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Production of Single Cell Protein with Three Agro-Shell Wastes Using *Bacillus subtilis*

*B.A. Omogbai and E.I. Obazenu

Department of Microbiology, University of Benin, Faculty of Life Sciences, University of Benin, Benin City
*Corresponding author E-mail: barryomogbai@yahoo.com

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ABSTRACT: In this study, *Bacillus subtilis* isolated from decaying pineapple fruit peel was identified by both phenotypic and molecular techniques and employed for the production of single cell protein from three agricultural wastes: melon, groundnut and walnut shells. Chemical analysis showed these agro-wastes to contain high amounts of carbohydrate which ranged from 61.26 - 65.12 %. Using submerged fermentation, maximum yield of cell biomass was produced from the groundnut shell followed by melon shell as substrate for fermentation. Among different nitrogen sources employed, yeast extract was the most suitable nitrogen source for optimum growth of *B. subtilis* at pH 7.0. The result of this study shows that bio-protein can be produced from these agro-wastes instead of discarding them into the environment indiscriminately to cause pollution.

Keywords: Agro-wastes, Fermentation, Inoculum size, Bacteria, Protein

Introduction

The rapid increase in world population has significantly led to shortage in food supply. The increasing demand for protein rich food led to the formulation of alternative protein sources which are particularly single cell proteins (SCP). Production of SCP is an alternative and an innovative way to successfully solve the global food problem (Adedayo *et al.,* 2011). SCP refers to cells or proteins derived from microorganisms such as bacteria, fungi, mold, algae and yeasts (Najafpour, 2007). SCP production technologies arose as a promising way to solve the problem of worldwide protein shortage. They evolved as bioconversion processes which turned low value by-products, often wastes, into products with added nutritional and market value. Microorganism can utilize waste materials that cause pollution problem and also sanitary hazards. The use of wastes would help in controlling pollution and also in solving waste disposal problems to some extent. SCP production has the potential for feeding the ever increasing world population at cheaper rates (Baldasso*et al*., 2011). The major protein sources such as meat are often not affordable by the common man. Therefore microbial proteins can be an alternative source to feed in the world in general. In other words, proteins are important for proper growth and development of all living beings (Adedayo *et al.,* 2011).

Recent research on groundnut shell has found its importance in production of substances such as ethanol and enzymes (tannase and cellulase) (Rashid *et al*., 2011). The shells of peanut contain all kinds of vitamins, crude protein, fibre, and fat. The chemical constituents (%) of groundnut shell are as follows: cellulose (65.7), carbohydrate (21.2), protein (7.3), and minerals (4.5) (Jambunathan, 1991). The main components of groundnut shell are lignocelluloses, however, three key structural polymers constitute it: hemicelluloses (a group of heteropolymers, which comprises of xylans and mannans), lignin (α-complex polyphenolic polymer) and cellulose (α-homopolymer built of D-glucosyl units).

The shell of walnut (*Juglans regia*) is a solid, non-hazardous and biodegradable material which can be employed as abrasive for gun polishing, castings, jewelry and metal object. It is an excellent media to recover crude oil from water (Srinivasan and Viraraghavan, 2008). The shells can be utilized as a carbonaceous sorbent for effective control of mercury from industrial liquid streams (Zabihi*et al.,* 2010). Flavonoids are antioxidant agents in walnut shell and have been evaluated by Akbari *et al.* (2012). Melon shells are the hard covering that are disposed after shelling of melon seeds (*Citrullus vulgaris*). It is a cucurbit crop that belongs to the Cucurbitaceae family (Abiodun and Adeleke, 2010). The crop is cultivated, harvested and processed in commercial quantity in central Nigeria. The shells from these crops may be a source of pollution to the environment. Thus it should be exploited properly as a substrate for the production of edible cellular biomass instead of dumping them as they can be used as a feed supplement for livestock.

Materials and methods

Source of microbial culture: Microbial culture used in all experiments was *Bacillus subtilis* isolated from decaying pineapple fruit peels. The isolate was identified using both phenotypic and molecular techniques.
Source of Agro-shells: Agro-shells used in this study include groundnut, walnut and melon shells respectively. Shells of groundnut were obtained from cottage industries producing fried bottled groundnuts. Melon shells were obtained from cottage industries producing melon seeds for commercial purposes. Walnut was obtained and the nuts were cracked to obtain the shell in the laboratory by aseptic mechanical means by the authors. The samples were air dried for 48 h and ground into powder with a household hand grinding machine (Model: LF80, Germany).
Proximate analysis of agro shells: The moisture content of each agro-waste sample were evaluated by drying to a constant weight (A.O.A.C., 1995). Ash content was obtained by dry ashing in a muffle furnace for 6 h at 600 ^{O}C. Lipid content of the samples were determined by standard chemical procedures with ether using a Soxhlet extractor (A.O.A.C., 1995). The residue obtained from ether extracted was further treated with 1.25 % sulphuric acid and 1.25 % of sodium hydroxide under heating for 30 min. The content was ashed in muffle furnace and re-weighed to obtain the fibre content. The Kjeldahl method was employed for protein determination. The amount of crude protein was calculated by multiplying the % nitrogen found by 6.25. Carbohydrate component was obtained by difference (A.O.A.C., 1995).
Optimization of process parameters: Different media such as sucrose, glucose, maltose, fructose, all mix together, were optimized for different variable parameters such as pH, fermentation period, carbon source and nitrogen source for the bacterium to determine the yield of single cell protein.
Effect of inoculum size: Different concentrations of 1.5 x 10^{8} Mcfarland inoculum sizes of 2, 3, 4, 5 and 6 % (v/v) at pH 6 for a period of 72 h at 120 rpm were used to analyze for the maximum production of single cell biomass of isolate.
Effect of fermentation period: Effect of fermentation period was studied by harvesting samples at 24, 48, 72, 96 and 120 h to check the maximum yield of biomass at the mentioned incubation period
Effect of initial pH of medium: Effect of different initial pH values of 4, 5, 6, 7 and 8 of growth media were checked before sterilization to enhance the maximum production of biomass of isolate.
Effect of different carbon sources: The shells were supplemented with different carbon source such as glucose, sucrose, maltose, fructose and the mixture of the four carbon source (glucose + sucrose + maltose + fructose) each at 0.5 % (w/v) to check for the maximum production of biomass.
Effect of different nitrogen sources: Different nitrogen sources and organic nitrogen sources (urea, peptone, yeast extract powder) and inorganic (ammonium nitrate, ammonium sulphate) were supplemented to the growth media, each at 0.25 % (w/v) to check the maximum production of biomass.
Fermentation: A batch fermenter was used for the production of single cell protein (SCP) from shells of melon, groundnut and walnut. The experimental set-up consisted of the fermenter, the air supply and the digital based data acquisition control system. The fermenter and all accessories were chemically sterilized with 2 % potassium metabisulphate solution and then washed with hot water several times. Each reactor was then filled with 1 L of mineral salt medium (MSM) each containing 20 g melon shell, walnut shell, and groundnut shell as a sole substrate and immediately inoculated with 40 mL of the inoculum. Cell suspensions were prepared from 24 h old cultures in sterile media, introduced aseptically into the 1 L flask containing MSM and the pH of the medium was adjusted to 7.0 for the bacterium before autoclaving. The pH was maintained at 7.0 with the addition of NaOH using digital based pH measurement and control system. The reactor was operated at 2 vvp at mixing speed of 300 rpm. The sample was taken after 48 h for maximum yield of biomass.

Isolation of single cell protein: 10 mL of the fermented media was centrifuged at 6000 rpm for 20 min. Then the supernatant was collected and analyzed. The supernatant obtained was subjected to purification. Ammonium sulphate precipitation or salting out method was used. Ammonium sulphate [40% (w/v)] was added to the cell free supernatant and was stirred for 4 h. The procedure was done at 4 ºC. The precipitate obtained was allowed to stand for 2 h and then collected by centrifugation at 15000 rpm for 20 min. The pellet obtained was dissolved in 2 mL of 2 mM glycine NaOH buffer, pH at 11 and protein was assayed by Lowry's method.

The blue colour developed by the phosphomolybdic phosphorus in the Folinciocalteau reagent by the amino acid tyrosine and the tryptophan present in the protein plus the colour developed by the Biuretic reaction of the protein with the alkaline cupric tartarate were measured by Lowry's method. Several dilutions of 0.3 mg/mL bovine serum albumin (BSA) was prepared in the same buffer having the unknown of 30 - 150 µg/mL. 1.0 mL was added to each dilution of standard, protein-containing unknown, or buffer (for the reference) to 0.90 mL Reagent A (0.1 N NaOH + 0.1N Na_2CO_3) in different test tubes and mixed. The test tubes were incubated for 10 min in a 50 ºC bath, and cooled to room temperature. A measured 0.1 mL Reagent B (0.02 M $CuSO_4.5H_2O$ + 0.052 M $C_4H_4O_6Na_2$) was dispensed into each test tube, mixed and incubated for 10 min at room temperature. This was followed by rapidly adding 3 ml of Reagent C (0.124 M $C_4H_4O_6Na_2.2H_20$) to each test tube, mixed and incubated for 10 min in the 50 ºC bath and cooled to room temperature. 5 mL of sample was added to each test-tube and 5 mL of distilled water was added to the blank test tube. It was thoroughly mixed and allowed to stand for 10 min. The 5 mL of the alkaline cupric tartrate ($CuC_4H_4O_6$) reagent was added to each tube including the blank. It was well mixed and incubated at room temperature in the dark for 30 min to allow for blue colour development. The reading was taken at 660 nm in the colorimeter. Working standard of tyrosine and tryptophan of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 1 mg/L were also prepared into the series of test tubes which were treated in the same way as the samples and run alongside with the samples.

Results

The result of the molecular identification of the culture of *Bacillus subtilis* used in the study with bands at 580 bp for the genus *Bacillus* and 200 bp for the species are shown in Plates 1 and 2. The proximate analysis of the three agro-wastes with respect to their percentage composition are shown in Figure 1. Groundnut shell had the highest crude protein content of 12.14 % followed by melon shell (9.26 %) and walnut shell (3.73 %). Moisture content analysis showed that melon shell was highest with 19.07 % followed by groundnut shell (13.57 %) and walnut shell with the least of 12.50 %. Walnut shell had the highest crude fibre content of 11.22 %, melon shell was next with 7.57 % and groundnut shell had the least of 6.45 %. The result of ether determination indicated that groundnut shell was highest with 1.14 %. Walnut shell had 0.49 % and melon shell had the least with 0.43 %. Crude ash content of walnut shell was highest with 6.50 % while groundnut shell had 2.79 % and melon shell had the least with 1.43 %. The three agro-wastes had high carbohydrate content, however, walnut shell had the highest with 65.12 % compared to groundnut shell (63.52 %) and melon shell (61.26 %).

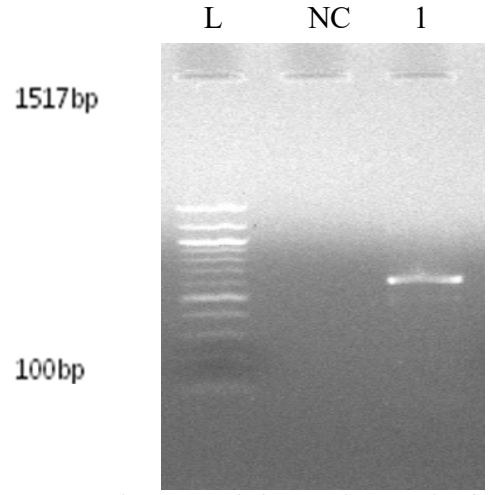

Plate 1: Polymerase chain reaction results for bacterial isolate analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517pb DNA ladder (molecular marker). Sample 1 is positive for *Bacillus genus* with bands at 580bp. NC is a no DNA template control.

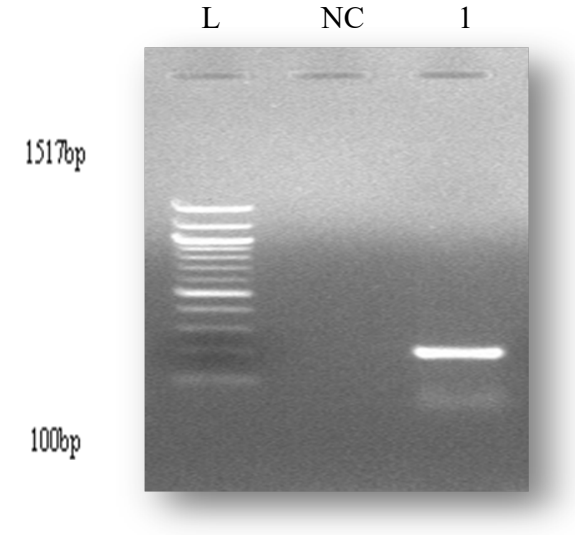

Plate 2: Polymerase chain reaction results for bacterial isolate analyzed with 1.5 % agarose gel electrophoresis stained with ethidium bromide. L is 100 bp-1517 pb DNA ladder (molecular marker). Sample 1 is positive for *Bacillus subtilis* with bands at 200 bp. NC is a no DNA template control.

The effect of initial pH in this study revealed that maximum cell biomass yield was produced at pH 7. The cell yield of 0.0842 mg/L was highest with melon shell as the substrate for fermentation. Next was 0.0772 mg/L cell yield obtained at pH 7 using groundnut shell containing medium (Table 1) while the least cell biomass (0.0156 mg/L) was produced with walnut shell as substrate for fermentation. In general a shift of acidic pH to neutral resulted in increase of the biosynthesis of protein in *Bacillus subtilis* during the fermentation. However as the pH increased to alkaline condition (pH 8-9) there was a decrease in the bio-protein production (Table 1).

Table 2 shows the optimization of inocula sizes for biosynthesis of protein. A gradual increase in inoculum size resulted in more bio-protein production which got to the peak at 4 mL for 1.5×10^8 cells/mL. A Further increase in inoculum size did not yield more biosynthesis. Among various inoculum sizes, 4 mL (v/v) inoculum size gave maximum yield of cell biomass (0.0733 mg/L) with groundnut shell as substrate for fermentation. This was followed

by cell yield of (0.0552 mg/lL) from melon shell containing medium. The least biomass obtained (0.0023 mg/L) was from walnut shell substrate inoculated with 2 mL (v/v) inoculum size as shown in Table 2.

72 h incubation period using groundnut shell as substrate gave maximum cell biomass of 0.1888 mg/Las shown in Table 3. The least cell biomass of (0.0105 mg/L) was produced after 24 h with melon shell containing medium.

Upon supplementation of the three substrates with organic and inorganic nitrogen sources, it was observed that the maximum cell biomass (0.4580 mg/Ll) was produced with yeast extract addition using groundnut shell as substrate for fermentation. This was followed by 0.3670 mg/L cell biomass from groundnut shell as substrate supplemented with ammonium sulphate (Table 4). Ammonium nitrate addition produced the least cell yield (0.0633 mg/L) from walnut shell containing medium.

Table 5 shows the effect of addition of different carbon sources to agro- waste media for protein production. The medium supplemented with glucose had the highest biomass yield of (0.3916 mg/L) after inoculating groundnut shell containing medium. The incorporation of all the carbon sources (glucose, maltose, sucrose and fructose) gave the next maximum biomass of (0.3830 mg/L after fermentation of the medium containing groundnut shell as substrate for fermentation. The least cell yield of (0.0800 mg/L) was obtained from melon shell containing medium that was supplemented with maltose.

Fig 1: Proximate composition of agro-wastes of groundnut, walnut and melon shells

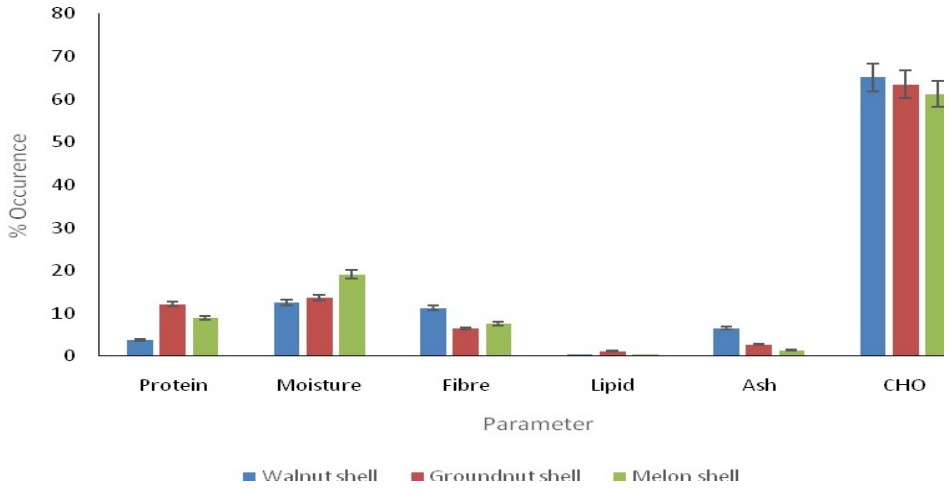

Table 1: Optimization of pH for the biosynthesis of single cell protein from walnut, groundnut and melon shells

pH	*Bacillus subtilis*			*p*-value
	Walnut shell (mg/l)	Groundnut shell (mg/l)	Melon shell (mg/l)	
5	0.0156±0.006^{A}	0.0254±0.040^{B}	0.0285±0.001^{B}	0.000
6	0.0418±0.007^{A}	0.0684±0.026^{B}	0.0656±0.022^{B}	0.000
7	0.0484±0.003^{A}	0.0772±0.014^{C}	0.0842±0.019^{D}	0.000
8	0.0484±0.006^{A}	0.0556±0.020^{B}	0.0721±0.008^{C}	0.000
9	0.0484±0.009^{A}	0.0242±0.006^{AB}	0.0320±0.002^{ABC}	0.008

Values are means of triplicates ± standard deviations (SD). Means with different alphabets are significantly different from each other across each row.

Table 2: Optimization of inoculum sizes for the biosynthesis of single cell protein from walnut, groundnut and melon shell

Inoculum size ($1.5x10^8$) McFarland (mL)	***Bacillus subtilis***			***p*-value**
	Walnut shell (mg/L)	**Groundnut shell (mg/L)**	**Melon shell (mg/L)**	
2	0.0023 ± 0.005^{A}	0.0034 ± 0.002^{AB}	0.0026 ± 0.002^{A}	0.184
3	0.0255 ± 0.127^{A}	0.0516 ± 0.014^{C}	0.0492 ± 0.004^{C}	0.000
4	0.0349 ± 0.004^{A}	0.0733 ± 0.019^{C}	0.0552 ± 0.016^{C}	0.000
5	0.0181 ± 0.001^{A}	0.0366 ± 0.019^{B}	0.0532 ± 0.002^{B}	0.001
6	0.0168 ± 0.007^{A}	0.0347 ± 0.006^{B}	0.0512 ± 0.002^{B}	0.000

Values are means of triplicates ± standard deviations (SD). Means with different alphabets are significantly different from each other across each row.

Table 3: Optimization of incubation period for the biosynthesis of single cell protein from walnut, groundnut and melon shell

Incubation period (h)	***Bacillus subtilis***			***p*-value**
	Walnut shell (mg/l)	**Groundnut shell (mg/l)**	**Melon shell (mg/l)**	
24	0.0245 ± 0.152^{A}	0.0170 ± 0.060^{A}	0.0105 ± 0.007^{A}	0.635
48	0.1029 ± 0.015^{B}	0.1748 ± 0.047^{E}	0.1468 ± 0.030^{D}	0.000
72	0.1088 ± 0.044^{B}	0.1888 ± 0.019^{E}	0.1576 ± 0.019^{D}	0.000
96	0.0512 ± 0.049^{AB}	0.1012 ± 0.015^{D}	0.0846 ± 0.035^{C}	0.000
120	0.0512 ± 0.017^{A}	0.0986 ± 0.100^{D}	0.090 ± 0.051^{CD}	0.000

Values are means of triplicates ± standard deviations (SD). Means with different alphabets are significantly different from each other across each row.

Table 4: Optimization of nitrogen sources for the biosynthesis of single cell protein from walnut, groundnut and melon shell

Nitrogen source	***Bacillus subtilis***			***p*-value**
	Walnut shell (mg/l)	**Groundnut shell (mg/l)**	**Melon shell (mg/l)**	
Yeast extract	0.2156 ± 0.005^{A}	0.4580 ± 0.035^{D}	0.3530 ± 0.038^{C}	0.000
Peptone	0.0703 ± 0.026^{A}	0.1166 ± 0.002^{A}	0.2716 ± 0.172^{B}	0.025
Urea	0.0900 ± 0.002^{A}	0.2120 ± 0.003^{C}	0.1346 ± 0.005^{B}	0.000
Ammonium nitrate	0.0633 ± 0.006^{A}	0.1470 ± 0.002^{C}	0.0923 ± 0.001^{B}	0.000
Ammonium sulphate	0.1680 ± 0.006^{A}	0.3670 ± 0.020^{C}	0.2503 ± 0.007^{B}	0.000
Control	0.0418 ± 0.007^{A}	0.0684 ± 0.026^{B}	0.0655 ± 0.22^{B}	0.000

Values are means of triplicates ± standard deviations (SD). Means with different alphabets are significantly different from each other across each row.

Table 5: Optimization of carbon sources for the biosynthesis of single cell protein from walnut, groundnut and melon shell

Carbon source (0.5%)	*Bacillus subtilis.*			*p*-value
	Walnut shell (mg/l)	Groundnut shell (mg/l)	Melon shell (mg/l)	
Glucose	0.2730 ± 0.036^{B}	0.3916 ± 0.020^{D}	0.3866 ± 0.004^{D}	0.000
Maltose	0.0843 ± 0.010^{A}	0.1250 ± 0.007^{AB}	0.0800 ± 0.001^{A}	0.006
Sucrose	0.1303 ± 0.001^{A}	0.2410 ± 0.060^{BC}	0.1420 ± 0.002^{A}	0.002
Fructose	0.0903 ± 0.011^{A}	0.1423 ± 0.004^{B}	0.1173 ± 0.029^{B}	0.000
G+M+S+F	0.2220 ± 0.015^{A}	0.3830 ± 0.006^{C}	0.2670 ± 0.030^{C}	0.000
Control	0.0418 ± 0.007^{A}	0.0684 ± 0.026^{B}	0.0655 ± 0.22^{B}	0.000

Values are means of triplicates ± standard deviations (SD). Means with different alphabets are significantly different from each other across each row.

Discussion

Proper optimization of culture conditions at laboratory scale play an important role in improving yield as well as reduction in production cost at commercial scale of bio-protein production. The groundnut, walnut and melon shells used as substrates for the production of single cell protein were rich in carbohydrate content providing the initial carbon source for the metabolism of *Bacillus subtilis* employed for bio-protein production. A large scale production of single cell protein for human and animal feed relies on cheap substrates with carbon source (Ezekiel *et al*., 2012).

The effect of pH of the growth media is very crucial for maximum biomass yield. Optimum pH for maximum biomass yield was found at pH 7 using melon shell with a value of 0.0842 mg/L. This result is corroborated by Halasz and Radomie (1991) who reported that the yield of yeast biomass increased by increasing the pH from 4 to 7. Rajoka *et al*. (2005) produced maximum biomass of *Candida utilis* at pH 6.0. Rosma and Ooi (2006) reported maximum yield of *Candida utilis* at pH 4.5. However, Adoki, (2007) reported that *Candida* spp was capable of growth over a wide pH range of 3.0 to 6.2 of growth media.

Among various inoculum sizes, 4 mL (v/v) inoculum size (v/v) gave maximum yield of biomass (0.0733 mg/L) with groundnut shell as the substrate for fermentation. The least yield of biomass (0.0023 mg/L) was obtained from walnut shell substrate at 2 mL (v/v) inoculum size. The decrease in growth can be attributed to limiting nutrients and oxygen, arising from their exhaustion of nutrients and oxygen (Kays and Vanderzant, 1980). However, different workers obtained maximum biomass production with different inoculum size. Rajoka *et al.* (2005) reported maximum yield of *Candida utilis* biomass with 10% (v/v) inoculum size on rice bran and Ravinder *et al.* (2003) obtained maximum *Aspergillus oryzae* biomass with 3% (v/v) inoculum size on deoiled rice bran.

Maximum production of biomass (0.1888 mg/L) was found after 72 h of incubation using groundnut shell as substrate for fermentation whereas the least biomass was (0.0105 mg/L) using melon shell as substrate. This result is supported by several researchers who studied single cell production with a cursory look at the optimum incubation period. Ravinder *et al*., (2003) studied the production of mutant *Aspergillus oryzae* SCP from deoiled rice bran and obtained maximum SCP after 3 days. Lubna *et al*. (2004) observed maximum cell biomass of *Aspergillus niger* after 120 h of incubation. These slight variations among all these findings might be due to difference in microorganisms as well as medium composition.

Following the investigation of the effect of different nitrogen sources on the three substrates utilized, it was observed that maximum cell biomass (0.4580 mg/L) was obtained with yeast extract (organic nitrogen source) using groundnut shell containing medium. This was followed by (0.3530 ml/L) with yeast extract using melon shell as substrate while the least cell biomass was (0.0633 mg/L) using ammonium nitrate with walnut shell as substrate for fermentation. This result is supported by Rajota *et al.,* 2004 who used organic nitrogen source for the production of single cell protein by using *Candida utilis*. These findings indicated that groundnut shell used as a substrate gave more yield.

The addition of all the carbon sources gave the maximum biomass yield of (0.383 mg/L) after fermentation using groundnut shell as substrate. The least biomass yield (0.0800 mg/L) was produced with maltose, using melon shell as the substrate. Glucose also produced the highest appreciable biomass yield of (0.3916 mg/L) with groundnut shell. Thus, utilization of glucose reported by Adoki (2007) on orange, plantain and banana processing residues corroborates results of the present study.

Conclusion

Results of the study show that higher yield of single cell biomass of *Bacillus subtilis* can be produced from groundnut shell supplemented with organic nitrogen source such as yeast extract at 0.4% concentration. Other parameters like incubation period, initial pH, incubation temperature and inoculum sizes have significant effect on the production of single cell biomass of *Bacillus subtilis*. By producing single cell protein from agro-waste like groundnut shell, environmental pollution resulting from their indiscriminate discharge will not only be minimized, feed derived from it will lead to economic boost when used for farm animals.

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