

UTILIZATION OF VARIOUS STARCH HYDROLYSATES AND DEFATTED PROTEINS BY *Bacillus cereus* FOR MICROBIAL SYNTHESIS OF METHIONINE IN SUBMERGED MEDIUM.

ABSTRACT

Three of the methionine-producing bacteria previously isolated from different soil ecovars in Owerri, characterized as *Bacillus cereus* no: DS13, RS16 and AS9, based on 16s rRNA sequencing, was screened for utilization agricultural products for production of methionine. Experimental studies on the *Bacillus* strains were carried out to examine the use of various starch hydrolysates from different agricultural products: wheat, sorghum, cassava, cocoyam, yam, plantain, millet, corn, potato, rice and defatted plant proteins: pigeon pea, cowpea, bambaranut, and groundnut as source of carbon and nitrogen respectively for the production of methionine. The result of the influence of various carbon and nitrogen sources on methionine production showed that 2.32mg/ml and 2.22mg/ml methionine accumulation in the culture broths of *Bacillus cereus* DS13 and *Bacillus cereus* RS16, when sorghum-starch hydrolysate/defatted groundnut meal and corn-starch hydrolysate/defatted groundnut meal respectively was used. *Bacillus cereus* AS9 utilized plantain-starch hydrolysate/bambaranut to produced 1.78mg/ml methionine. Methionine was accumulated in the fermentation broth of the *Bacillus* strains after a fermentation period of 96h. This study indicates the possible utilization of agricultural products as substrates for L-methionine production in submerged medium especially when considering the limitations of producing methionine by fermentation processes.

Key words: *Bacillus* strains, agricultural products, submerged medium, methionine production.

INTRODUCTION

Amino Acids are significantly known as the building blocks of the body involves in building cells, repairing tissues and also intermediates on the pathway {genetic to the protein level} [1]. They also form antibodies to combat invading foreign bodies such as bacteria and viruses [2]. The L-form of methionine is used expansively in human medicine for a diversity of therapeutic purposes, including pH and electrolyte balancing, parental nutrition, pharmaceutical adjuvant, and other applications [3].

Since the 1950s the amino acids production by fermentation has become an essential part of industrial microbiology, which leads to several studies to comprehend and improve the metabolic conditions leading to overproduction of amino acids.

To synthesize amino acids processes such as Chemical, enzymatic and fermentation methods have been used, and the benefit of microbial methods is that the amino acids produced are purely optically active [4].

L-Methionine belongs to the essential amino acids which both human and animal metabolism is incapable to produce. It's important which are normally used as supplement in foods and feeds. The influence and effects of its absence on animal nutrition and nutritive feed as additive have been investigated over the years [5]. It has been observed in poultry that the stability of egg shells decreases just as the milk production in cow decreases [6,7]. The production of methionine from bacteria have reported by numerous researchers such as [8,2,9,10,3,11,12,13]. Most natural strains cannot produce industrially substantial amounts of L-methionine in the broth culture due

to their various metabolic regulation mechanisms. Some attempts have been made to overproduce biologically active L-methionine using fermentation methods [14,15,16,8,10,17,12,13].

Ezemba, *et al* [18], noted that due to the large production of some of agricultural products in Nigeria, methionine production by fermentation process may likely be more economical. Agricultural residues are rich in bioactive compounds. Vastrad and Neelagund, [19], reported that some of these agricultural industrial residues contain abundant nutrients (hemicelluloses cellulose, proteins and starch), relatively cheap and can also be utilized as alternative fermentation substrates. The used of carbohydrates like sugarcane juice, molasses, banana, cassava and coconut water as sources of carbon for methionine production was reported by [20]. Also [18] conducted the influence of agricultural products using native starches and proteins for methionine accumulation by *Bacillus cereus* S8 in submerged fermentation. Ekwealor and Orafu [21], concluded that use of native starches and protein sources are the most economical and practicable means of producing lysine by fermentation methods. Numerous researches have been carried out using agricultural products in methionine production as their basic carbon and nitrogen sources [21,22,23,19]. The major properties of these fermentation media are that they are low cost; due to this products used in production of amino acids are economical, rich in nutrient and free of toxins. It has been reported that Nigeria has a large production of cassava, cocoyam, millet, potato, plantain, yam, rice, corn, wheat, sorghum, soy bean, pigeon pea, cowpea, bambara and groundnut [24]. This present investigation was to examine the influence of various native starches and protein on methionine accumulation by different strains of *Bacillus* species which has been previously studied by [25] for methionine stimulation using synthetic media. These residues can be used as an alternate source for the production of different products by microorganisms.

2. MATERIALS AND METHODS

2.1 Microorganisms

The microorganisms used was *Bacillus cereus* DS13, *Bacillus cereus* RS16, and *Bacillus cereus* AS9, previously recovered from soil ecovars in Owerri, South Eastern Nigeria, and have been evaluated for methionine production by Dike and Ekwealor,[25]. The organisms were tested for pure strains before been maintained on Nutrient agar slant (Lab M) slants at 4°C.

2.2. Agricultural products and Enzymes

Cassava tubers were obtained from Nnamdi Azikiwe University agricultural farm at Awka, Cocoyam, groundnut, cowpea sorghum, wheat, plantain, sweet potato, yam, corn, rice, millet, pigeon pea and bambaranut were purchased from Onitsha and Awka markets, of Anambra State. The enzymes, α -amylase (Termamyl) and amyloglucosidase were obtained from Life Breweries Onitsha, Anambra state.

2.3. Preparation of native starches for Carbon Sources:

Native starches used include wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), cocoyam (*Alocasia amazonica*), yam (*Dioscorea rotundata*), plantain (*Musa sapientum*), millet (*Panicum miliaceum*), corn (*Zea mays*), potato (*Ipomoea batata*), and rice (*Oryza sativa*). These were prepared according to the method described by Odibo [26]. Cassava, cocoyam, potato and plantain samples were first peeled, washed and cut into small cubes before being homogenized with water in a Moulinex blender. Sorghum, corn and millet

were soaked for 48h to soften the seeds and then homogenized with water. Each homogenate mixed with excess water was tied in a cheese cloth and placed on a tripod stand overnight, to allow for the leaching out of starch into a clean plastic bowl. The supernatant was decanted and the sedimented starch dried at 50°C for 48h. The resultant flakes were ground into powder and used as native starch.

2.4. Saccharification of Starch

The method described by [27] was used for the saccharification of native starches. A 500ml flask containing a mixture of 30g of native starch and 100ml of water was heated for 15min at 95°C in a water bath to gelatinize the starch. The beaker was covered with an aluminium foil after adding 1.0ml of α -amylase and heated again in a water bath for 10min at 95°C to effect liquefaction. After cooling the liquefied starch to 60°C, 1.0ml amyloglucosidase enzyme was added before replacing the beaker in the water bath at 60°C for 48h, for saccharification to take place.

2.5. Preparation of Native Proteins for Nitrogen Sources

The proteins used include: pigeon pea (*Cajanus cajan*), cowpea (*Vigna* sp.), bambaranut (*Voandzeia subterranean*), and groundnut (*Arachis hypognea*). The method described by [27] was used in preparing native starches. They were milled in a Moulinex blender and some fractions of the homogenized native proteins were then defatted by Soxhlet extraction method using the solvent diethyl ether. The meals obtained after extraction were oven-dried at 34-35°C for 20h and then ground into fine powder. The defatted meals were then oven-dried at 50°C for 20h.

2.6. Production of Methionine by the *Bacillus* Strains in Submerged Medium

2.6.1. Inoculum preparation

The medium for seed inoculum consists of peptone: 10.0g; yeast extract, 10.0g; NaCl, 5.0g; distilled water, 1L. The pH was adjusted to 7.2 with 1N NaOH. One loopful of a 24h old culture of the isolate on Nutrient agar was inoculated into 1ml of the sterile seed medium in a test tube and incubated for 24h on a orbital shaker (VWR DS2-500-2) at 160rpm and 30°C.

2.6.2. Fermentation experiment

Fermentation was done following the method described by [28]. The composition of the fermentation medium was: KH_2PO_4 , 0.05g; K_2HPO_4 , 0.05g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001g; CaCO_3 , 20g; starch hydrolysates, 20g; defatted nitrogen sources, 10g; distilled water 1L. The pH was adjusted to 7.2 with 1N NaOH. Twenty millimeter of the medium in 100ml Erlenmeyer flask was sterilized in an autoclave at 115°C for 10min, cooled and then inoculated with 1ml (OD 3.00) of the seed inoculum. The flask was incubated for 72h on an orbital shaker at 160rpm and 30°C. Duplicate flasks were used and uninoculated flasks served as control. Methionine accumulation in the broth culture was determined.

2.6.3. L-Methionine Assay

Quantitative determination of methionine in the broth culture without purification was carried out following the method described by Greenstein and Wintz [29]. To 5ml of the supernatant after centrifugation at 5000xg for 15min was added 1ml of 5N NaOH and 0.1ml of 10% sodium

nitroprusside solution. The tube was thoroughly shaken and the mixture allowed to stand for 10min. Then 2ml of 3% aqueous solution of glycine was added to the reaction mixture with frequent shaking over a period of 10min. After an additional 10min interval, 2ml of concentrated orthophosphoric acid was added drop wise to the mixture and the test tube properly shaken. Colour development was allowed to proceed for 5min and colour intensity measured at 540nm in a spectrophotometer (PerkinElmer Lambda 35 UV-VIS). The methionine yield was extrapolated from a standard methionine curve.

Statistical data analysis

Where necessary, the data obtained were subjected to statistical analysis (ANOVA) using Statistical Package for Social Science (SPSS) 15.0 for Windows Evaluation, Version (2006). P-values <0.05 were considered statistically significant while P-values >0.05 indicates that there is no significant difference.

RESULTS

Effects of starch hydrolysates and defatted cowpea on methionine accumulation by *Bacillus* Strains

Figure 1 shows the effects of different starch hydrolysate and cowpea on methionine production by the *Bacillus* strains. As presented in Fig.1, yam and cowpea gave the methionine yield of 1.78mg/ml by *B. cereus* DS13, while potato and cowpea gave the least (1.08mg/ml). In Fig.1, wheat and cowpea gave the highest methionine accumulation (1.85mg/ml) while cowpea and potato gave the least yield (0.85mg/ml) by *B. cereus* RS16. Figure 1, shows that *B. cereus* AS9 gave highest methionine yield of 1.75mg/ml with sorghum and least yield with yam (0.85mg/ml). P-value = 0.000, indicating that there is significant difference in methionine production when different starch hydrolysate and defatted cowpea were used.

Effects of starch hydrolysates and defatted bambara-nut on methionine production by *Bacillus* Species

The effects of different starch hydrolysate and bambara on methionine production by *B. cereus* was evaluated in Fig.2. As presented in Fig. 2, corn and bambara gave the highest methionine yield of 2.13mg/ml by *B. cereus* DS13, while rice and bambara gave the least yield (1.08mg/ml). In Fig.2, cocoyam and bambara gave the highest methionine accumulation (1.86mg/ml) while bambara and millet gave the least yield (1.02 mg/ml) by *B. cereus* RS16. Fig. 2 shows that *B. cereus* AS9 gave highest methionine yield of 1.78mg/ml with plantain and least with sorghum (0.93mg/ml). There is significant difference in methionine production by the microorganism (mos) when starch hydrolysate and defatted bambara is used (p=0.00). There is strong positive interaction between the medium and microorganism on methionine production.

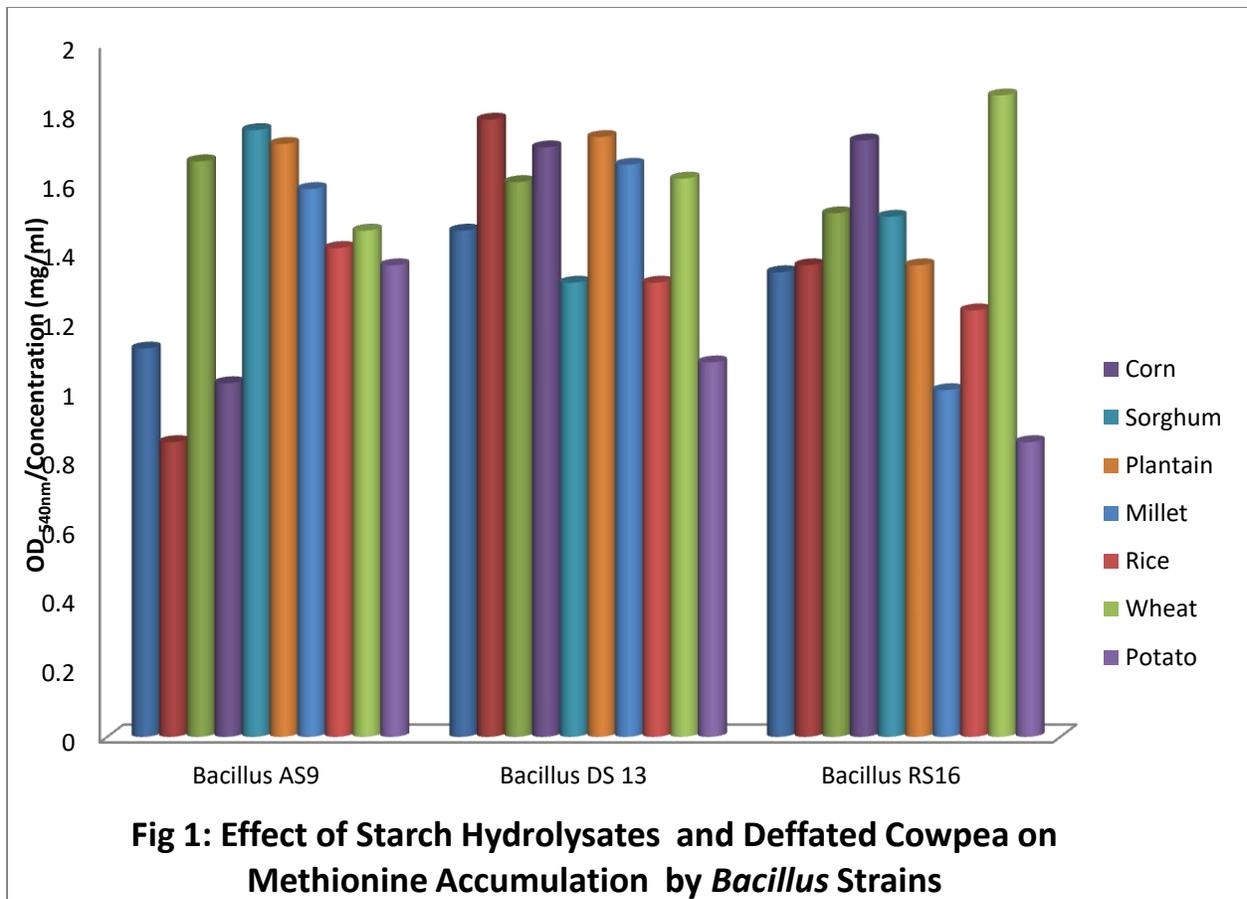
Effects of starch hydrolysates and defatted groundnut on methionine accumulation by *Bacillus cereus*.

Figure 3 shows the effects of different starch hydrolysate and defatted groundnut on methionine production by *B. cereus*. As presented in Fig.3, sorghum and groundnut gave the highest methionine yield of 2.32mg/ml by *B. cereus* DS13, while potato and groundnut gave the least

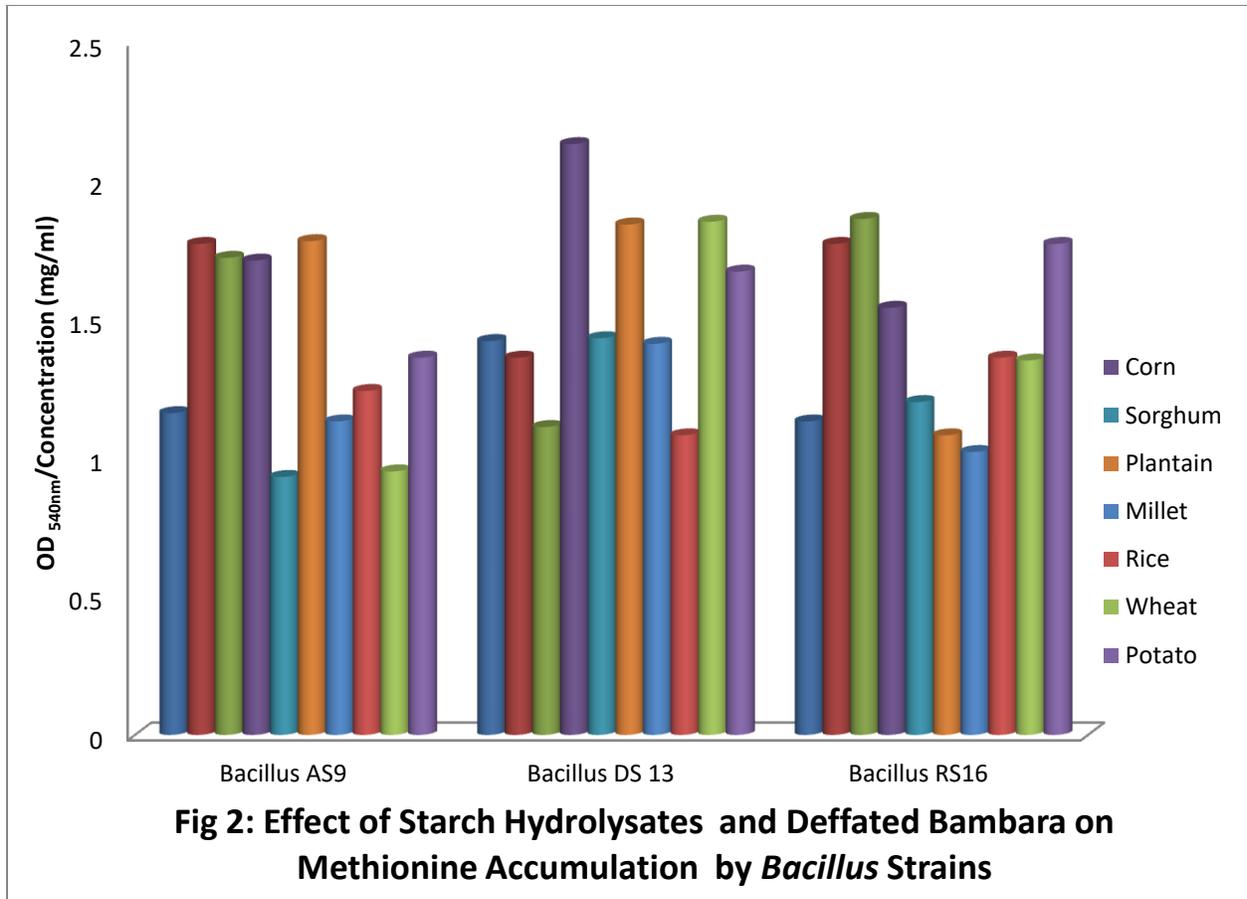
(0.92mg/ml). In Fig.3, corn and groundnut gave the highest methionine accumulation (1.99mg/ml) while groundnut and wheat gave the least yield (0.87mg/ml) by *B. cereus* RS16. Figure 3, shows that *B. cereus* AS9 gave highest methionine yield of 1.76mg/ml (corn) and least with wheat (0.84mg/ml). Statistical analysis (Anova), showed a significant difference when starch hydrolysate and defatted groundnut is used ($p=0.00$). Statistically, interaction between carbon and microorganism interactions on methionine production was positive ($r=0.000$). Although groundnut is good, other nitrogen sources can be used based on the microorganism to be used.

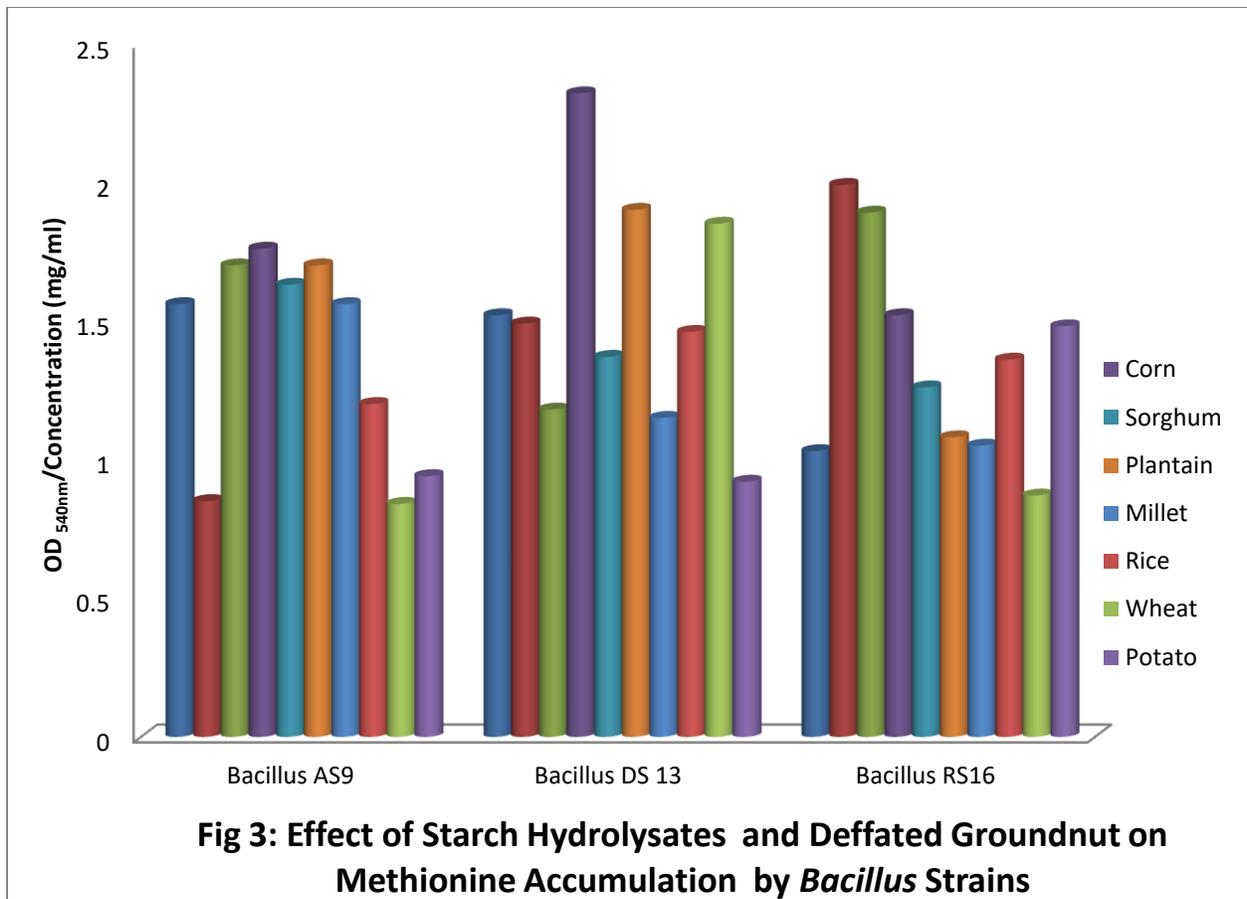
Effects of starch hydrolysates and defatted pigeon pea on methionine accumulation by *Bacillus cereus*

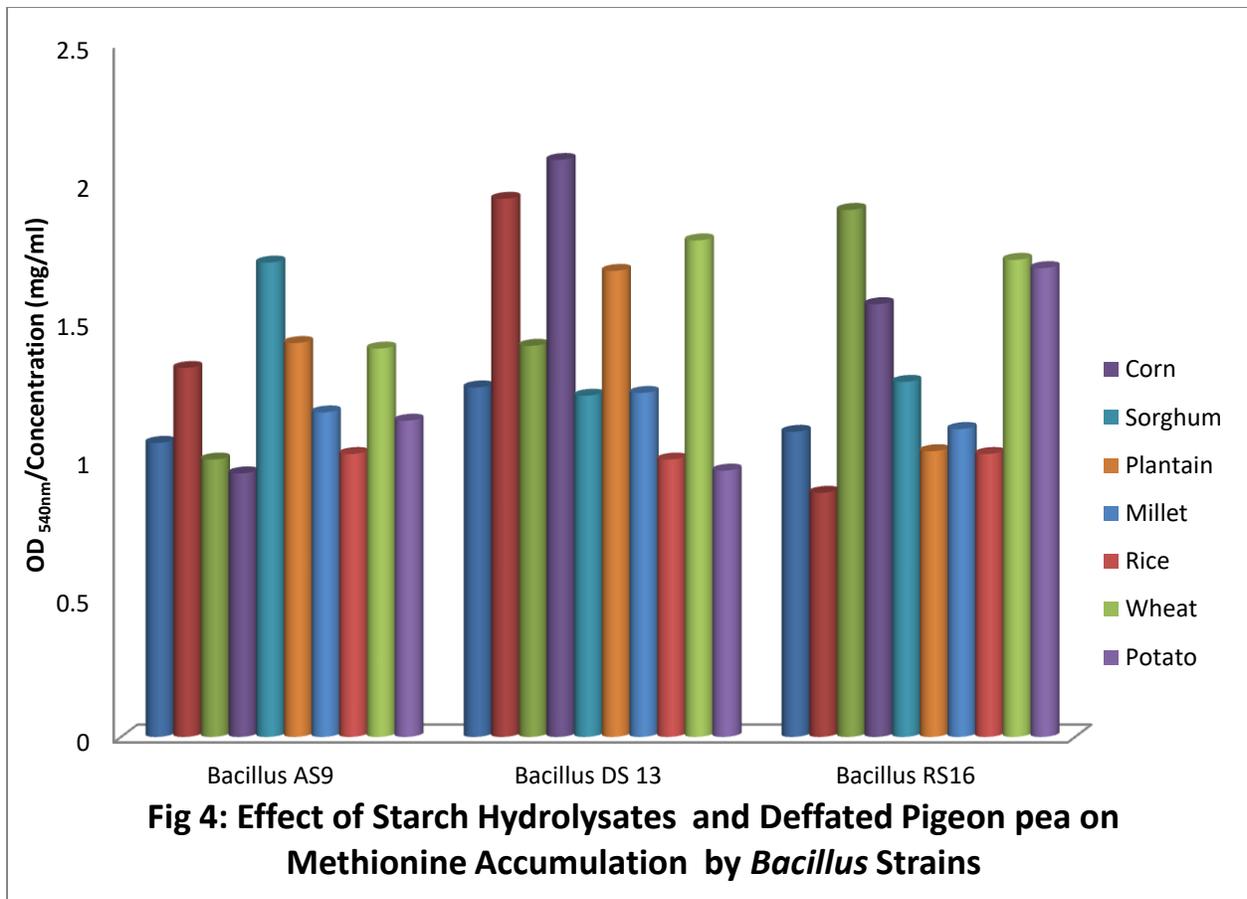
The effects of different starch hydrolysate and pigeon pea on methionine production by *B. cereus* was evaluated in Fig.4. As presented in Fig.4, corn and pigeon pea gave the highest methionine yield of 2.08mg/ml by *B. cereus* DS13, while potato and pigeon pea gave the least (0.96mg/ml). In Fig.4, cocoyam and pigeon pea gave the highest methionine accumulation (1.90mg/ml) while pigeon pea and yam gave the least yield (0.88 mg/ml) by *Bacillus cereus* RS16. Figure 4, shows that *B. cereus* AS9 gave methionine yield of 1.71mg/ml with sorghum. Statistically, P-value = 0.000, indicating that there is significant difference in methionine produced with the different starch hydrolysate and pigeon pea used.



UNDER REVIEW







UNDER REVIEW

DISCUSSION

Bacillus strains which have been reported to produce methionine using synthetic medium [25], has been observed to utilize agricultural products for the accumulation of methionine in submerged medium.

The ability of *Bacillus* species to produce methionine has been reported by many workers [30,18,12,13,3,31,2].

Although, Gomes and Kumar (2005), reported that *Bacillus* species are not yet recognized as overproducers of methionine as *Corynebacterium* sp. and *Brevibacterium* sp.

Methionine accumulation by *Bacillus* strains using starch hydrolysate and defatted cowpea are shown in Fig. 1. Wheat stimulated a high yield of 1.85mg/ml by *B. cereus* RS16. This observation is in concordance with the work of [33], who also observed that wheat bran (1.25%), pearl millet starch (1%) with nutrient broth was the best substrate for α -amylase production by *Bacillus licheniformis*. Ellaiah *et al.* [22], also reported an improved neomycin production under solid state fermentation by *Streptomyces marinensis* NUV 5, when wheat bran was solid support material in the medium. Nwokolo *et al* [34] worked on production of lysine by *Alcaligenes aquatilis* from agricultural products (banana and soyabean), which showed that maximum lysine yield (1.158mg/mL and 1.279mg/mL) for the fermentation period of 96h was obtained. Hang and Woodams [35], noted that wheat bran is also utilized as substrates for citric acid production by solid state fermentation using *Aspergillus* spp.

However, contrary to the report in this study, Akcan [36], reported that wheat starch, wheat flour and rice flour repressed amylase production while arabinose, sucrose, corn flour and corn starch enhanced the production with casein as nitrogen source by *Bacillus* specie. However, [27] observed that cowpea/millet, gave a lysine yield of 5.60mg/ml by *Bacillus laterosporus*.

The methionine secretions by the *Bacillus* species using starch hydrolysate and defatted bambaranut are presented in Fig. 2. Corn gave a high yield of 2.13mg/ml by *Bacillus cereus* DS13. This is in support of the work by Ezemba, *et al* [18] who observed that corn and soyabean gave a highest methionine production by *Bacillus cereus* S8 when used as carbon and nitrogen respectively. But in contrast with the work of Umerie *et al.* [27] who reported that corn/soyabean low lysine yield while millet/bambara nut enhanced lysine accumulation of 5.50mg/ml in *Bacillus laterosporus*.

The maximum production of methionine using starch hydrolysate and defatted groundnut (Fig. 3) was achieved by *Bacillus cereus* DS13. This observation is in accordance with the work of [27], who also reported millet/groundnut as the best nitrogen sources for lysine production (5.64mg/ml) by *Bacillus laterosporus*. However, they also observed that low lysine yields were obtained when sorghum is used as a carbon source.

Effect of various starch hydrolysate and defatted pigeon pea, (Fig. 4) on methionine production, showed that corn and pigeon pea gave a high yield of 2.08mg/ml by *Bacillus cereus* DS13. Statistical analysis (Anova), showed a significant difference between starch hydrolysate and all the defatted protein is used ($p=0.00$). Interaction between carbon and microorganism interactions on methionine production was positive ($r=0.000$). Although soyabean meal is good, other nitrogen sources can be used based on the microorganism to be used.

The result obtained with regard to utilization of native starches and proteins for methionine production (Fig.1-4). It shows that there was an increase in methionine accumulation than synthetic medium reported by Dike and Ekwealor, [3]. This innovation is in agreement with the work of [20], who reported the use of 250g/l of molasses diluted by coconut water and

2% w/v of ammonium sulphate by *Corynebacterium glutamicum* ATCC21608 as source of carbon/nitrogen for methionine production.

The markedly enrichment of microorganism with native starches compared with synthetic carbon implies a very high rate of carbon utilization. This could be attributed to slowly- utilized carbon sources and low protein dispersion index (PDI) of nitrogen source [19]. He also reported that relatively cheap agro industrial by-products such as hemicelluloses, cellulose, proteins and starch, have a great potential to be utilized as alternative substrates for fermentation because they contain abundant nutrients. Protein enrichment has been obtained by growing *Aspergillus niger* on agricultural wastes in solid state fermentation [37].

The ability of these *Bacillus* species to utilize native starches and defatted proteins are supported by the work of Ezemba, *et al.* [18], who studied on various carbon and nitrogen sources and reported that yields of 2.05mg/ml and 2.04mg/ml were achieved with plantain-starch hydrolysate/groundnut meal and corn-starch hydrolysate/defatted bambaranut meal when *Bacillus cereus* S8 were used as an inoculants in a submerged fermentation. Also Umerie *et al.* [27], used millet/soyabean meal as carbon/ nitrogen sources for accumulation of 5.67g/l lysine by *Bacillus latesporus*. Ekwealor and Orafu [21], reported the secretion of high lysine yield by *Bacillus megaterium* SP-14 and *Bacillus circulans* TX-22 using natural proteins as a nitrogen source. Trifonova *et al.* [38] used agricultural product fruits and vegetable raw materials but sugar beet gave lysine yield of 25.9g/l, when used as substrate by *Brevibacterium* spp.

Apart from this, other various agricultural raw materials have been employed as carbon/nitrogen source for other production, they include: Javed *et al.* [39] and Vandenberghe *et al.* [40] observed citric acid production by *Aspergillus niger* using molasses and cassava bagasse respectively.

Economou *et al.* [41], produced single cell oil (lipid) from rice hulls hydrolysate by oleaginous fungus *Mortierella isabellina* under nitrogen limited conditions while [42,43,44], used agricultural by- products for single cell protein production. Buzzini *et al.* [45], also produced gluconic acid by fermenting agricultural by-products (grape must) using *Aspergillus* spp and *Gluconobacter oxidans*.

Vastrad and Neelagund [19], produced 2765µg/g of neomycin by actinomycete *Streptomyces fradiae* in solid state fermentation using apple pomace.

Comparing the methionine production of all the three *Bacillus* species, evaluated from Fig. 1-4, the results show that sorghum/groundnut for *B. cereus* DS13, corn/groundnut for *B. cereus* RS16 and plantain/bambaranut for *B. cereus* AS9 were the carbon/nitrogen sources of choice for methionine synthesis. This study reveals that plant foods were better than synthetic source (glucose/ ammonium sulphate) when compare with the report of Dike and Ekwealor (2013), who used the same organism for methionine production using synthetic source for the same *Bacillus* sp. This could be the selection of appropriate carbon and nitrogen sources as suggested by Konsoula and Kyriakides [46], who noted that it was one of the most critical stages during the development of an efficient and economic process.

The use of starch hydrolysates and various defatted nitrogen plant sources in this study, enhanced methionine production. This is in line with the work of Ezemba, *et al.* [18] and Umerie *et al.* [27] who observed that natural nitrogen sources enhanced methionine and lysine accumulation respectively than synthetic nitrogen sources.

CONCLUSION

This preliminary study and the successful utilization of agricultural products as substrates for L-methionine accumulation by *Bacillus* strains hold a promising future for L-methionine production in the country. Since the agricultural by-products are good sources of carbon /nitrogen and rich in fermentable substrates, cheap and available, methionine production by fermentation processes will be very economical, especially when considering the limitations of producing methionine by fermentation processes. Therefore this microbiological fermentation process of methionine production if well developed and established will reduce the importation of already made product or drugs into the country and makes it more readily available. Also these residues can be used as an alternate source for the production of different products by microorganisms.

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