Journal of Applied & Environmental Microbiology, 2020, Vol. 8, No. 2, 39-45
Available online at http://pubs.sciepub.com/jaem/8/2/1
Published by Science and Education Publishing
DOI:10.12691/jaem-8-2-1

Stimulation of Lysine Accumulation in the Broth Culture of *Bacillus* Species Isolated from Nigerian Fermented Food Condiments Using Agro-products

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Received September 10, 2020; Revised October 11, 2020; Accepted October 20, 2020

Abstract Fermented food condiments serve as taste enhancers in African diets and these products are broken down during fermentation by microorganisms to proteineous substances, releasing free amino acids like lysine. Lysine, an essential amino acid added to animals feed, is produced by *Bacillus* species. This study was undertaken to evaluate the stimulatory effects of agro-products on lysine production by *Bacillus* species isolated from fermented food condiments. The effects of synthetics carbon sources: Sucrose, maltose, galactose, glucose, lactose, fructose, and nitrogen sources: KNO_3, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$ on lysine production by *Bacillus subtilis* and *Bacillus* licheniformis were examined. The influence of non-synthetic carbon sources: cassava, cocoyam, yam, plantain, millet, corn, potato, rice and nitrogen sources: soybean, cotton seed, cowpea, bambara nut, groundnut on lysine accumulation by the *Bacillus* species, were studied. Medium containing carbon and nitrogen sources (2:1), was inoculated with the *Bacillus* sp and incubated for 72h on a shaker at 160rpm and 30°C. Lysine was determined from the broth culture. The *Bacillus* species produced lysine levels above 0.6mg/ml in all the synthetic medium except lactose and galactose medium of *Bacillus licheniformis*. A maximum yield of 1.0 mg/ml was accumulated by *Bacillus licheniformis* in a medium of fructose and $(NH_4)_2SO_4$. The use of non-synthetic carbon and nitrogen sources stimulated lysine yields by the *Bacillus* species. Lysine accumulations of 3.63mg/ml and 3.73mg/ml were observed in culture broths of *Bacillus subtilis* and *B. licheniformis* respectively, containing rice hydrolysates and defatted groundnut meals. The stimulatory effect of agro-products on lysine production by the *Bacillus* species has proved that they are good substrates for use in the fermentation industry.

Keywords: *agro-products, fermentation, Bacillus, lysine, condiments*

Cite This Article: Madukwe Ebelechukwu Judith, Mbah Anthonia Nkiruka, Ekwealor Chito Clare, Ajogwu Tochukwu Maximilian Cajetan, and Ekwealor Ikechukwu Amechi, "Stimulation of Lysine Accumulation in the Broth Culture of *Bacillus* Species Isolated from Nigerian Fermented Food Condiments Using Agro-products." *Journal of Applied & Environmental Microbiology*, vol. 8, no. 2 (2020): 39-45. doi: 10.12691/jaem-8-2-1.

1. Introduction

Fermented food condiments play very important role in the diet of many Africans. They are used to enhance the flavor of many dishes including soups and sauces, to improve nutrient values of foods [1,2] and are known to be good sources of proteins and vitamins [3]. The substrates for the fermentation of these condiments, which include African locust bean, Fluted pumpkin, Castor oil seeds, African bread fruit, African oil bean seed and Soybean, are known to harbor diverse microorganisms from the environment [4,5].

Studies on the microbiology of the fermentation of African food condiments have identified *Bacillus* species as the main microorganism responsible for fermentation [5,6,7,8]. The biochemical changes during the fermentation have shown that proteo-lysis is the main activity leading to a pronounced increase of free amino acids such as lysine [9]

Lysine is an essential amino acid added as supplements for animal feed [10]. L-Lysine is usually recognized as the primary limiting amino acids in various grains and is produced mainly by submerged fermentation. It represents around 80% of world market, and in 2015, the world market for L-lysine was around 2.2 million tons per year [11]. The major costs involved in L-lysine production are due to raw materials [12]. Preliminary investigation has shown that lysine can be produced by microbiological process using available raw materials such as agro-products [13]. The bioconversion of the carbohydrate and protein from agricultural products or by-products into lysine may increase the economics importance.

This study was, therefore, conducted to evaluate the effects of agro-products on lysine production by *Bacillus* species isolated from Nigerian fermented food condiments.

2. Materials and Methods

2.1. Microorganisms

Two strains of *Bacillus* species, namely *Bacillus subtilis* and *Bacillus licheniformis*, recovered from Nigerian fermented food condiments, okpeye *(Prosopsis africana*), and dawadawa (*Parkia biglobosa*), African locust bean respectively were used for lysine production. They were maintained on Nutrient agar slants at 4°C.

2.2. Seed Inoculum

The seed medium consists of peptone, 10.0g;yeast extract, 10.0g; NaCl, 5.0g; H_2O, 1L; pH 7.2 adjusted with 1N NaOH. Two loopfuls of a 24h culture of the *Bacillus* sp was used to inoculate 5ml of the seed medium in a test tube and incubated on a Water bathing Constant Temperature Vibrator (SHA-C shaker, B Bran Sci. Comp. UK) at 120rpm for 18h and 30°C.

2.3. Fermentation Experiment

Basal medium for fermentation was composed of KH_2PO_4, 1.0g; $MgSO_4.7H_2O$, 0.4g; $MnSO_4.H_2O$, 2.0mg; $FeSO_4.7H_2O$, 2.0g; $CaCO_3$, 50.0g; carbon source, 20.0g; nitrogen source, 10.0g; H_2O, 1L; pH adjusted to 7.2, and the medium sterilized at 121°C for 15min. A 100ml Erlenmeyer flask containing 30ml of the fermentation medium was inoculated with 3ml (Ca. $3.2x10^9$ cells/ml) of the seed inoculum and the flask incubated for 72h on a Stuart orbital incubator (Bibby Sterilin Ltd, UK) at 160rpm and 30°C. The flasks were prepared in triplicate and uninoculated flasks served as control. Lysine accumulation was determined from the broth culture.

2.4. Lysine Accumulation

Lysine in the broth culture was determined following the acidic ninhydrin method [14].

2.5. Carbon Sources

The synthetic carbon sources used for fermentation include, sucrose, maltose, galactose, glucose, lactose, fructose. Agricultural products (non-synthetic) used include, cassava (*Manihot esculenta*), cocoyam *Colocasia esculenta*), yam (*Dioscorea rotundata*), plantain (*Musa sapientum*), proso millet (*Panicum miliaceum*), corn (*Zea mays*), potato (*Ipomoea batatas*), rice (*Oryza sativa*), which served as natural starches.

2.5.1. Preparation of Natural Starch

The following process was used for starch preparation [15]. Cassava, cocoyam, yam, potato, and plantain samples were peeled and reduced to pulp using a hand grater. The pulp and the grains (millet, rice, corn, soaked for 48h in water) were separately homogenized in a Warring blender. Each homogenate, mixed with excess water (1g/100ml), was placed inside a bag of fine white cloth, properly tied up and suspended on a tripod stand placed inside a clean glass vat, to allow for the leaching out of the starch suspension. The crude starch sediment in the vat was collected by decanting the supernatant. The starch was dried in an oven at 50°C for 48h and the resultant flakes grinded into fine powder and used as natural or native starches.

2.5.2. Saccharification of the Starch

The starch was saccharified following a modified method [16]. A-250ml Elenmeyer flask containing 30.0g of the native starch in 100ml of water was heated for 15min at 95°C in a water bath, to gelatinize the starch. To the starch slurry was added 0.4ml thermostable α-amylase (Termamyl 120, Novo industries, Bagsvared, Denmark) produced from a strain of *Bacillus licheniformis.* The slurry was adjusted to a pH of 6.5, and 50ppm $CaCl_2$ added before heating the flask in a water bath at 90°C for 2h. The liquefied starch was cooled to 60°C and the pH adjusted to 4.5 with IN HCl. For saccharification, 0.5ml of fungal glucoamylase (AME 300, Novo industries, which has activity of 300AUG/ml) was added to the flask and heated for 20h at 60°C. The reaction was stopped by heating the flask to 90°C for 10min and the saccharified starch filtered using Whatman No1 filter paper. The starch hydrolysate was oven-dried at 45°C

2.6. Nitrogen Sources

Synthetic nitrogen sources used are Potassium nitrate (KNO_3), Ammonium nitrate (NH_4NO_3), Ammonium chloride (NH_4Cl) and Ammonium sulphate ($(NH_4)_2SO_4$), while the agro-products (natural proteins) which served as non-synthetic nitrogen sources include Soybean (*Glycine max)*, Cotton seed (*Gossypium sp)*, Cowpea (*Vigna sp)*, bambara nut (*Vigna subterranean*) and groundnut (*Arachis hypogaea)*

2.6.1. Preparation of Natural Protein

The natural proteins were milled in a Moulinex blender into fine powders. Some fractions of the milled proteins were defatted by Soxhlet extraction method using the solvent diethyl ether. The meals obtained after extraction were dried at 35°C for 20h and then grinded into fine powder.

2.7. Effects of Carbon and Nitrogen Sources on Lysine Production by the *Bacillus* species

The effects of synthetic carbon and nitrogen sources on lysine accumulation in the culture broths of *Bacillus subtilis* and *Bacillus licheniformis* were examined. The basal fermentation medium containing carbon and nitrogen sources in the ratio of 2:1 respectively, was inoculated with the *Bacillus* sp. The fermentation process and lysine determination was carried out as previously described.

The lysine accumulation by the *Bacillus* species using agro-products (non-synthetic) as carbon and nitrogen

sources in the fermentation broth was similarly treated as previously described.

3. Results

The effects of synthetic carbon and nitrogen sources on lysine production by the *Bacillus* species are as presented in Figure 1a, b. Lysine yields above 0.6mg/ml were accumulated in the culture broths of *Bacillus subtilis* with various carbon sources and NH_4Cl (Figure 1a). Similar effects were observed with *Bacillus licheniformis* (Figure 1b), although other carbon and nitrogen sources produced lysine levels above 0.60mg/ml. Maximum lysine concentration of 1.0mg/ml was accumulated by *Bacillus licheniformis* when fructose and $(NH_4)_2SO_4$ were the substrates in the fermentation medium.

Lysine accumulation by the *Bacillus* species in a fermentation medium containing starch hydrolysates with non-defatted proteins (Figure 2a,b) and starch hydrolysates with defatted proteins (Figure 3a, b), as carbon and nitrogen sources respectively, showed improved lysine yields when compared with the synthetic substrates. Lysine level of 2.60mg was observed in the culture broth of *Bacillus subtilis* prepared with rice hydrolysate and non-defatted soybean (Figure 2a), while *Bacillus licheniformis* accumulated 2.84mg/ml lysine in a fermentation medium having millet hydrolysates and non-defatted bambara-nut (Figure 2b) as the substrates.

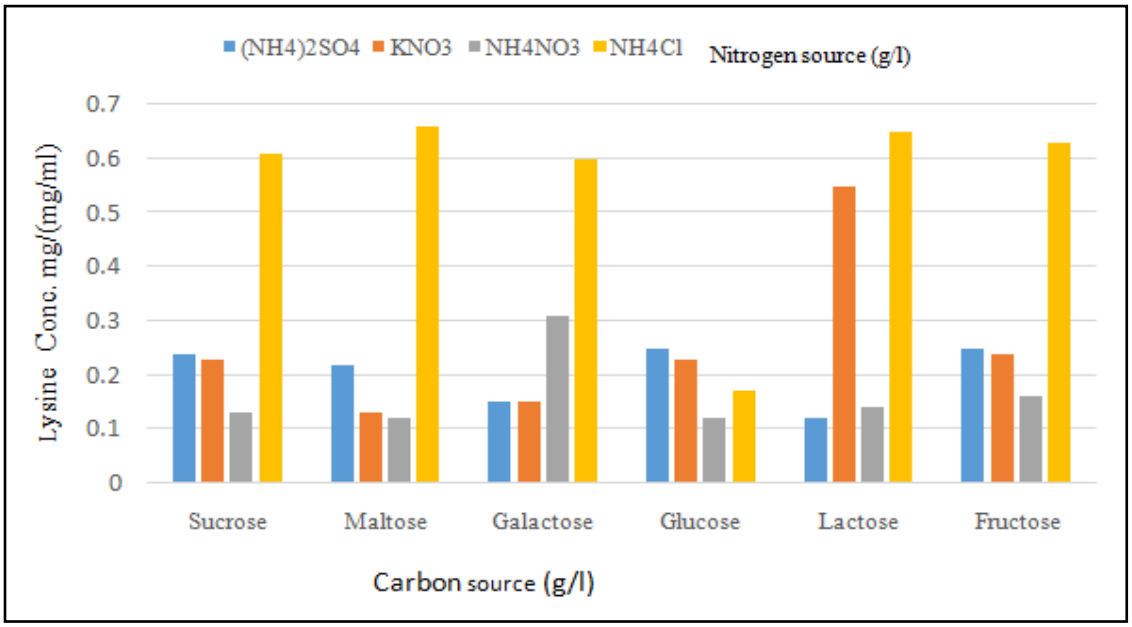

Figure 1a. Effects of synthetic carbon and nitrogen sources on lysine production by *Bacillus subtilis*

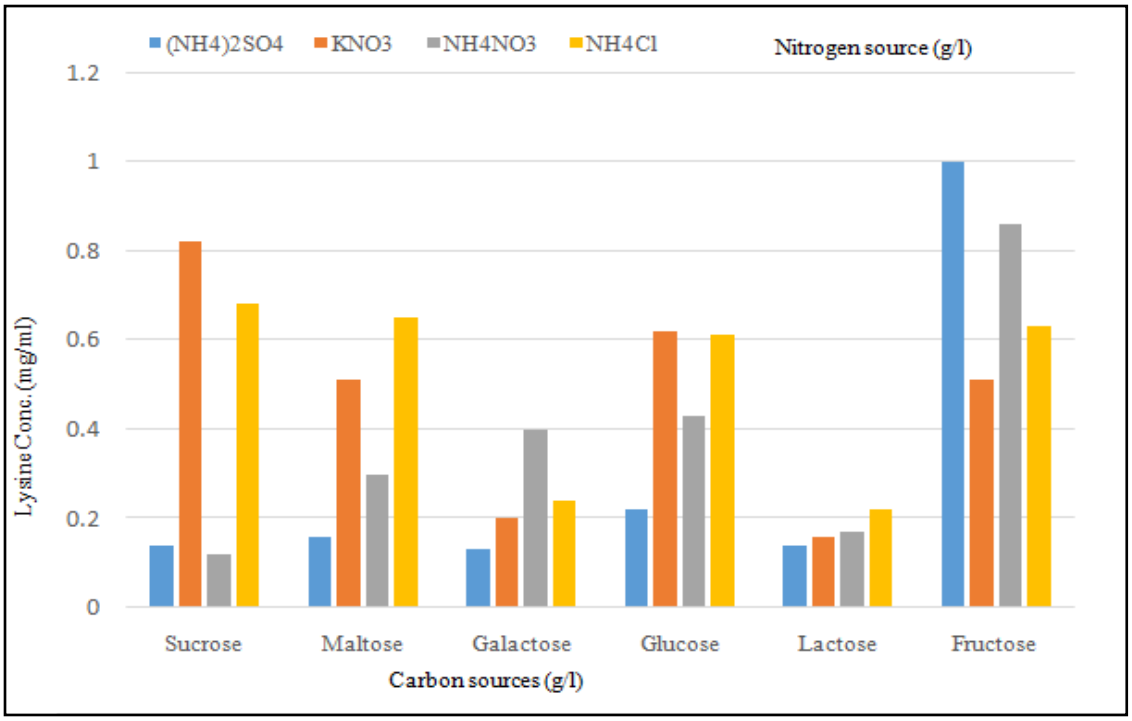

Figure 1b. Effects of synthetic carbon and nitrogen sources on lysine production by *Bacillus licheniformis*

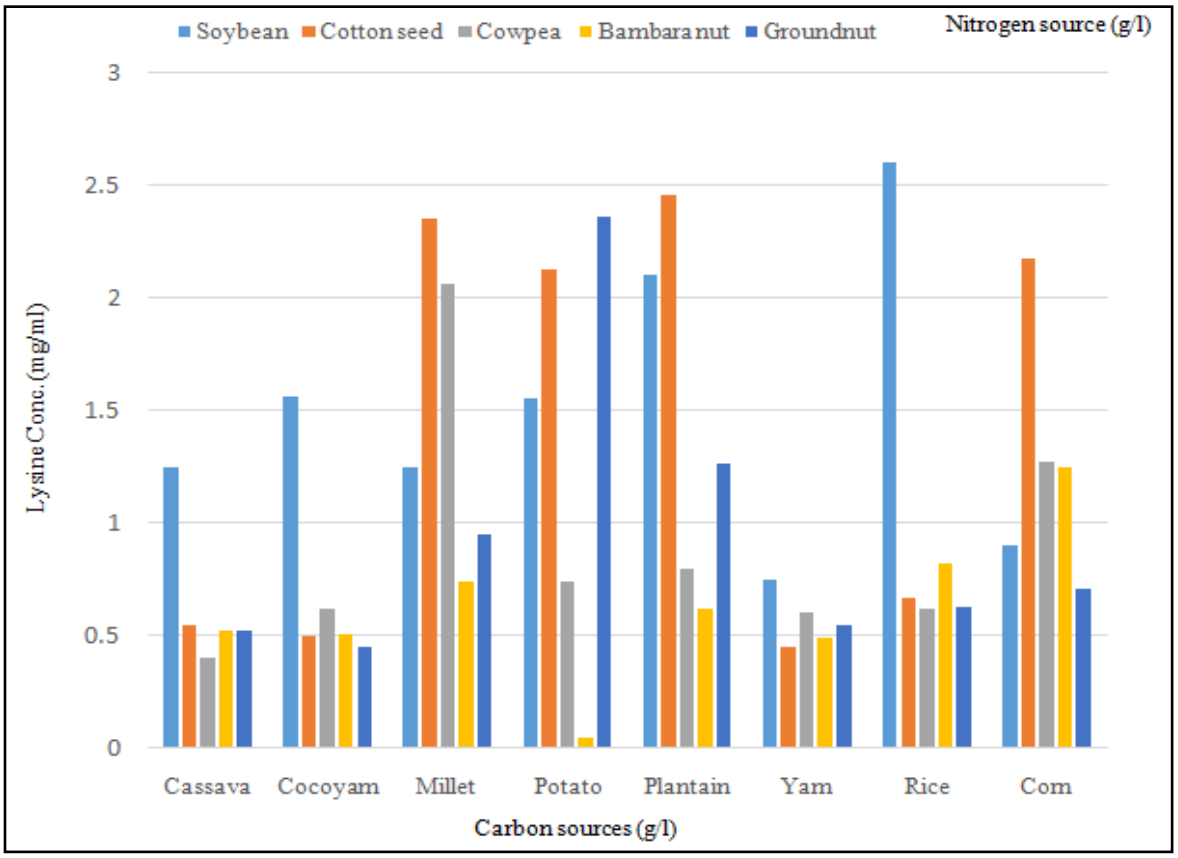

Figure 2a. Effects of starch hydrolysates and non-defatted seed meals on lysine accumulation by *Bacillus subtilis*

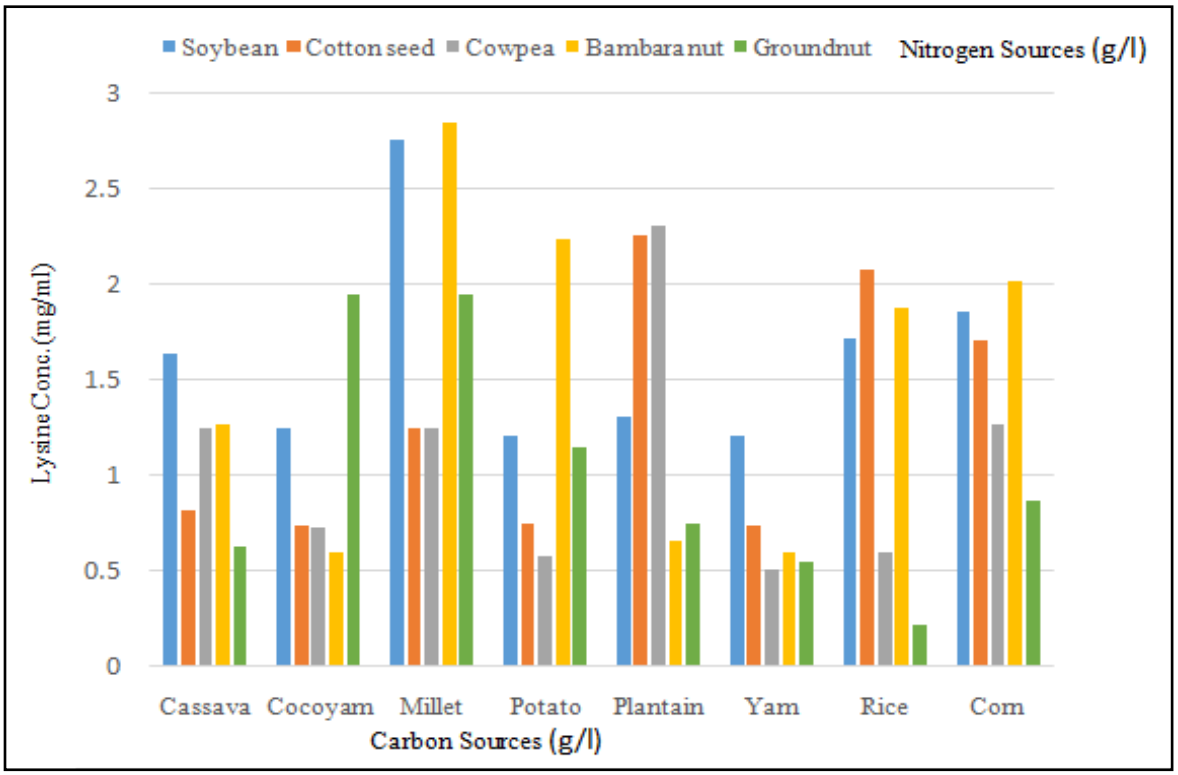

Figure 2b. Effects of starch hydrolysates and non-deffated seed meals on lysine accumulation by *Bacillus licheniformis*

Figure 3a and Figure 3b, showed enhanced lysine yields in the culture broths of the *Bacillus* species prepared with starch hydrolysates and defatted proteins. Lysine levels of 3.63mg/ml and 3.73mg/ml were produced by *Bacillus subtilis* and *Bacillus licheniformis* respectively in the fermentation medium containing rice hydrolysate and defatted groundnut meal as carbon and nitrogen sources (Figure 3a, b).

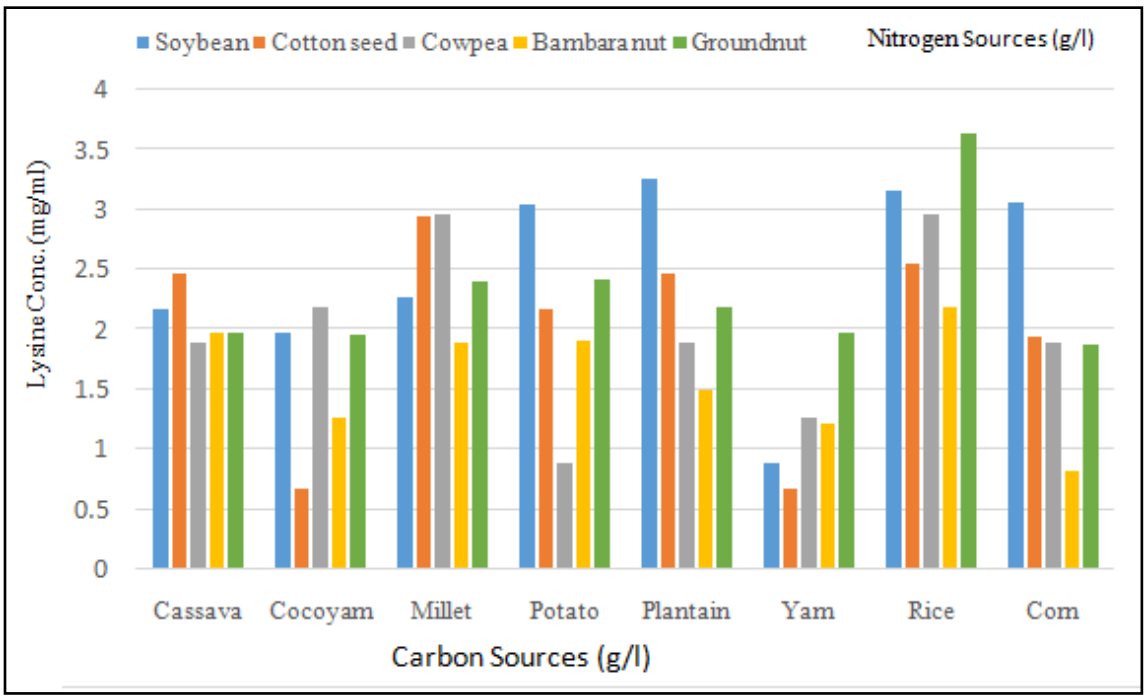

Figure 3a. Effects of starch hydrolysates and defatted seed meals on lysine accumulation by *Bacillus subtilis*

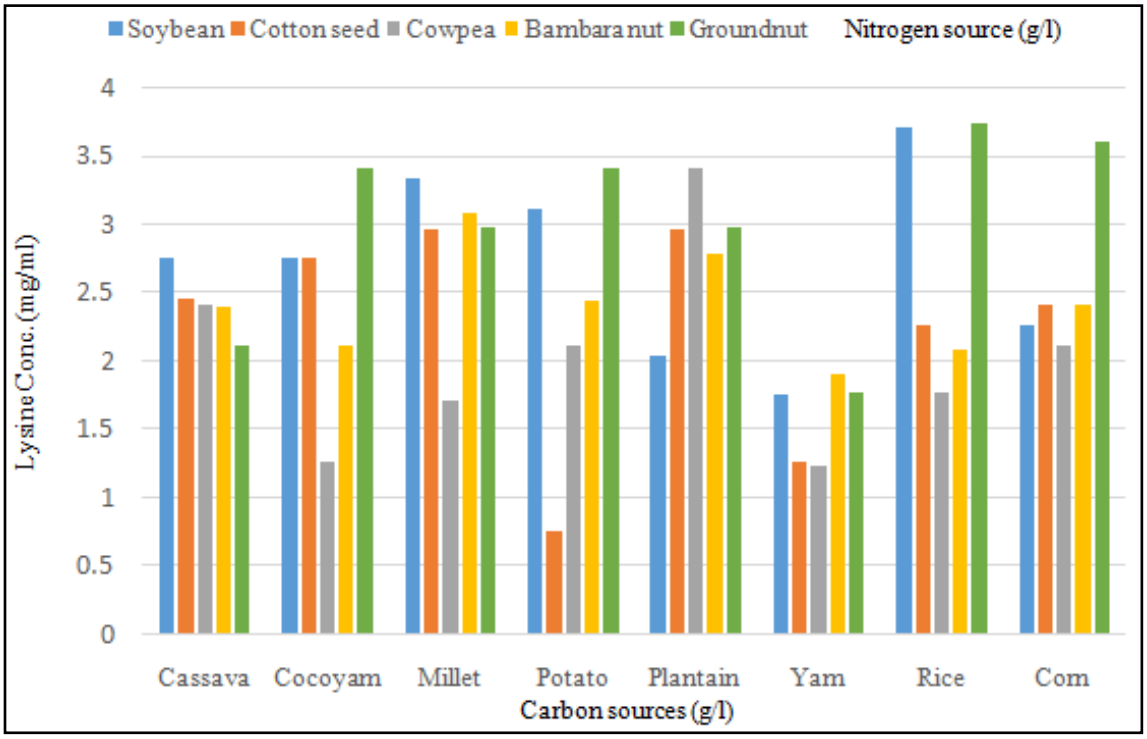

Figure 3b. Effects of starch hydrolysates and defatted seed meals on lysine accumulation by *Bacillus licheniformis*

4. Discussion

Fermented food products are commonly considered as condiments, and are used as taste enhancers in traditional African dishes. Natural and spontaneous fermentation usually occur during the preparation of various traditional fermentation products, which leads to occurrence of mixed microbial population and differences in product quality [5,17].

Many researchers have shown that *Bacillus* species are the main microorganism responsible for fermentation, indeed they are regarded as the dominant bacterial workhorses in microbial fermentation [18,19]. More so, members of *Bacillus* and related genera are also known for the synthesis of a wide range of medical, agricultural, pharmaceutical and industrial products [20,21].

The use of *Bacillus* species, which is an emerging organism in the production of lysine, has been reported by many workers [13,16,22,23], although their lysine yields are not as high as those observed in *Corynebacterium* and *Brevibacterium* species [24] The low lysine yield from *Bacillus* species is not unexpected because not much work has been directed towards strain improvement or optimization of the fermentation parameters.

This study shows that *Bacillus subtilis* and *Bacillus licheniformis* isolated from Nigerian fermented food condiments were capable of accumulating lysine in a fermentation medium containing synthetic and non-synthetic carbon and nitrogen sources. More so, it is an indication that *Bacillus* species for lysine production can be isolated from various sources of nature.

Factors like carbon, nitrogen sources and their concentrations have always been of great interest to researchers in the industry, for the low cost media design. However, investigation of the impact of carbon and nitrogen supplements has revealed that not all carbon and nitrogen sources act as enhancers for the production of microbial metabolites [25,26,27].

As observed in Figure 1a, b, the lysine yields produced by the *Bacillus* species in the synthetic medium are quite low. The low lysine levels obtained may have been as a result of the low concentrations of the carbon and nitrogen sources used in the fermentation medium. Use of high concentrations of carbon and nitrogen sources to produce high lysine yields, would likely lead to high cost of media formulation.

The stimulatory effects of the agro-products on lysine production by the *Bacillus* species are as presented in Figure 2a, b, Figure 3a, b. They showed higher lysine yields than those accumulated by the *Bacillus* species in the synthetic medium. This observation supports the work of other researches [28,29,30], who employed agro-products as carbon and nitrogen substrates in submerged fermentation. They observed that application of agro-products for bioprocesses yielded higher titres of microbial products.

The high lysine yields accumulated in the culture broths of the *Bacillus* species containing starch hydrolysates and defatted proteins (seed meals) (Figure 3a, b) as substrates, may have been influenced by the extraction of oil from the seed meals. This view is in line with the reports of many workers [31,32]. They noted that defatted seed meals have higher protein contents than non-defatted seed meals, and also have the potential to serve as good nitrogen sources for microbial products. Although a good number of the starch hydrolysates and defatted proteins improved lysine yields (Figure 3a,b), a combination of rice hydrolysate and groundnut meal have proved to be good substrates for fermentative production of lysine by the *Bacillus* species.

From the experimental study, lysine yields by the *Bacillus* species using synthetic carbon and nitrogen sources as substrates are quite low, and the cost of the substrates does not make them economical for use in the fermentation industry. The *Bacillus* species, however, can be exploited for lysine production using agro-products as substrates. These substrates are inexpensive and can serve as rich renewable sources of energy. Strain improvement and optimization of the production parameters can further enhance lysine yields by the *Bacillus* species.

Statement of Competing Interest

The authors have no competing interest.

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