.90	24.50	24.20	20.70
1.5	25.40	24.90	21.50	22.00	25.40	22.70	20.80	19.40
2.0	24.30	23.40	19.80	19.70	21.60	20.40	19.60	18.00
Control		**42.20**				**28.70**		

Table 11: Cellulase Activity in Crude and HCl Pretreated Eggshell Media at 60°C for 60 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)				*Bacillus cereus* (IU)			
	Crude eggshell	HCl pretreated eggshell			Crude eggshell	HCl pretreated eggshell		
		5%	10%	15%		5%	10%	15%
0.5	19.50	16.10	15.70	15.00	18.00	16.60	13.80	14.20
1.0	15.70	14.50	13.70	12.40	15.10	13.00	12.90	14.70
1.5	14.00	13.70	12.10	13.40	13.30	12.10	11.90	10.80
2.0	12.60	10.70	11.20	12.30	12.60	10.10	11.00	8.90
Control		**26.40**				**22.20**		

Table 12: Cellulase Activity in Crude and HCl Pretreated Eggshell Media at 40°C for 120 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)				*Bacillus cereus* (IU)			
	Crude eggshell	HCl pretreated eggshell			Crude eggshell	HCl pretreated eggshell		
		5%	10%	15%		5%	10%	15%
0.5	5.70	5.30	3.90	3.70	5.50	5.50	3.70	3.70
1.0	5.00	4.90	3.70	3.60	5.00	5.00	3.60	3.20
1.5	3.80	3.70	3.60	3.60	4.70	3.80	3.50	2.80
2.0	3.30	3.20	3.10	3.00	3.60	3.60	3.00	2.60
Control		**9.10**				**7.60**		

Table 13: Cellulase Activity in Crude and HCl Pretreated Eggshell Media at 50°C for 120 Minutes Assay Period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)				*Bacillus cereus* (IU)			
	Crude eggshell	HCl pretreated eggshell			Crude eggshell	HCl pretreated eggshell		
		5%	10%	15%		5%	10%	15%
0.5	18.10	17.80	17.10	14.90	17.90	17.30	15.80	12.60
1.0	15.40	14.90	14.50	13.50	15.50	16.60	15.30	12.50
1.5	15.00	14.40	14.30	12.70	14.60	14.90	13.10	12.10
2.0	14.40	13.20	12.70	12.40	13.80	14.40	12.40	12.00
Control		**29.90**				**25.10**		

Table 14: Cellulase activity in crude and HCl pretreated eggshell media at 60°C for 120 minutes assay period (IU= 10^{-6})

Weight of eggshell (g)	*Pseudomonas aeruginosa*(IU)				*Bacillus cereus* (IU)			
	Crude eggshell	HCl pretreated eggshell			Crude eggshell	HCl pretreated eggshell		
		5%	10%	15%		5%	10%	15%
0.5	11.10	10.60	10.60	10.40	10.90	10.00	8.40	8.40
1.0	10.90	10.00	9.60	8.90	10.00	9.90	8.10	7.80
1.5	7.60	7.50	6.90	6.70	9.80	7.50	7.40	7.20
2.0	7.20	7.00	7.00	6.60	7.00	6.40	6.30	6.20
Control		**25.20**				**24.90**		

DISCUSSION

The results obtained from this research work showed that the activities of cellulase produced by eggshell supplemented medium is not favoured by a low and higher temperatures of 40°C and 60°C respectively. The enzyme cellulase produced in eggshell (crude and HCl pretreated) supplemented medium acts best at an optimum temperature of 50°C. This is in agreement with the findings of Elshafei *et al*., (2008) and also correlates with the report of Pardo and Forchiassin (1999). A clear distinction in the enzyme activities with change in temperature is an indication that enzymes are highly sensitive to a slight change in temperature (Fagade and Bamigboye, 2010).

At 60 minutes reaction time i.e. assay period, cellulase activities in both crude and HCl pretreated eggshell supplemented media were higher when compared with its activities at 120 minutes reaction time. This implies that activities of cellulase enzyme are reduced with an increase in reaction time. (Elshafei *et al*., 2008). In the control (Nutrient broth), cellulase produced by *Pseudomonas aeruginosa* showed the highest activity of 42.20 IU at 50°C for 60 minutes while the least activity of 7.50IU was observed in cellulase produced by *Bacillus cereus* at 50°C for 60 minutes.

The enzyme production media supplemented with crude eggshell powder as the nitrogen source had activities of its cellulase increased when compared with those of HCl pretreated eggshell powder. As observed from the results, higher and lower dosages of acid concentrations eggshell powder were not suitable for the production of cellulase, however, pretreating the powder with 5% HCl at 50°C for 60 minutes reaction time (Table 3 and 11) showed a better yield than 10 and 15% pre-treated eggshell powder at the same temperature and reaction time. It was therefore observed that the higher the concentration of acid used for pretreatment, the lower the yield and the lower the cellulase activities, that is, an increase in the concentration of the acid reduced the activities of the enzyme. The HCl might have impacted a deproteinizing effect on the eggshell powder, or rather, the residual HCl might have posed inhibitory effect on the bacteria, thereby reducing cellulase production which may in turn lead to low cellulase activity (Dalia and Ahmad, 2013).However, this is at variance with the findings of Nitin (2012) in production of cellulase from *Neurospora crassa*.

In this study, cellulase activity decreased with an increase in the weight of eggshell (crude and HCl pretreated) powder in the medium from 0.5g to 2.0g and *vice-versa*. This may probably be as a result of an increase in protein concentrations which may have inhibitory effect on microbial activity, leading to low enzymatic activity (Garg and Neelakantan, 1982). It was observed that when the microorganisms were grown in the commercial nutrient broth medium (Fluka) used as control, highest cellulase activity of 42.20 IU/ml (Tables 3 and 10) was recorded at 50°C in 60 minutes reaction time in cellulase produced by *Pseudomonas aeruginosa* while the least activity of 7.50 IU/ml (Table 1, 3, 5 and 7) was also recorded in 40°C temperature and at 60 minutes reaction time in cellulase produced by *Bacillus cereus.* This showed that the enzyme cellulase acts best at 50°C, this is in agreement with the findings of Sethi *et al.,* (2013).

It could be observed also that there is a reduction in cellulase activity with an increase in reaction time in both the crude and pretreated eggshell powder. This implies that at 60 minutes reaction time, cellulase activity of eggshell supplemented medium was reduced.

Generally, at different temperature, time of reaction, concentration of HCl, and weight of eggshell used, it was observed that cellulase from *Pseudomonas aeruginosa* had higher activities than those of *Bacillus cereus* in the various eggshell supplemented media with few exceptions, these conforms with the findings of Sethi *et al.* (2013) that *Pseudomonas aeruginosa* has the ability to produce high cellulase enzyme.

CONCLUSION

Based on this research finding, it is therefore recommended that eggshell powder could be used as protein substitute in bacteriological media composition and more research work could be done on how to further increase the surface area of the eggshell powder and its solubility in bacteriological media. Utilizing eggshell as protein supplement in media composition could be added to the list of benefits of eggshell waste, thereby reducing its effect on environmental pollution. This study has proved that sustainability of economic development as well as waste management can be enhanced.

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