Studies on Optimal Production of Keratinase by *Bacillus cereus* 35 Isolated from Feather Dump Sites in Enugu Metropolis, Enugu State, Nigeria

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Abstract: Chicken feathers are usually difficult to degrade due to their hard keratinous nature. Their inadequate methods of disposal have brought about environmental problems and loss of possible essential amino acids. Keratinases produced by bacteria including *Bacillus* spp. could be used to alleviate most of the disposal problems hence, the need to optimize the submerged fermentation process for maximum keratinase production from *Bacillus cereus* 35 was crucial. The *Bacillus cereus* 35 was isolated and identified previously using spread plate method and was subjected to various submerged fermentation process parameters assay for maximum keratinase production through one-factor-at-a-time method. The *Bacillus cereus* 35 keratinase was produced optimally with 1% inoculum concentration at 30ºC for 8 days in a fermentation medium of pH 8.0 containing 1% whole chicken feather and 0.1% casein as carbon and nitrogen sources. A higher keratinase yield (111.2%) was achieved after optimization when compared to the pre-optimization yield. The result indicates that the assessed fermentation parameters positively influenced the keratinase production by *Bacillus cereus* 35.
Key words: Fermentation, Keratinase, Optimization, *Bacillus cereus* 35.

INTRODUCTION

Keratin is an insoluble rigid complex macromolecule with high structural stability and resistant to microbial degradation but rich in essential amino acids. Keratin is basically a structural component of the skin of animals, hair, feathers, hooves, horns, cloves, beaks, nails, reptilian osteoderm, and slime (McKittrick *et al*., 2012). Feathers constitute majority of keratin wastes in the environment as poultry industries produce several tones of feathers per year (Korniłłowicz-Kowalska and Bohacz, 2011). Chicken feathers account for around 5–7% of the entire weight of a chicken (Babalola *et al*., 2020). The vast quantity of feathers produced causes accumulation of wastes and environmental pollution (Nnolim *et al.,* 2020). Sometimes, these feathers are treated at high temperature and pressure and homogenized to produce dry feather powder for use as feed supplements for livestock. This kind of treatment is expensive and destroys the essential amino acids (Ekta and Rani 2012).

The use keratinolytic microorganisms is an ecofriendly approach for recycling keratinous wastes into useful products thereby avoiding environmental pollution and also maintaining the nutritive value of these chicken feathers (Sharma and Devi 2018). Keratinase is proteolytic in nature and are mostly serine protease or metalloprotease. They had been proved to be effective in management of keratinous wastes, production of organic fertilizers, protein supplements, personal care products as well as in dehairing processes and enhanced drug delivery in nails and acnes (Kshetri and Ningthoujam 2016). Generally, microbial keratinases are useful because of their action particularly on insoluble keratin substrates, and other broad range of protein substrates (Sharma and Devi 2018).

Many Microbes have ability to produce keratinase enzyme in the presence of keratin (Srivastava *et al*., 2019). A lot of keratinolytic microorganisms such as *Bacillus licheniformis, Bacillus pumilis, Vibrio* sp. *strain* Kr2*, Actinobacteria, Streptomyces pactum, Aspergillus* spp., *Rhizomucor* spp., *Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton gallinae, Microsporum canis* and *Microsporum gypseum*) have been isolated (Gupta *et al*., 2013).

Consequently, there is a need for continuous search for a potent keratinase producer that will maximize production for possible utilization in Nigeria as there is little or no report on the use of ecofriendly approach for feather disposal. Hence, this study was aimed at enhancing keratinase produced by *Bacillus cereus* 35 isolated from feather dump sites.

MATERIALS AND METHODS

Sources of Substrates

Keratinous substrates such as chicken feathers (10g), hooves (10g), horns (10g), hair (10g), were utilized for the study. The feathers, hooves and horns were obtained from animal slaughter houses while the hair was collected from Barber shops in Enugu metropolis.

Preparation of Substrates:

The keratinous substrates were prepared using the method of Ire and Onyenama (2017) by washing with water to remove dirt and debris followed by drying them in a hot air oven at 60℃ and later milled to powder using manual grinder (Corona).

Source of Inoculum

Previously identified ***Bacillus cereus* 35** (Ezeme-Nwafor *et al.,* 2020b) was utilized for this study. The isolate was reconfirmed using Feather meal agar ((g/L): NH_4Cl 0.5, NaCl 0.5, K_2HPO_4 0.3, KH_2PO_4 0.3, $MgCl_2.6H_2O$ 1, yeast extract 0.1, feather 1.0, Agar 1.5)**.** Clear zones of hydrolysis on feather meal agar was an indication of Keratinase production.

Initial Production of Keratinase by the Isolate

Bacillus cereus 35 previously identified by Ezeme-Nwafor *et al.* (2020b) was utilized for the assay of various process parameters. The shake-flask fermentation was performed in 250 ml Erlymeyer flask containing 100 ml of the feather basal medium consisting of (g/L): NH_4Cl 0.5, NaCl 0.5, K_2HPO_4 0.3, KH_2PO_4 0.3, $MgCl_2.6H_2O$ 1, yeast extract 0.1, feather 1.0 at 30℃ /160 rpm for 7 days. The fermentation broth was centrifuged at 10000 rpm to obtain the crude keratinase. The activity of recovered crude enzyme was determined by Lowry *et al*. (1951) as described by George-Okafor and Odibo (2011) with a slight modification of using 4.2% Na_2CO_3 instead of 2% of Na_2CO_3. Equal volume of 1% feather powder in a 0.2M phosphate buffer (pH 7.0) and the crude enzyme was incubated for 60 min at 30°C. The reaction mixture was terminated by the addition of 2 ml of 10% trichloroacetic acid (TCA). This was followed by centrifugation at 10000 rpm for 10 min. Thereafter, 5 ml of 4.2% sodium carbonate (Na_2CO_3) was added to 1ml of the recovered supernatant followed by the addition of 0.5 ml of 3-fold diluted Folin's reagent. The reaction mixture was allowed to stand for 15 min. Absorbance was read at 660 nm. A reaction mixture without the enzyme served as the control. All assays were done in duplicate. One unit of keratinase activity was defined as the amount of enzyme that released one microgram of tyrosine per minute under assayed conditions.

Optimization Study of Keratinase Production

The following parameters were assayed for optimal enzyme yield using one-factor-at-a-time technique.

Effect of Temperature on Keratinase Production

A modified method of Sivakumar *et al.* (2013) was employed. The constituted fermentation medium was utilized for the assay. One percent (1%) of 24 h culture of *Bacillus cereus* 35 was utilized for shake-flask fermentation at 160rpm for 7 days under varied temperature ranging from 20°C to 60°C. The fermented liquor was centrifuged at 10000 rpm to recover the crude keratinase and assayed as earlier described. The temperature with maximum keratinase production was utilized for further fermentation studies.

pH Profile on Keratinase Production

The feather basal medium was prepared as earlier described at varying pH of 3 to 12 maintained with 0.2M phosphate buffer.

The fermentation proceeded with 1% 24h inoculum (*Bacillus cereus* 35) but maintained at determined optimal temperature. The pH at which the maximum enzyme was produced was utilized for subsequent optimization assays.

Influence of Test Carbon Substrates on Keratinase Production

One percent (%) of various carbon sources (glucose, galactose, mannitol, starch and fructose) was assayed for optimum keratinase production first by substituting each with the initial carbon source of the formulated medium using a modified method of Sivakumar *et al*. (2013). The best carbon source with the highest keratinase activity was utilized. Fermentation at this point was at determined optimal conditions.

Keratinase Production Profiles in Relation to Test Nitrogen Substrates

The nitrogen sources assayed were defatted groundnuts, milled bambara nut, yeast extract, casein, soybeans and $NaNO_3$ at 1% concentration in a constituted fermentation medium under earlier determined optimal conditions. The recovered keratinases were assayed as earlier described. The best nitrogen source with maximum yield was used for further fermentation studies.

Effect of different keratinous Substrates on Keratinase Production

Keratinous substrates (chicken feathers, hooves, nails and hair) were prepared as previously described and screened separately in each constituted fermentation medium at optimal conditions. The keratinous substrate with maximum keratinase activity was selected and its concentrations varied (0.1%-2.5%).

Inoculum Concentration Profile

The fermentation medium was respectively inoculated with varied concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0% of *Bacillus cereus* 35 and was subjected to fermentation. The inoculum concentration with the optimal keratinase yield was subsequently utilized.

Fermentation Period for Keratinase Production

Fermentation medium at optimum pH containing the best carbon and nitrogen concentrations was utilized for keratinase production at 160 rpm and 30ºC for 10 days. At every 24h, a fermentation flask was pooled for keratinase determination.

Produced Keratinase After Optimization of Process Parameters

Fermentation at this stage was carried out with 1% inoculums at 30ºC for 8 days with a fermentation medium containing %(w/v): NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.03, $MgSO_4.6H_2O$ 0.01, casein 0.1, chicken feather 1.5. The fermentation was carried out at 160rpm and assayed for keratinase production.

RESULTS AND DISCUSSION

In the present study, previously isolated *Bacillus cereus* 35 was subjected to various fermentation study for optimal keratinase production. Keratinase production of *Bacillus cereus* 35 was found to be optimal at 30°C (fig.1). Further increase in temperature to 60°C reduced the enzyme production. This could be due to inactivation of the keratinase inducers in the medium at higher temperatures or that metabolic activities of the isolate was affected as the organism is mesophilic. Similar reports have also been documented for keratinase production by *Bacillus* spp. at mesophilic temperatures (Dada and Wakil, 2020). The optimal production of keratinase at 30°C is also similar to the report of Agrahari and Wadhwa (2010) that had maximum keratinase production at 30°C for *Bacillus thuringenesis, B. megaterium,* and *B. pumilus.* A closer range to 30ºC was observed by Kansoh *et al*. (2019) and Venkata *et al.* (2013) whose report indicated maximum keratinase production was at 37°C and 35°C from *B. megaterium*.

The optimum pH for *Bacillus cereus* 35 keratinase production was at pH 8.0 with a yield of 33.00U/mL) while the least was obtained at pH 3.0 (fig.2). This could be that the accessibility of the raw feathers or

enzymatic hydrolysis by bacteria was favoured more under alkaline environment than acidic range. A previous report has also documented maximum keratinase production at pH 8.0 by *Bacillus thuringenesis*, *Microsporum gypseum* and other *Bacillus* sp. (Srivastava *et al*., 2011). . However, there are other reports of maximum enzyme production at higher pH of 10 and 11 by *Bacillus thuringiensis*, *Bacillus megaterium*, *Bacillus cereus, Bacillus licheniformis* 1269 and *Bacillus subtilis* (Venkata *et al*., 2013; Mousavi *et al.,* 2013; Sivakumar *et al.*, 2012; Mazotto *et al*., 2011). The alkaline environment has been reported to make feathers more accessible for keratinase hydrolysis (Huang *et al.*, 2015).

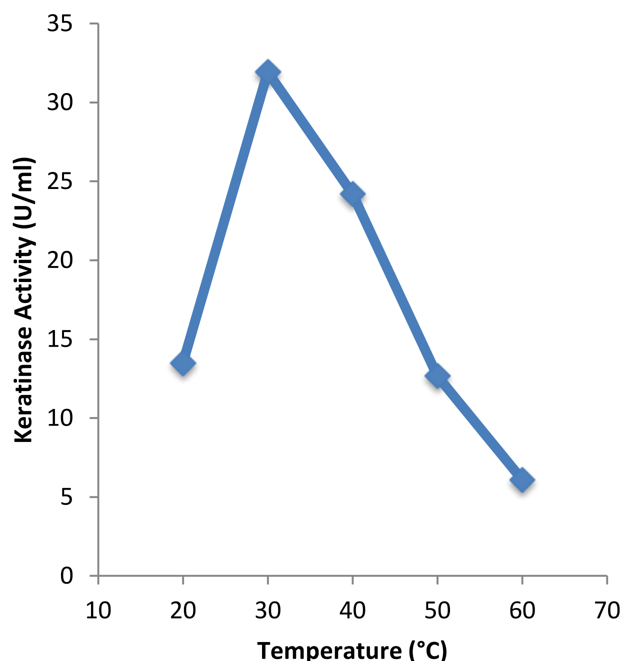

Fig.1: Effect of Temperature on Keratinase Production

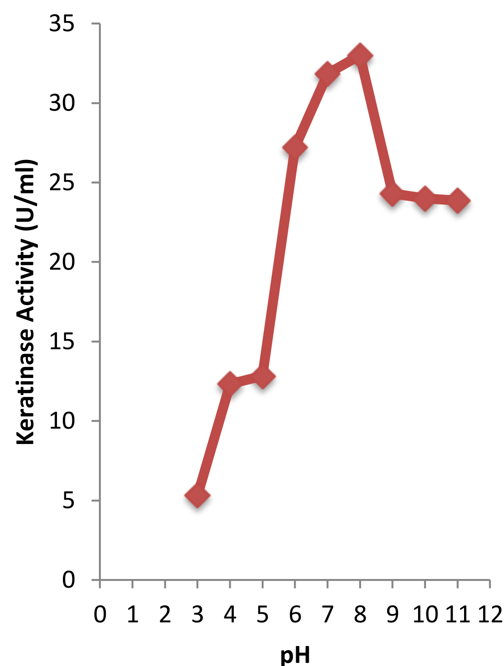

Fig.2: Effect of pH on Keratinase Production

Table 1: Effect of Carbon Sources on Keratinase Production

Carbon Sources	Enzyme Activity (U/mL)
Galactose	13.81
Fructose	28.52
Glucose	11.99
Mannitol	12.32
Starch	32.10

Table 2: Effect of Nitrogen Sources on Keratinase Production

Nitrogen Sources	Enzyme Activity (U/mL)
Deffatted nut	26.77
Bambara nut	24.55
Soy bean	25.16
Caesin	40.00
Yeast extract	33.43
Sodium Nitrate	13.00

Table 3: Effect of Different Keratinous Substrates on Keratinase Production

Keratinous Substrates	Enzyme Activity (U/mL)
Chicken Feathers	37.33
Hooves	24.88
Hair	24.87
Nail	22.45

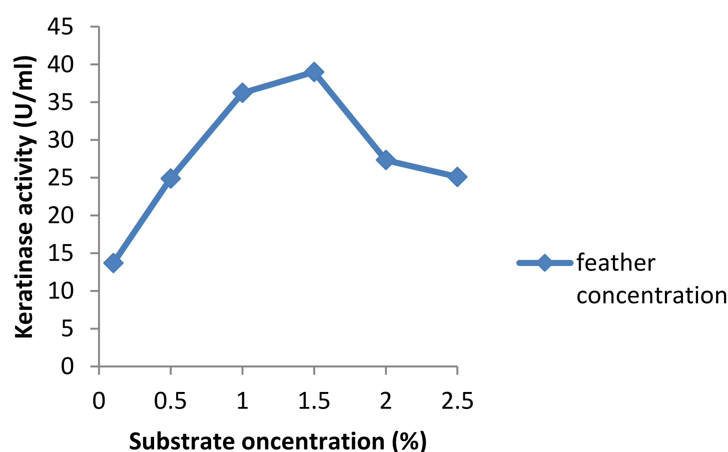

Fig.3: Substrate Concentrations on Keratinase production

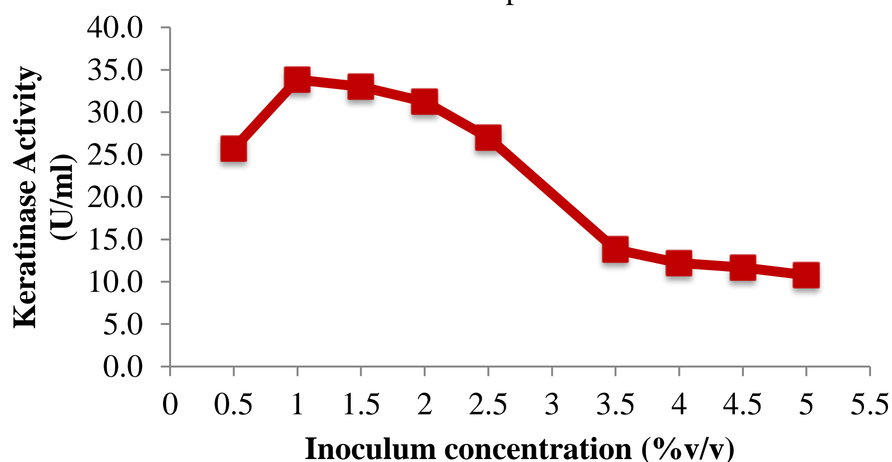

Fig.4: Effect of Inoculum Concentrations on Keratinase Production

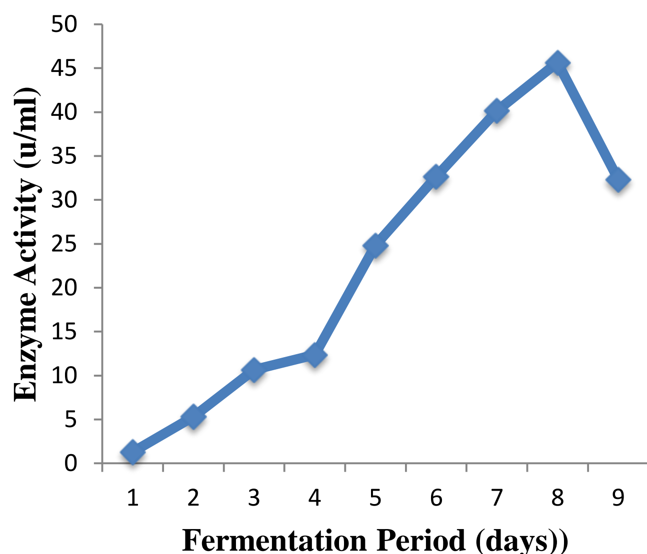

Fig.5: Effect of Fermentation Period on Keratinase Production

Table 4: **Keratinase Production Profile of *Bacillus cereus* 35**

	Enzyme Activity (U/mL)	Relative Enzyme Yeild (%)
Pre-optimization	32.21	100
Post- optimization	68.03	211.21
% Increase	35.82	111.21

Interestingly, glucose registered the least keratinase production for the isolate (table 1). The reduced keratinase production in the presence of glucose might be as a result of catabolic repression which in many cases have been shown to demonstrate inhibitory effects on protease synthesis by microorganisms. A similar observation was reported in another study by Dipak *et al.* (2012) who observed a reduced keratinase production with glucose (1%) in the fermentation medium. However, glucose was found to have a positive effect on *Bacillus subtilis* keratinase in some other studies (Sivakumar *et al*., 2012; Ramnani and Gupta, 2006).
Results on effect of different nitrogen sources on enzyme production by *B. cereus* 35 showed that optimal keratinase production was achieved with casein (Table 2). Generally, the microbial utilization of nitrogen sources varies. For instance, reports of Lakshmi *et al.* (2013) indicated a maximum keratinase yield from *Bacillus cereus* with groundnut cake (Defatted peanut) as nitrogen source while Venkata *et al*. (2013) and Sivakumar *et al*. (2012) recorded maximum keratinase production with 0.1% yeast extract and peptone for *B. megaterium* and *B. thuringiensis*.
Among the test keratinous substates screened for optimal keratinase production, feathers gave a higher yield as shown in table 3. This is an indication that keratinases could be inducible enzymes. Similarly, *Pseudomonas stutzeri* K4 keratinase isolated from feather dump sites had been reported to show high substrate specificity for chicken feathers, and low specificity for collagen, casein and hair (Chaturvedi *et al*., 2014).
The study on the effect of substrate concentration indicated that 1.5% chicken feather gave the highest keratinase yield with a decreasing trend as the concentration increased (fig.3).

The decrease could be attributed to substrate repression on keratinase production as the increased concentration of chicken feathers in the medium decreased the enzyme production suggesting catabolic repression (Armin *et al*., 2015). A higher substrate concentration may also have increased the medium viscosity which could result in oxygen limitation for the bacterial growth. This result was in conformity with Lin and Yin (2010) and Saibabu and Sunil (2013) that reported optimal keratinase production at about 1.5% feather concentration. Contrary, Ire and Onyenama (2017), Venkata *et al*. (2013), Sivakumar *et al*. (2012) and kainoor and Naik (2010) reported maximum keratinase production at 1% feather concentration for *B. licheniformis* PWD-1, *Bacillus* sp. JB 99, *B. megaterium* and *B. thuringiensis* respectively.

The fermentation study showed that maximum enzyme production of 32.00U/ml was attained on the 8th day *B. cereus* 35 (fig.5). This can be compared to another study with maximum keratinase yield from *Bacillus licheniformis* Yj4 on the 7th day (Lin and Yin, 2010). On the contrary, a shorter fermentation periods of 2-5days for optimal keratinase production by *B. megaterium*, *Bacillus licheniformis, Bacillus subtilis* and *B.cereus* have been reported (Dina and Thanaa, 2017; Saibabu *et al*., 2013; Han *et al.,* 2012). Maximum enzyme activities are known to be recorded at the exponential growth phase of bacteria which has been reported to vary among bacteria. The optimum fermentation period of 8 days recorded by the isolate in this study, could be related to the fact that bacterial isolates do not show exact same growth phase due to their individual expression of genes in a culture as reported by Dipak *et al.* (2012). It could also be due to the utilization of whole feather instead of its homogenized powdered form for the formulation of the fermentation medium.

The optimized processes gave an increase in keratinase yield of 111.21% as compared to the initial production (table 4). This is an indication that the optimized parameters enhanced the production of keratinase from *B. cereus* 35.

CONCLUSION

The results from this study indicates that *B. cereus* 35 have great potential for keratinase production using cheap nutritional sources. The fermentation conditions of *Bacillus cereus* 35 for optimal keratinase production were as follows: pH 8.0, temperature 30°C, feather concentration 1%, 0.1% casein and fermentation period 8 days. The findings from this study suggests that the keratinolytic enzyme from the *B. cereus* 35 strain belongs to alkaline serine protease family. Therefore, keratinase produced from *Bacillus cereus* 35 when adequately optimized with the above optimal process parameters could be useful in large scale production for industrial and bioremediation purposes.

Conflict of Interests

The authors hereby declare that there is no conflicts of interests.

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