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

ABSTRACT

Three of the methionine-producing bacteria isolated from different soil ecovars in Owerri and characterized as *Bacillus cereus* DS13, *Bacillus cereus* RS16, *Bacillus cereus* AS9, based on 16s rRNA sequencing, were screened for methionine production using agricultural products. Experimental studies on the *Bacillus* strains were carried out to examine the use of various starch hydrolysates from different agricultural products: 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)*, rice *(Oryza sativa)*.) and defatted plant proteins: pigeon pea *(Cajanus cajan)*, cowpea *(Vigna* sp*.)*, bambaranut *(Voandzeia subterranean)*, and groundnut *(Arachis hypognea)* as carbon and nitrogen sources respectively for the production of methionine. The influence of the various carbon/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 were used. *Bacillus cereus* AS9 utilized plaintain-starch hydrolysate/bambaranut to produce 1.78mg/ml methionine. Methionine was accumulated in the culture 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.

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

INTRODUCTION

Amino Acids are considered as the building blocks of the body. Amino acids are not only involved in building cells, repairing tissues and intermediates on the pathway from the genetic to the protein level (Anon, 2009), but also form antibodies to combat invading foreign bodies like bacteria and viruses (Ali, 2008). The L-form of methionine is used extensively in human medicine for a variety of therapeutic purposes, including pH and electrolyte balancing, parental nutrition, pharmaceutical adjuvant, and other applications (Dike and Ekwealor, 2012b).

Since the 1950s the production of amino acids by fermentative methods has become a very important part of industrial microbiology, which leads to numerous studies to understand and improve the metabolic conditions driving to amino acid overproduction.

Chemical, enzymatic and fermentation processes have been used to synthesize amino acids, and the advantage of microbial methods is that the amino acids are purely optically active (Kinoshita *et al.,* 1957).

L-Methionine belongs to the essential amino acids which human and animal metabolisms are not capable to produce (New World Encyclopedia, 2008). It’s an important amino acid used as food and feed supplement. The impact of L-methionine on animal nutrition and the consequences of its absence as nutritive feed additive have been investigated (Campbell, 2001). It has been observed in poultry that the stability of egg shells decreases just as the milk production in cow does (Noftsger *et al.,* 2003; Keshavarz, 2003).

Several researchers have reported the production of methionine from bacteria by (Kumar *et al*., 2003; Ali, 2008; Anike and Okafor, 2008; Reershemius, 2008; Dike and Ekwealor, 2012b; Arunkumar *et al*., 2014; Anakwenze, *et al*., 2014a,b). Most natural strains cannot produce industrially significant amounts of L-methionine in the culture broth due to various metabolic regulation mechanisms. Attempts have been made to overproduce biologically active L-methionine using fermentation (Roy *et al.*, 1989; Kase and Nakayama, 1975; Mondal *et al.*, 1996; Kumar *et al.,* 2003; Reershemius, 2008; Dike and Ekwealor, 2013; Anakwenze, *et al*., 2014a,b).

Ezemba, *et al* 2016, noted that due to the large production of some of agricultural products in Nigeria, methionine production by fermentation process may likely be more economical. Vastrad and Neelagund, *(*2011), reported that these relatively cheap agricultural industrial residues contain abundant nutrients (hemicelluloses cellulose, proteins and starch) and have a great potential to be utilized as alternative fermentation substrates. Pharm *et al.* (1992), used the carbohydrates like sugarcane juice, molasses, banana, cassava and coconut water as sources of carbon for methionine production. Also Ezemba, *et al.*(2016) conducted the influence of agricultural products using native starches and proteins for methionine accumulation by *Bacillus cereus S8* in submerged fermentation. Ekwealor and Orafu (2003), concluded that use of native starches and protein sources are the most economical and practicable means of producing lysine by fermentation methods. Several researches have been carried out using agricultural products in production as basic carbon and nitrogen sources (Ekwealor and Orafu, 2003; Ellaiah, *et al.*, 2004; Ajila, 2007; Vastrad and Neelagund *et al.*, 2011).

The important properties of these fermentation media are that they are low cost, and products used in production 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 (Anon, 2011). This present investigation was conducted to examine the influence of various native starches and protein on methionine accumulation by *Bacillus* species.

2. MATERIALS AND METHODS

2.1 Microorganisms

The microorganisms used for this study were *Bacillus cereus* DS13, *Bacillus cereus* RS16, and *Bacillus cereus* AS9, recovered from soil ecovars in Owerri, South Eastern Nigeria,and have been screened for methionine production on solid agar medium (Dike and Ekwealor, 2012a). The organisms were maintained on Nutrient agar (Lab M) slants at 4°C.

2.2. Agricultural products and Enzymes

Cassava tubers were obtained from the agricultural farm at Nnamdi Azikiwe University, 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. Carbon Sources: Preparation of native starches:

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)*. The native starches were prepared according to the method described by Odibo (1987). 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 Umerie *et al* (2000) 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^{o}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 saccharifcation to take place.

2.5. Nitrogen Sources: Preparation of Native Proteins

The proteins used include: pigeon pea *(Cajanus cajan)*, cowpea *(Vigna* sp.*)*, bambaranut *(Voandzeia subterranean),* and groundnut *(Arachis hypognea)*. The method described by Umerie *et al*. (2000) 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^{o}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 carried out following the method described by Ozulu *et al* (2012). The composition of the fermentation medium was: KH_2PO_4, 0.05g; K_2HPO_4, 0.05g; $MgSO_4.7H_2O$, 0.1g; $MnSO_4.4H_2O$, 0.001g; $FeSO_4.7H_2O$, 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 (1961). 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 stastitically 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 *Bacillus 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 *Bacillus cereus* RS16. Figure 1, shows that *Bacillus 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 *Bacillus cereus* was evaulated in Fig.2. As presented in Fig. 2, corn and bambara gave the highest methionine yield of 2.13mg/ml by *Bacillus 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 *Bacillus cereus* RS16. Fig. 2 shows that *Bacillus 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 hdrolysate 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 *Bacillus cereus*. As presented in Fig.3, sorghum and groundnut gave the highest methionine yield of 2.32mg/ml by *Bacillus 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 *Bacillus cereus* RS16. Figure 3, shows that *Bacillus 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 *Bacillus 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 *Bacillus 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 *Bacillus cereus* AS9 gave methionine yield of 1.71mg/ml with sorghum. Satistically, P-value = 0.000, indicating that there is significant difference in methionine produced with the different starch hydrolysate and pigeon pea used.

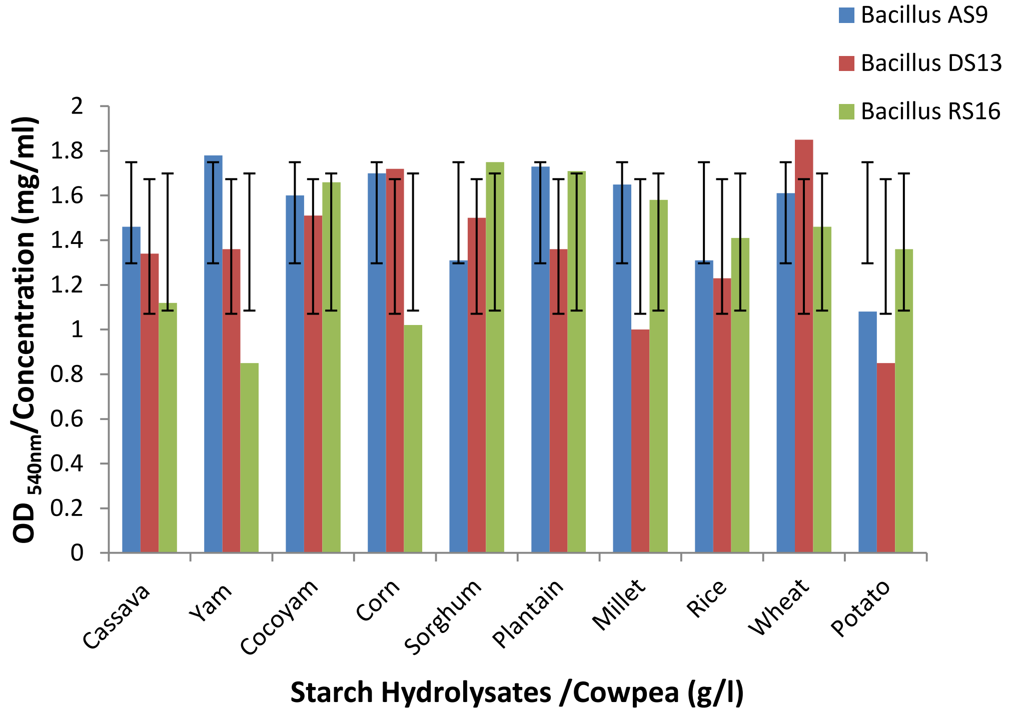

Fig 1: Effect of Starch Hydrolysates and Deffated Cowpea on Methionine Accumulation by *Bacillus* Strains

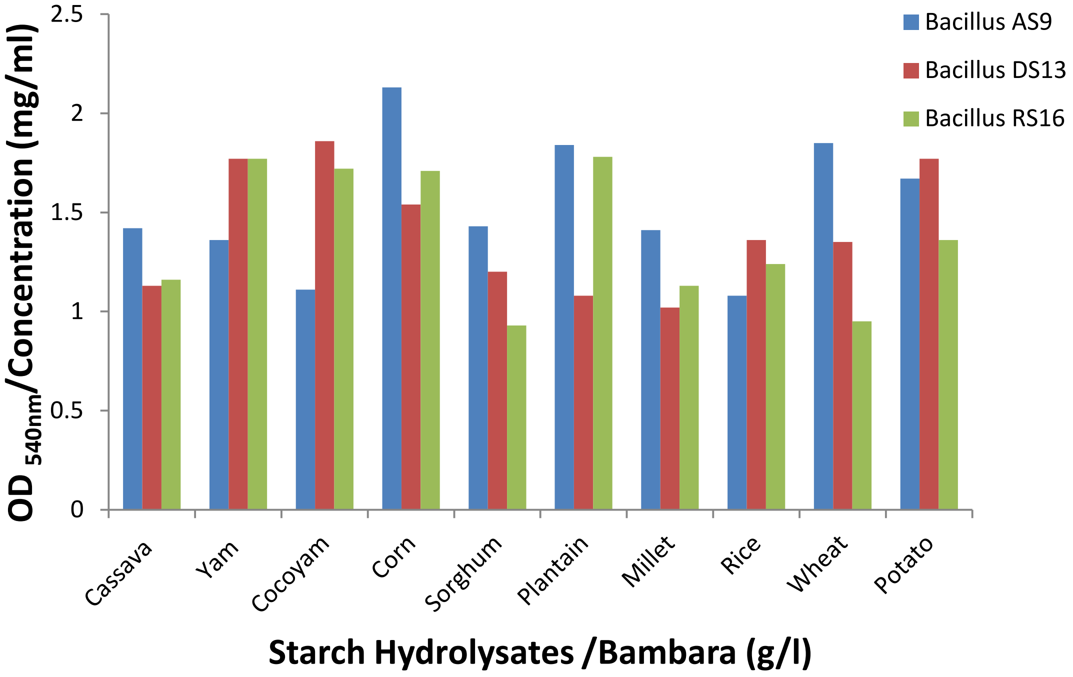

Fig 2: Effect of Starch Hydrolysates and Deffated Bambara on Methionine Accumulation by *Bacillus* Strains

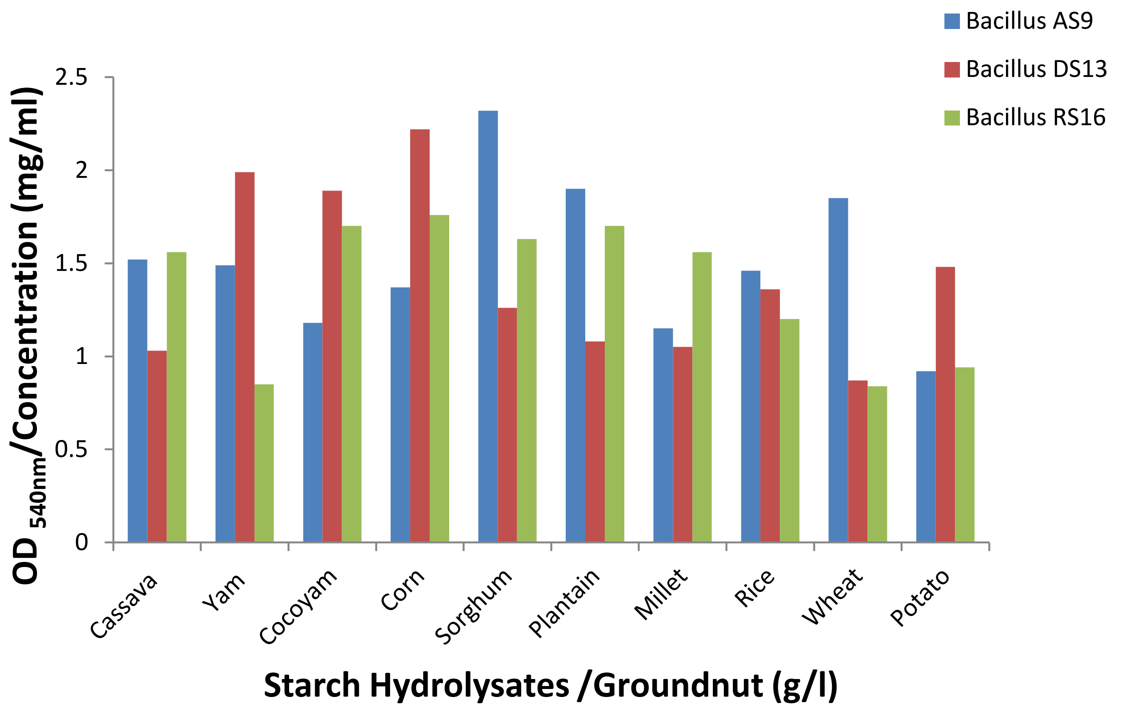

Fig 3: Effect of Starch Hydrolysates and Deffated Groundnut on Methionine Accumulation by *Bacillus* Strains

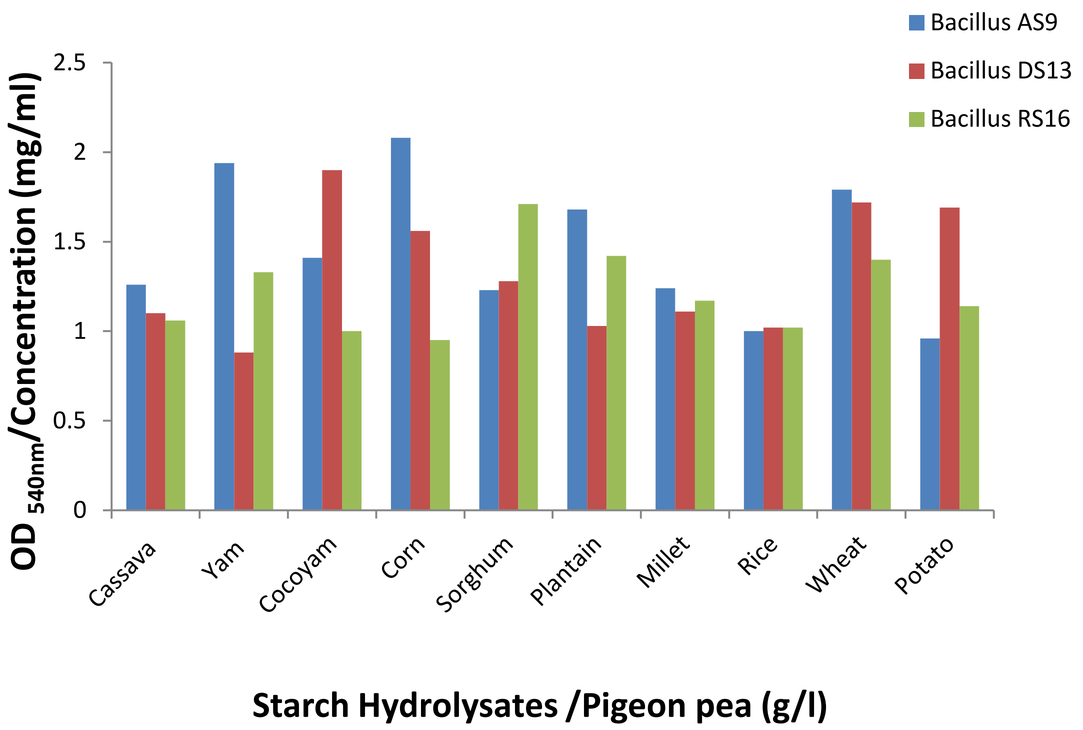

Fig 4: Effect of Starch Hydrolysates and Deffated Pigeon pea on Methionine Accumulation by *Bacillus* Strains

DISCUSSION

Bacillus strains which have been reported to produce methionine using synthetic medium (Dike and Ekwealor, 2012a), has been observed to utilized agricultural products for the accumulation of methionine in submerged medium.

The ability of *Bacillus* species to produce methionine has been reported by many workers (Ezemba, *et al* 2018; Ezemba, *et al* 2016; Anakwenze *et al* 2014a,b; Dike and Ekwealor, 2012b; Yamashiro *et al*. 1988 and Ali 2008).

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

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 *Bacillus cereus* RS16. This observation agrees with the work of Ikram-ul-Haq *et al.* (2003), who also reported wheat bran (1.25%), pearl millet starch (1%) with nutrient broth as the best substrate for α-amylase production by *Bacillus licheniformis*. Ellaiah *et al*. (2004), also observed an improved neomycin production under solid state fermentation by *Streptomyces marinensis* NUV 5, when wheat bran was is solid support material in the medium. Hang and Woodams (1987), noted that wheat bran is also utilized as substrates for the production of citric acid by solid state fermentation using *Aspergillus* spp.

However, contrary to the report in this study, Akcan (2011), 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, Umerie *et al*. (2000) observed that cowpea/millet, gave a lysine yield of 5.60mg/ml by *Bacillus latersporus.*

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* 2016 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*. (2000), who reported that corn/soyabean low lysine yield while millet/bambara nut enhanced lysine accumulation of 5.50mg/ml in *Bacillus latersporus*.

The maximum production of methionine using starch hydrolysate and deffated groundnut (Fig. 3) was achieved by *Bacillus cereus* DS13. This observation agrees with the work of Umerie *et al*., (2000), who also reported millet/groundnut as the best nitrogen sources for lysine production (5.64mg/ml) by *Bacillus latersporus*. However, they also observed that low lysine yields were obtained when sorghum is used as a carbon source.

Effect of various starch hydrolysate and deffated 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 synethtic medium reported by Dike and Ekwealor, (2012b). This innovation is in agreement with the work of Pham *et al*. (1992), who reported the use of 250g/l of molasses diluted by coconut water and 2%w/v of ammonium sulphate by *Corynebacterium glutamicium* 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 (Vastrad and Neelagund 2011). He also reported that relatively cheap agro industrial by-products (hemicelluloses, cellulose, proteins and starch) have a great potential to be utilized as alternative fermentation substrates because they contain abundant nutrients. Protein enrichment has been obtained by growing *Aspergillus niger* on agricultural wastes in solid state fermentation (Davey *et al.*1981).

The ability of these *Bacillus* species to utilize native starches and defatted proteins are supported by the work of Ezemba, *et al.* (2016), who studied on various carbon and nitrogen sources and reported that yields of 2.05mg/ml and 2.04mg/ml were achieved with plaintain-starch hydrolysate/groundnut meal and corn-starch hydrolysate/defatted bambaranut meal when *Bacillus cereus* S8 where used as an inoculants in a submerged fermentation. Also Umerie *et al.* (2000), used millet/soyabean meal as carbon/ nitrogen sources for accumulation of 5.67g/l lysine by *Bacillus latersporus*. Ekwealor and Orafu (2003), 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.* (1993) 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, various other agricultural raw materials have been employed as carbon/nitrogen source for other production, they include: Javed *et al*. (2011) and Vandenberghe *et al*. (2008) observed citric acid production by *Aspergillus niger* using molasses and cassava bagasse respectively.

Economou *et al.* (2011), produced single cell oil (lipid) from rice hulls hydrolysate by oleaginous fungus *Mortierella isabellina* under nitrogen limited conditions while Krishnamurth (1980), Nicoloni *et al.(*1987) and Nigam (2000), used agricultural by- products for single cell protein production. Buzzini *et al.* (1993), also produced gluconic acid by fermenting agricultural by-products (grape must) using *Aspergillus* spp and *Gluconobacter oxidans.*

Vastrad and Neelagund (2011), 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 *Bacillus cereus* DS13, corn/groundnut for *Bacillus cereus* RS16 and plantain/bambaranut for *Bacillus 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 (2007), who noted that it is one of the most critical stages in 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* (2016) and Umerie *et al*. (2000), 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 processs will be very economical. Therefore this microbiological process of methionine production if well developed will reduce the importation of this product into the country and make it more readily available.

REFRENCES

Ajila, C.M. (2007). Isolation and characterization of valuable components from the mango peel and black gram husk. Ph.D thesis. Department of Biochemistry and Nutrition, University of Mysore. India.

Ali, N. M. (2008). Study on bacterial isolates for amino acid production: improvement in production of amino acids of commercial importance. PhD Thesis. Department Of Zoology Government College University, Lahore.

Akcan, N. (2011). High level production of extracellular α-amylase from *Bacillus licheniformis* ATCC 12759 in submerged fermentation. *Romanian Biotechnological Letters*. 16:6.

Anakwenze, V. N.; Ezemba, C.C. and Ekwealor, I.A. (2014a). Improved cultural conditions for methionine accumulation in submerged cultivation of *Bacillus cereus* S8. *British Microbiology Research Journal*. 4(8):885-895.

Anakwenze, V. N.; Ezemba, C.C. and Ekwealor, I.A. (2014b). Optimization of fermentation conditions of *Bacillus thuringiensis* ECI for enhanched methionine production. *Advances in Microbiology*. 4:344-352.

Anike, N. and Okafor, N. (2008). Secretion of methionine by microorganisms associated with cassava fermentation. *African Journal of Food Agriculture Nutrition and Development*. 8(1): 77-90.

Anonymous (2009). Amino acids peptide guide. www.peptideguide.com. Accessed May, 2009.

Anonymous (2011). Staple crop production and consumption; Nigeria on the way to food self-sufficiency. Special report Inter-reseaux development rural. www.inter-reseaux.org/nigeria/staple. Accessed May, 2011.

Arunkumar, N.; Sundaram, S.P.; Anahdham, N.O. Gopal and Balakrishnan, S. (2014). Studies on methionine secreting micro-organisms from sago industrial wastes and standardization of growth parameters for maximum methionine secretion. *International jounarl of Agricultural sciences.* 10(1):29-35.

Buzzini, P.; Gobbertti, M.; Rossi. J. and Ribaldi, M. (1993). Utilization of grape must and concentrated rectified grpe must to produce gluconic acid production by *Aspergillus niger* in batch fermentations. *Biotechnology letters*, 15:151-156.

Campbell, K.C.M. (2001). Therapeutic use of D-methionine to reduce the toxicity of platinum-containing anti-tumor compounds and other compounds, US Patent. 6187817.

Dike, K. S and Ekwealor, I. (2012a). Studies on process and physical parameters for the production of L-methionine from newly isolated *Bacillus cereus* strains. *Asian Journal of Biological Sciences*. 5(2) 96- 104.

Dike, K. S and Ekwealor, I. (2012b). Production of L-methionine by *Bacillus cereus* isolated from different soil ecovars in owerri, south east Nigeria. *European Journal of Experimental Biology*. 2(2)311-314.

Dike, K. S.; Ekwealor, I. and Eziuzor, S. C. (2013) Influence of antibiotics and surfactants addition on growth and methionine productivity by *Bacillus cereus*. *Advances in Microbiology*. 3:26-31.

Davey, C.; Eng, A. E. C. and Chem, E. (1981). Food industry wastes In: Disposal and recovery. Herzka.A. and Boot R. G. edition. Applied science publishers, Lonodon. p.219.

Economou, C. N.; Aggelis, G.; Pavlou, S. and Vagenas, D.V. (2011). Single cell oil production from rice hulls hydrolysate. Bioresource Technology. 102:9737-9742.

Ekwealor, I. A. and Orafu, A. E. (2003). Preliminary study of L-lysine production by *Bacillus species* using various agricultural by- products. *Nahrung/Food*. 47:226-227.

Ellaiah, P., Srinivasulu, B. and Adinarayana, K. (2004). Optimization studies on neomycin production by a mutant strain of *Streptomyces marinensis* in solid state fermentation process. *Biochemistry*. 39:529.

Ezemba,C.C; Akagu, S and Uchefuna, N.(2018). Isolation, Screening and Methionine Production by Bacillus species isolated from Poultry Waste. *Abasyn Journal of Life Sciences.* 1 (2): 95-101.

Ezemba, C.C.; Anakwenze, V.N.; Archibong, E.J.; Anaukwu, G.C.; Obi, Z.C.; Ekwealor C.C. amd I.A. Ekwealor (2016). Methionine production using native starches and proteins in submerged fermentation by *Bacillus cereus* S8.*World Journal of Pharmacy and Pharmaceutical sciences*, 5(4):2056-2067. www.wjpps.com. ISSN 2278-4357.

Greenstein, J. P. and Wintz, M. (1961). Methionine. *Chemistry of the amino acid*. John Wiley & Sons. Hoboken, New york. pp: 2125–2155.

Gomes, J. and Kumar, D. (2005). Production of L-methionine by submerged fermentation: A review. *Enzyme and Microbial Technology*. 37:3–18

Hang ,Y. D. and Woodams, E. E.(1987). Production of cellulase by agroindustrial by-product. *Applied Environmental Microbiology*. 33:1293-1296.

Ikram-ul-Haq; Ashraf, H.; Iabal, J. and Qadeer, M.A. (2003). Production of α-amylase by *Bacillus Licheniformis* using an economical medium. *Bioresources Technology*. 87(1):57-61.

Javed, S.A.; Sigher, M.; Nasmaz, H. and Jamil, A. (2011). Enhanced citric acid production by *Asprgillus niger* EB-3 mutant using an inert soild support in molasses medium. *African Journal of Biotechnology*. 10(55):11784-11791.

Kase, H. and Nakayama, K. (1975). O-Acetylhomoserine as an intermediate in methionine biosynthesis in *Arthrobacter paraffineus*, *Corynebacterium glutamicum* and *Bacillus* species. *Agricultural and Biological Chemistry*. 39(3):687-693.

Keshavarz, K. (2003). Effects of reducing dietary protein, methionine, choline, folic acid and vitamin B12 during the late stages of the egg production cycle on performance and eggshell quality. *Poultry Science*. 82:1407–1414.

Kirshnamurth, G. U. (1980). In proceedings of the symposium on waste from food processing Industry:Utilization and disposal. CFTRI, Mysone, India.

Kinoshita, S.; Udaka, S. and Shimono, M. (1957). Amino acid fermentation. 1. Production of L-glutamic acid by various microorganisms. *Journal of General and Applied Microbiology*. 3:193–205.

Konsoula, Z. and Liakopoulou-Kyriakides, M. (2007) Co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates. *Bioresource Technology*. 98 150-157.

Kumar, D.; Garg, S.; Bisaria, V.S.; Sreekrishan, T.R. and Gomes, J. (2003). Production of methionine by a multi-analogues resistant mutant of *Corynebacterium lilium. Process Biochemistry*. 38(8):1165–1171.

Mondal, S., and Chatterjee, S.P. (1994). Enhancement of methionine production by methionine analogue ethionine resistant mutants of *Brevibacterium heali. Acta Biotechnology*. 14 (2):199-204.

Mondal, S.; Das, Y.B. and Chatterjee, S.P. (1996). Methionine production by microorganisms. *Folia Microbiology*. 41:465–72.

Noftsger, S. and St-Pierre, N.R. (2003). Supplementation of methionine and selection of highly digestible rumen undegradable protein to improve nitrogen efficiency for milk production. *Journal Dairy Science*. 86: 958–969.

Nicoloni, L.; Humolstein, V.C and Corilli, A. (1987). Solid state fermentation of orange peed and grape stalk by *Pleurotus ostreatus*. Agrocy be Aegerita and armilloriella mellea. *Journal of Applied Microbiology and Biotechnology*, 26;95-98.

Nigam, J. N. (2000). Cultivation of *Candida langeronii* in sugar cane bagasse hemicellulosic hydrolyzate for the production of single cell protein. *World Journal of Microbiology Biotechnology*. 16;367-372.

Odibo, F. J. C. (1987). Production and charactreization of a pullulanase and a protease from *Thermaactinomyces thalpophilus*. Ph.D thesis. Department of Microbiology, University of Nigeria, Nsukka, Nigeria.

Ozulu, U. S.; Nwanah, O. U.; Ekwealor, C. C., Dike, S. K.; Nwikpo, C. L. and Ekwealor, I. A. (2012) A new approach to screening for methionine-producing bacteria. *British Microbiology Research Journal*. 2(1):36-39.

Pham, C. B.; Galvez, C. F. and Padolina, W. G. (1992). Methionine fermentation by batch fermentation from various carbohydrates. *ASEAN Food Journal*. 7:34–37.

Reershemius, K.H. (2008). Production of L-methionine with *Corynebacterium glutamicum*. Ph.D Dissertation. Von der Fakultät für Lebenswissenschaften der Technischen Universität Carolo Wilhelmina zu Braunschweig zur Erlangung des Grades eines Germany.

Ross, B. (2003). The aminos depression part 2B. www.ross bishop.com. Assessed on May, 2013.

Roy, D. K. and Chatterjee, S. P. (1989). Production of glutamic acid by *Arthrobacter globiformis*. Influence of culture condition. *Folia Microbiology*. 34 : 11-24.

Triforinova (1993). Microbial utilization of agro-by-products. Environmental protection strategies for sustainable development : Malik, A and Grohmann, E. pp. 605.

Umerie, S. C.; Ekwealor, I. A. and Nawabo, I. O. (2000). Lysine production from various carbohydrates and seed meals. *Bioresource Technology*. 75:249–252.

Vandenberghe, L. P. S.; Soccol, C. R.; Padro, C. and Pandey, A. (2008). Comparision of citric acid production by solid-state fermentation in flask, column, tray and drum bioreactors. Applied Biochemistry/ Biotechnology- Part A Enzyme eng. Biotechnology. 118(1-3):293-303.

Vastrad, B.M. and Neelagund, S. E. (2011). Optimization and production of neomycin from different agro industrial wastes in solid state fermentation. *International Journal of Pharmaceutical Sciences and Drug Research*. 3(2): 104-111

Yamashiro, A.; Kubota, K. and Yokozeki, K. (1988). Mechanism of stereospecific production of L-amino acids from the corresponding 5- substituted hydantoins by *Bacillus brevis*, *Agricultural and Biological Chemistry*.52:2857-2863.