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EFFECTS OF PH, FEATHER PARTICLE SIZE AND FEATHER CONCENTRATION ON THE GROWTH AND HYDROLYSIS OF SOME FEATHER-DEGRADING BACTERIA ISOLATED FROM FEATHER WASTE

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ABSTRACT
Owing to growing concern for cheap and alternative source of animal feeds, the use of microorganisms capable of breaking down feather keratin for the utilization of feather as protein feed was investigated. Eight (8) bacterial isolates from chicken feather-waste dump were screened for feather degradation by growing them with feathers as their primary source of carbon, nitrogen and energy. One of the isolates designed as D4 was characterized and selected for further studies based on its superior keratinolytic activity. The time course of study on bacterial growth and feather hydrolysis in liquid culture medium having raw feather as sole source of carbon and nitrogen was followed by measuring the release of free amino nitrogen (FAN), total non-protein nitrogen (TNPN), and medium optical density. Growth, TNPN, and FAN were influenced by feather particle size, pH and medium feather concentration. Maximum growth, FAN and TNPN occurred at 6 –7% (w/v) medium feather concentration. The feather particle size diameter of ≤90µM and pH of 10.0 gave the optimum growth, yield of TNPN and FAN in the fermentation medium. These tests indicated that chicken feathers can be used in the local production of amino acid concentrate based on the isolate and also improve feather- based protein feeds for animal nutrition.

KEYWORDS: Biodegradation, keratin, Lysine, Protein, Fermentation.

INTRODUCTION

Feathers are made of keratin; the same tough tightly wound protein fibers, which make up hairs, which make up hairs, fingernails, and hooves. In domestic fowl, they represent 5 – 7% of the body weight. They are of insulatory, structural and locomotory importance to the birds. Poultry feathers accumulate as waste after processing the chicken for human consumption thus the waste carries potent polluting implications especially with burgeoning global poultry production. Feathers could be put into good use in the manufacture of consumer goods, replacing wood pulp and other expensive fibers (Schmidt, 1998).

It is estimated that a year worth of feathers could replace approximately 25% of the wood pulp used annually for diapers. The environmental impact would go beyond saving trees as feathers require much less processing than wood pulp and unlike paper pulp, do not require bleaching (Schmidt, 1998).

Production of feather- meal, a feed additive, gives poultry processors another way to rescue feathers. Feathers lock up large amount of beneficial protein and amino acids that could be beneficially harnessed as animal feed additive. The keratins found in feathers are insoluble, not easily degradable by common proteolytic digestion are consequences of the high degree of cross-linking of the polypeptide chain caused by extensive formation of disulphide bonds and hydrolyzing the feather into cyteine-rich high product, which is 60% digestible, produces feather meal; it involves hydrothermal degradation whereby feathers are steam-pressure-cooked, a method similar to autoclaving, followed by drying. But producing feather meal through the above requires significant energy and destroys certain amino acids; it causes also the formation of non-nutritive amino acids such as lysino-alanine and lanthionine (Papadopoulos, 1989; Latshaw *et al*., 1984; Wang and Parson, 1997).

In view of the thermo energetic cost of the conventional processing of feathers against the backdrop of its limited nutritional improvement, investigations into alternative technology with prospects for nutritional enhancement that will turn feathers into better feed product seems justifiable. Recently, emphasis has shifted to biodegrading by microorganism – possessing keratinolytic activity. The objectives of this study include: (a) isolate bacteria from feather waste dump sites (b) screen the bacterial isolates for the presence of kerantinolytic activity (c) harness the possibility of producing amino acid concentrate and improved feather based protein feeds for animal nutrition.

MATERIALS AND METHODS

Collection of sample and isolation of microorganism:

Feathers were collected from several sites within a local poultry farm in Abakaliki, Ebonyi State, Nigeria. The feathers were incubated in $5gl^{-1}$ peptone solution at 30^oC for 24 h. Bacterial suspensions were streaked on feather meal agar plates ($15gl^{-1}$ feather meal, $0.5gl^{-1}$ NaCl, $0.3gl^{-1}$ K_2HPO_4, $0.4gl^{-1}$ KH_2PO_4 and $15gl^{-1}$ agar) which were incubated at 37^oC for 1-5d. Single colonies were isolated and screened for their ability to hydrolyze keratin in feather meal agar plates.

Collection and Procession of Chicken Feathers

Fresh chicken feathers were obtained from chicken rendering house in Abakaliki, Ebonyi State, Nigeria. Strong and healthy ones (quill feathers) were selected in the laboratory and washed very well with sterile distilled-water. Washed feathers were dried at 50^{o}C in a forced draught oven (Gallenkamp, Ltd UK). The dried feathers were ground into fine fractions (<90, 90, 150, 300, 425 and 850µM) with test sieves of appropriate diameters.

Screening for Feather Degradation

Degradation of feathers by the isolates was demonstrated in large test tubes (20 x2.5cm) using the method of Williams *et al*, (1990). Each test tube containing 29mL mineral salt medium (MSM), 2g of dried native feather and plugged with cotton wool was sterilized at 121^{o}C for 15min. The tubes with their contents were each inoculated with equal amounts of washed cells of the bacterial cultures. Inoculated controls were incubated at 50^{o}C in the water bath for 7 days under static-conditions. All the isolates that shows the highest ability to utilize chicken feathers in the process were collected. One of the isolates designated as D4 which showed the highest ability to utilize chicken feathers in the medium were used for further work. Pure culture of the isolate was grown at room temperature (25^{o}C) in mineral salt medium (growth medium) containing feather meal, after washing by centrifugation it was re-suspended in sterile saline to a McFarland nephelometer standard of 0.5 containing 1x10^{5} cells (Baron and Finegold, 1990). For the studies described below 1% $^{V}/_{V}$ of the growth medium was used.

Effects of medium feather concentration on the growth of isolate D4 and hydrolysis of chicken feather.

Ground feather (particle size 300µM) was added at level of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5g (1-7w/v) to **seven** set of quadruplicate 250ml Erlenmeyer flask each containing 50ml of the MSM described above and then sterilized at 121^{o}C for 10 minutes.Thereafter, the medium was inoculated with fleshly prepared inoculums of the isolate D4. Incubation was carried out in a kottermann water bath under similar condition of temperature and duration. About 5ml samples were aseptically withdrawn 24 hourly for analysis on biomass changes, total nitrogen, and free amino acids.

Effect of feather particle size diameter on the growth of Isolate D4 and hydrolysis of chicken feather

Twenty four (24) 250ml Erlenmeyer flasks containing 50ml MSM each arranged into six sets. Five percent ground feather particle size <90, 90, 150, 300, 425, and 850µM were after then added into the flask respectively and sterilized at 121^{0}C for 10 min. The sterile medium was inoculated with freshly prepared inoculums. Incubation was carried out at 50^{0}C for 7days in water bath. Exactly 5 ml of the sample was aseptically withdrawn on daily basis for analysis on free amino acid, total non-protein nitrogen, and biomass change.

Effect of medium pH on the growth of Isolate D4 and hydrolysis of chicken feather

MSM medium (50ml) was dispensed into each of 10 duplicate 250ml Erlenmeyer flasks. The pH of the duplicate flasks were adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0 with 0.1N HCl and 0.1n NaOH, using a pye Unicam pH meter (model 90MK2, Cambridge, UK). Then, 2.5g of ground feather (particle size 300µM) were added to each flask before sterilization at 121^{o}C for 10 min. Another set of 10 duplicate, 250ml Erlenmeyer flask was prepared as above. All the flasks were inoculated with saline suspension of isolate D4. One set was incubated in water bath shaker, while the other set was incubated under static condition in kottermann water bath. Samples were collected on daily basis and analyzed for free amino group, total non-protein nitrogen and biomass change.

Assay for free α-amino nitrogen (FAN).

FAN assay was done by the European brewing congress, ninhydrin procedure(I.O.B) method of analysis,1982. A colony from the overnight cultures was clarified by centrifugation at 5oog for 15mins. The supernatant (0.1ml) was diluted with 0.2ml of distilled water an d then mixed with 0.6ml of ninhydrin reagent (containing in g/l) $Na_2HPO_4.12H_20$, 100; KH_2PO_4, 6; Ninhydrin, 5; Fructose, 3. The mixture was incubated in a boiling water bath (100^{o}C) then cooled at 20^{o}C for 20 mins following diluting with 2ml of FAN diluting reagent ($KI0_3$, 2g; ethanol, 400ml and distilled water, 600ml). Sample absorbance was taken at 570nm with a spectrophotometer against a blank prepared as the test but with distilled water in place of the sample.

$$\text{FAN content in mg/l was calculated as FAN (mg/l)} = \frac{\text{Absorbance of test solution at 570nm}}{\text{Mean absorbance of glycine standard}}$$

Assay for total Non-Protein Nitrogen (TNPN)

Nitrogen in the culture filtrate was measured by the semi-micro kjeldahl method as reported by Pearson (1976). 1ml aliquot of culture filterate was digested in kjeldahl digestion flask (50ml) using 0.8g of catalyst mixture (96 % anhydrous Na_2SO_4; 3.5 % $CuSO_4$, and 0.5% SeO_2) and 2ml concentrated H_2SO_4. The digest was diluted with 15ml distilled water and transferred to a stream out apparatus, made alkaline with concentrated aqueous NaOH (15ml of 40%NaOH). The ammonia steam was distilled into 10ml boric acid solution (2%) and 0.2ml of screened methyl red indicator (0.016%) methyl red and 0.033% bromocresol green in alcohol for 10 min. Thereafter, the receiving flask was detached and allowed to stand for further 5 mins followed by the titration of the content (distillate) with 0.01m H_2SO_4. Nitrogen in the sample was calculated as 1ml of 0.01m H_2SO_4= 0.0014gN.

Statistical analysis: The data were analyzed using paired 't test' as stipulated by Zar (1984).

RESULTS

Screening of isolates for feather degradation

The result of the screening test of the isolates on feathers (Table 1) show that eight (8) of the 10 bacterial isolates

recovered from the samples were able to grow on the whole chicken feather as its primary source of carbon, nitrogen and energy, but the degree of degradation varied with each isolate after 7 days of incubation at room temperature (25^{o}C). The isolates were designated as k1, K2, k3, k4, D1, D2, D3 and D4. Isolates- K1, K2, & K3, degraded feather vanes extensively, and hydrolyzed shaft slightly, while isolate K4,D1, D2 & D3 showed extensive degradation of both feather vanes and shafts. Only Isolate D4 alone degraded both feather vanes and shafts as well as quill.

TABLE 1. Feather degradation by isolates

S/N	Isolate designation	Extents of feather degradation
1	K1	++
2	K2	++
3	K3	++
4	K4	+++
5	D1	+++
6	D2	+++
7	D3	+++
8	D4	++++

++ = Degradation of feather vanes plus slight degradation of feather shaft

+++ = Extensive degradation of feather vanes and shaft.

++++ = Extensive degradation of feather vanes and shaft as well as quill.

Characterization of the Isolates

The isolate D4 which showed the most degradation of feather vanes, shafts and quill was characterized. The result of the characterization showed that isolate D4 was a gram positive, spore- forming straight rods occurring singly or in pairs and were motile. Collectively, these characteristics indicated that the isolate was of the genus *Bacillus* (Krieg and Holt, 1984). It was catalase positive; hydrolyzed starch, showed a negative reaction in arginine test, positive reaction in nitrate reduction and voges-proskaur test. It also produced acid from mannitol fermentation. It grew well on nutrient agar medium incorporated with 7% sodium chloride (NaCl) salt. The isolate was therefore identified as a *Bacillus* species based on the outline in Bergey's manual of systematic bacteriology (1986).

Effect of medium feather concentration on isolate D4 growth

Maximum growth or biomass yield on the effect of medium feather concentration on the time course of isolate D4 growth is presented in Figure.1 for both shake flask and static cultures. From Figure 1, it could be seen that isolate D4 grew better at highest medium feather concentration irrespective of the mode of cultivation. Growth was faster and more on shake flask cultures except at feather concentration level of 1&2 % w/v

The effect of feather concentration and incubation time on the release of free amino nitrogen is presented in Fig 2, for static and shake-flask cultures. Amino acid level rose gradually to peak on the 3rd and 4th day of fermentation.

Effect of medium Feather Concentration on Non-protein Nitrogen release

The effects of incubation time, feather substrate concentration and mode of cultivation on the release of non-protein nitrogen by isolate D4 is shown in Fig. 3. More non-protein nitrogen was liberated at higher substrate level for both static and shake flask cultures. Irrespective of the feather substrate level and the mode of cultivation, pattern of non-protein nitrogen release by isolate D4 was biphasic and with 2 peaks each. The total non-protein nitrogen values were generally high in static cultures.

Effect of medium feather concentration and cultivation mode on time course of amino acid production

Effect of Feather Substrate Particle Size on Growth of Isolate D4

Figure 4 shows that, growth in both cultures was biphasic irrespective of the feather particle size. Shake flask cultures with particle size diameters of <90µM show a sharp increase in microbial biomass from the beginning of fermentation with maximum growth after 2 days of cultivation. Growth in static culture followed a similar pattern except that the biomass was less than that observed for shake flask cultures. Overall, optimal growth rate was achieved with the smallest substrate particle size [i.e. <90µM] under shake-flask culture.

FIGURE 1. Effects of medium feather concentration and cultivation mode on isolate D_4 growth

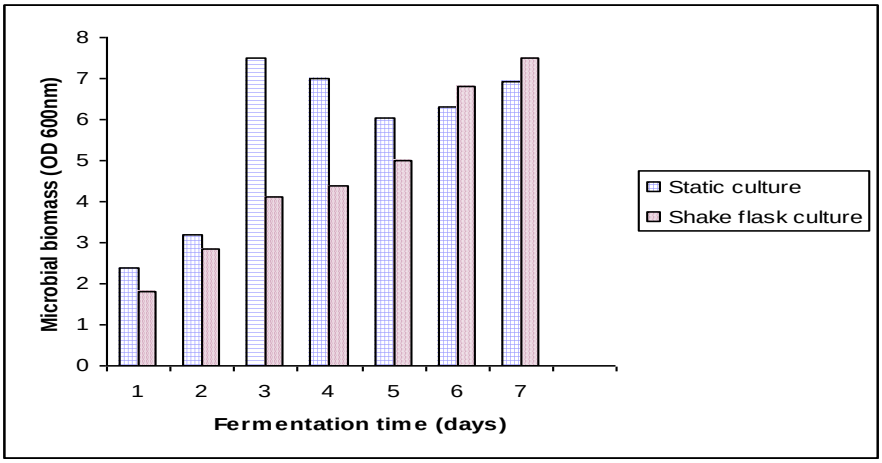


FIGURE 2. Effects of feather concentration and cultivation mode on time course of free amino acid release by isolate D_4.

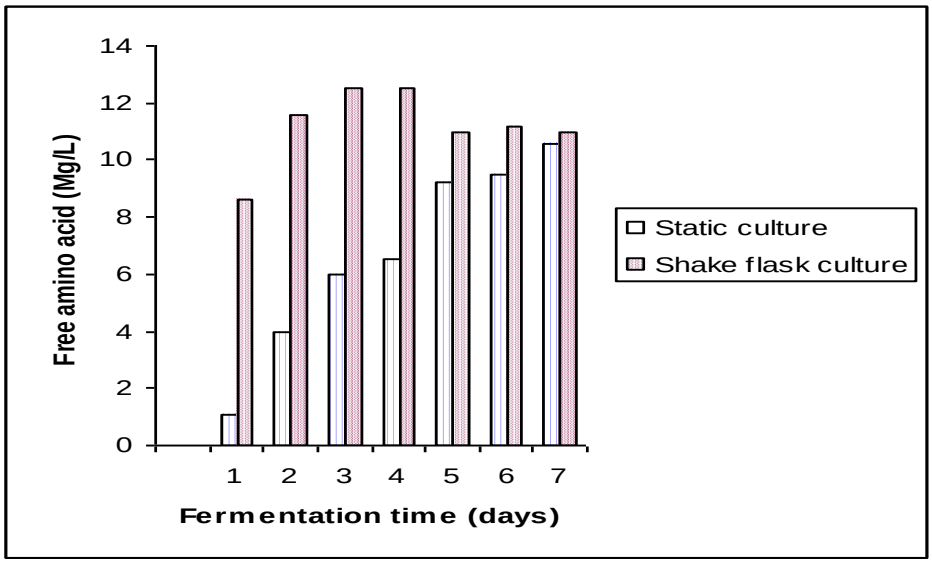


FIGURE 3. Effects of feather concentration and cultivation mode on time course of non – protein nitrogen release by isolate

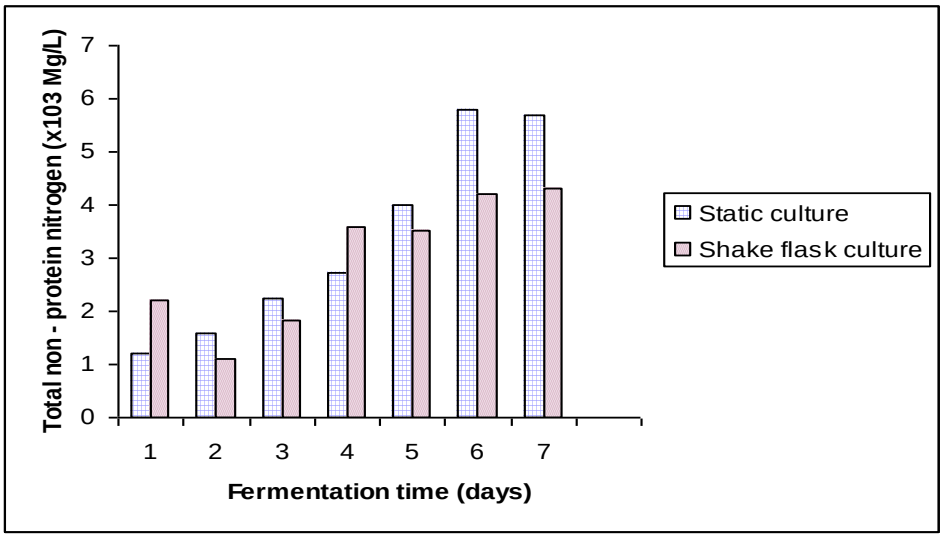


FIGURE 4. Effect of Feather Particle Size and Cultivation Mode on Isolate D_4 growth

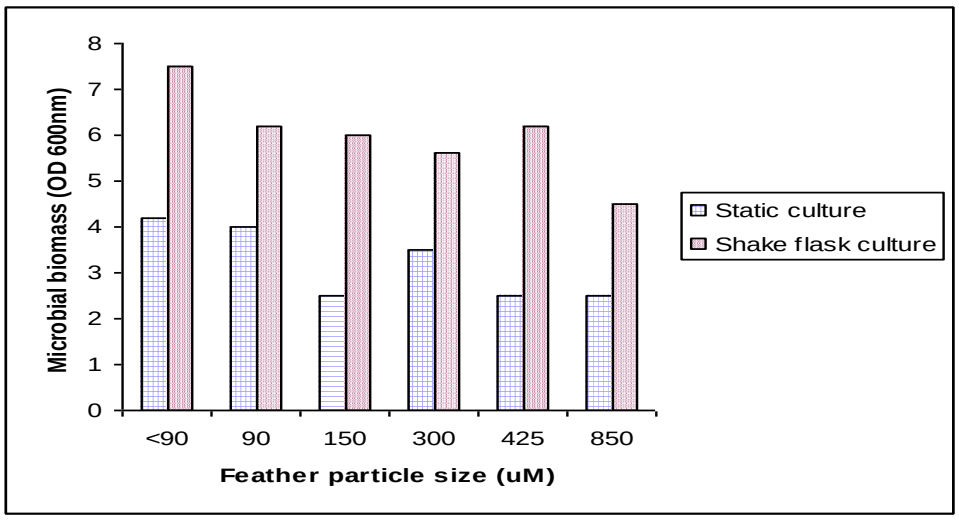


FIGURE 5. Effects of feather particle size diameter on amino acid production by isolate D4

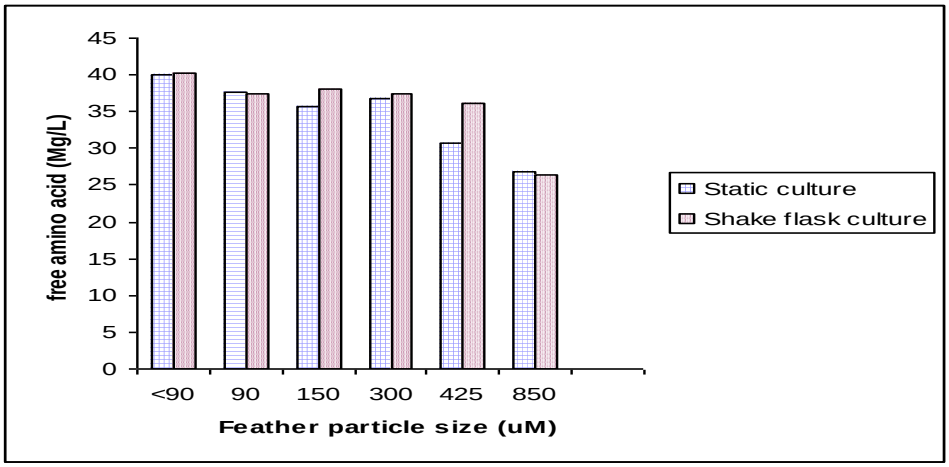


FIGURE 6. Effects of feather particle size on non – protein nitrogen release by isolate D_4

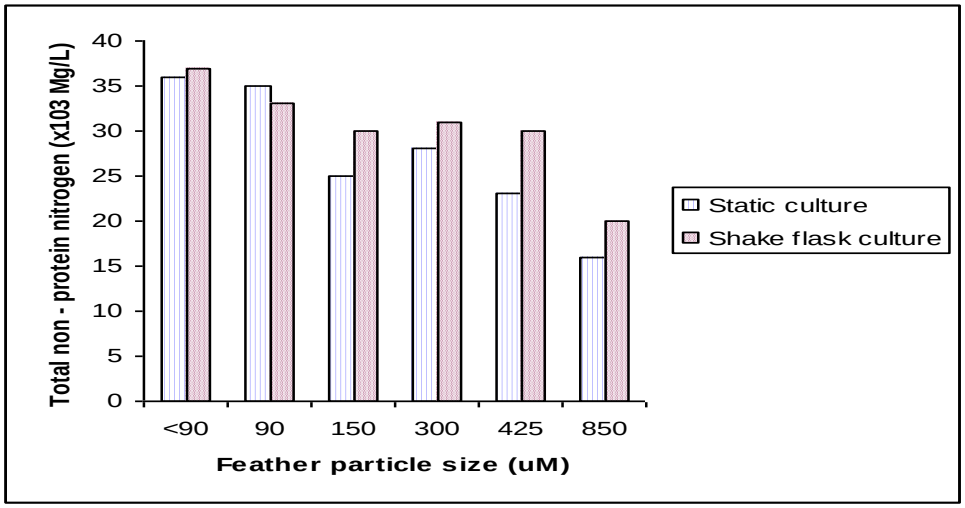
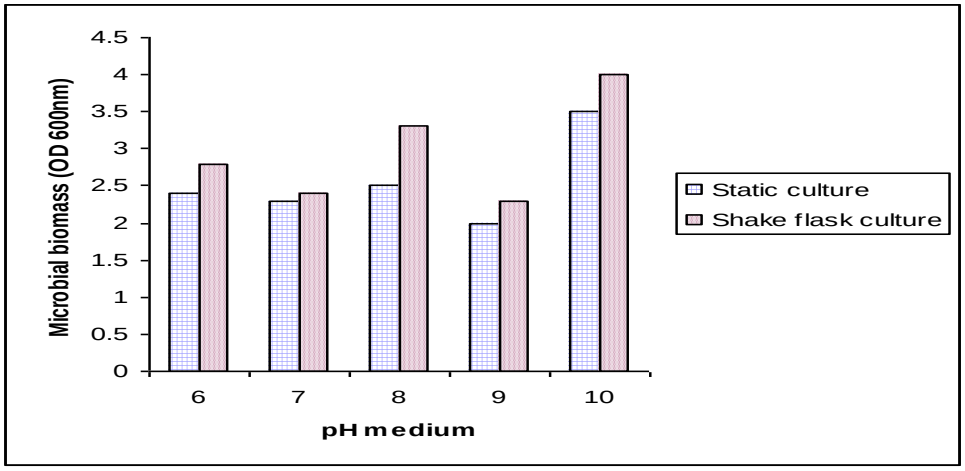


FIGURE 7. Effect of initial medium pH and cultivation mode on isolate D_4



Effect of feather particle size diameter on amino acid production

The effect of feather particle size diameter on amino acid production by isolate D4 is shown in Fig 5, it could be seen that feather particle size of <90µM has the most positive influence on amino acid production, this was because of the faster rate of amino acid release from both cultures. Also, amino acid production and rates of amino acid release with

every particle size were almost the same for both shake flask than static cultures.

Effect of feather particle size diameter on total non-protein nitrogen release

The maximum non-protein nitrogen is shown in Figure 6. Feather particle size affected rates of non-protein nitrogen release. Also non-protein nitrogen release was high for both shake flask and static cultures with maximum NPN level being reached between 5 – 7 days fermentation. Highest NPN release was given by feather substrate particle size of <90μM in shake flask.

Effect of initial medium pH of growth of Isolate D4

Maximum growth or biomass yield is shown in Figure 7. Initial medium pH influenced growth of isolate D_4 on feather. Rates of microbial growth rose as initial medium pH increased from pH 6.0 upwards reaching peak at pH 10.00 for both culture. Shake flask cultures gave more biomass than static cultures irrespective of pH

FIGURE 8. Effect of initial medium pH on free amino acid release by isolate D_4

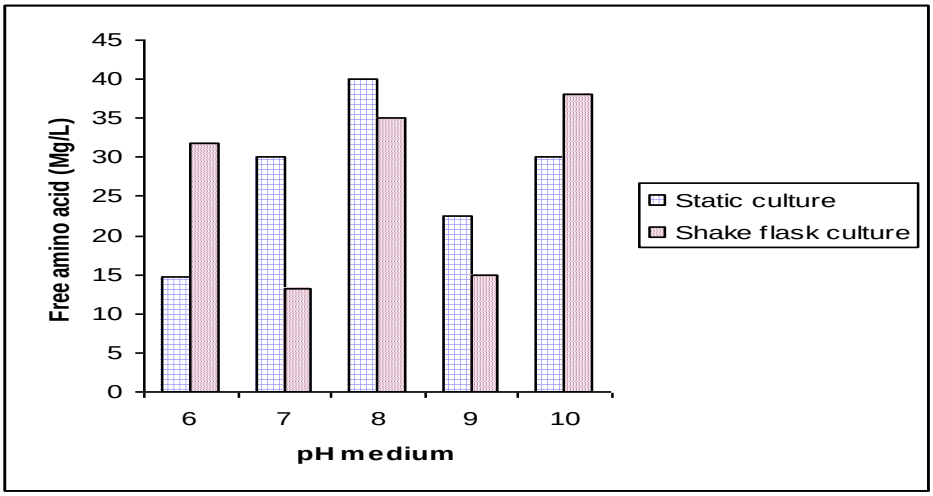
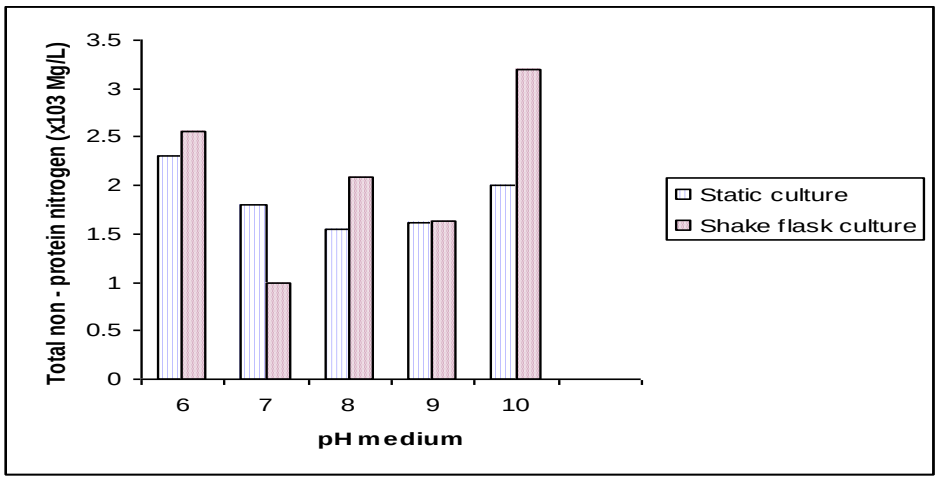


FIGURE 9. Effect of pH medium on total non – protein nitrogen release by isolate D_4



Effect of initial medium pH on amino acid production

Maximum amino acid production for shake flask and static cultures is shown in figure 8. Maximum amino acid production was attained at pH 10.0 (38.1mg/1) for shake flask, while that of static cultures was attained at pH 8.0 (40.0mg/1). In general, rate of release of amino acid were higher in shake flask grown cell than in static cultures.

Effect of Initial Medium pH on Total Non-Protein Nitrogen Production (NPN)

The effects of medium pH on the rate of non-protein nitrogen release by isolate D_4 is presented in figure 9. Then maximum NPN production was achieved at pH 10.0 for shake –flask culture, while maximum NPN production was achieved at pH 6.0 for static culture.

DISCUSSION

Eight (8) of the ten bacterial isolates were found to be capable of hydrolyzing feathers. The isolate that showed the most pronounced clearing zones on ball-mild feather agar plate was further characterized to be *Bacillus* species. The isolate grew optimally and hydrolyzed chicken feather at a thermophilic temperature of 50^0C. Aerobic growth of the isolate on chicken feather yielded appreciable levels of degradation of the untreated feather substrate as measured by the release of free amino acids in the fermentation broth

as well as by the visual observation of the physical – disintegration of the feather substrate. This suggests the possible liberation, within the culture medium, of extra cellular keratinolytic enzymes as has been described by Williams *et al.*, 1990 and Takami *et al*., (1992). Substrate concentration has a profound influence on the growth of and liberation of products by microorganisms. The most efficient production of free amino acids and total non-protein nitrogen in both agitated and non-agitated culture was within a semi-solid fermentation medium of 7 parts feathers substrate to 100 parts liquid culture incubated for two and four days respectively. The high rates of increase in free amino acids and TNPN at increasingly higher substrate level may have been due to faster degradation and keratinous matter probably occasioned by higher contact rates between enzymes and feather substrates (Singh, 1997). Amino acid liberation was slower and lower under static conditions; generally higher levels of TNPN were recorded for agitated cultures. It is possible that growth in agitated cultures, because of higher substrate: enzymes contact rates encouraged a more rapid catabolism of newly released non-free amino acid and non-protein nitrogen.

The particle sizes of substrates have been considered important determinants of microbial productivity in culture (Malathi and Chakraborty, 1991). In this study the finest variety of feather (<90) was the best substrate for isolate D_4 growth with increasingly lower growth velocities and maximum biomass level obtained with substrates of progressively coarser variety. Improvement in growth velocity with the finer feather varieties many be due to an improved digestibility of the feather substrate at the finer particle sizes, a higher availability of readily utilizable growth substrate such as amino acids and small peptides. The improved availability of free amino acid and non-protein nitrogen growth substrates could be due to an enhancement in keratinous substrate hydrolysis probably occasioned by the ever greater overall surface area afforded by contact between the microbial keratinolytic system and feather substrates of increasingly finer quality. The resistance of keratin to proteolytic digestion has been associated with their super-coiled helical structure (Lin *et al.*, 1992; Onifade *et al.*, 1998 and Lin, *et al*., 1996). Ball milling is one of the methods that is employed in the conversion of keratin containing substances such as chicken feather to a more degradable product. The milling process modify the recalcitrant native keratinous structure of feather to a form that is readily attacked and degraded by enzymes which might not be specific for chicken feather e.g. trypsin. Since the milling of the feather substrates involves the degradation of its complex structure, it is assumed that the ever more rigorous mechanical process involved in the preparation of increasingly finer feather substrates types would have resulted in progressively more extensive keratinous structure modification. The bacterial isolate used in this study is essentially aerobic. Williams *et al*. (1990) observed heavier growth of *Bacillus licheniformis PWD-1* under aerobic conditions. It is therefore additionally possible that better aeration under agitated culture conditions could, in part be responsible for the better microbial growth observed throughout this work under shake flask conditions.The pH of microbiological growth media is one of the most important factors that modulate microbial fermentative productivity (E1-shoara *et al.,* 1992). The growth on feather by isolate D_4 was studied in relation to the pH of the liquid fermentation medium. The growth velocities of the isolate were higher at increasingly more alkaline medium pHs except for pHs 7.0 and 9.0. The fall in rates of microbial growth at both pH could be due to lowered enzyme stability under these conditions. This view is supported by data on enzyme pH stability which reveal that enzyme grew increasingly unstable as pH become more alkaline and may also explain the lower free amino acid TNPN levels recorded at that ranges of pH. Growth under static culture condition was also optimal at pH of 10.0. Amino acid release was also influenced by the pH of the fermentation medium. High rate of amino acid and TNPN release observed at pH of 10.0 may have been the results of improved feather substrate digestibility emanating from exposure to the alkaline condition of the fermentation medium. Reports exist of the traditional use of alkaline treatment to bring about vast improvements in feather digestibility for farm animals (Steiner *et al.*, 1983).

CONCLUSION

The results revealed that a thermophilic bacterial isolate from the poultry waste dump was used in the degradation of feather keratin using feathers as a primary source of energy, carbon and sulphur. The isolate was classified as *Bacillus* species.

When the isolate was grown aerobically on feather medium, it yielded appreciable degradation products measured by the release of free amino acids and TNPN in the fermentation medium. The released free amino acids are essential to animal nutrition, suggesting the possible use of biotechnological process based on the isolate in local production of animal nutrition.

There is no significant difference at $p<0.05$ in the cultivation modes in all the processes in exception of "the effect of Feather concentration on the amino acid production" yet no significant difference at $p<0.01$ with this process.

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