Peer-Reviewed /ISSN: 2616-9754 *A J Life Sci. 2018, 1 (2): 95-101*

Original Research Article

Abasyn Journal of Life Sciences

Open Access

Journal home: www.ajlifesciences.com

Isolation, Screening and Methionine Production by Bacillus species isolated from Poultry Waste

C.C. Ezemba[1,*], S. Akagu[2] and N. Uchefuna[3]

[1]*Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University Anambra State, Nigeria.*
[2]*Department of Microbiology, Caritas University Emene Enugu State, Nigeria.*
[3]*Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University Awka Anambra State, Nigeria*

Abstract

The current study was focused on isolating and identifying methionine producing bacteria from poultry waste. The bacteria were isolated from poultry waste and the ones which specifically produced methionine on solid agar medium, using a methionine requiring auxotroph (*Escherichia coli*) were kept for further analysis. Methionine assay was also carried out after fermentation to quantify methionine produced by each isolate. A total of 10 bacteria were isolated of which 2 were confirmed as active methionine producers after halo growth was observed on the solid agar medium. These 2 isolates which produced higher amount of methionine were ultimately selected for further investigation. The methionine producing isolates which were designated PW_1 and PW_3 were identified as *Bacillus* sp. respectively after biochemical tests were carried out. The test carried out include; gram staining, motility test, catalase test, citrate test, hemolysis test, indole test, penicillin sensitivity, oxidase test and crystal colony formation. Where PW_3 (*Bacillus* sp.) produced greater amount of methionine (0.54 mg/l), while PW1 (*Bacillus* sp.) produced 0.20 mg/l after the completion of the methionine assay.

Keywords: Keywords: Methionine production, *Bacillus* species, poultry waste.

Article Info:
Received:
June 1, 2018
Received Revised:
September 14, 2018
Accepted:
September 14, 2018
Available online:
September 25, 2018

Corresponding author:
constancechinyere790@yahoo.com

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

1. INTRODUCTION

Importance of amino acids cannot be over emphasized, as these primary metabolites do not only play the role of building blocks of proteins, cells, tissues, intermediate on the genetic pathway[14]. Methionine is an amino acid which can be applied in the biosynthesis of different vital proteins. This amino acid is important in poultry based diets for the growth. It is known that some diets are limited in nutrients so important amino acids are added to the feed. Furthermore, it is observed that due to lack of methionine, egg shell stability decrease[22,10]. Lack of methionine or deficiency have adverse effects, and it is linked with many diseases and physiological conditions like muscle paralysis, loss of hair, depression etc[29]. It is observed that methionine deficient in the diet can be covered by adding it exogenously in the feed[27].

Recently, synthesis of important amino acids has become a vital area for industries. Amino acid methionine is produced in different ways; by hydrolysing proteins or by chemical synthesis methods. But these processes are costly due to some chemicals[9]. L- Methionine (Fig. 1) can be produced by the enzymatic or microbial fermentation process but these processes require substrates, and it requires a long period to produce and purify these compounds. Hence, there is large interest to produce methionine via microbial fermentation[1, 8, 25, 30]. Different amino acids such as histidine, threonine, etc. have been produced by microbes [13, 19, 16, 23, 24]. There are increasing interests to produce methionine via fermentation process [18, 21, 5, 15]. Also, it is observed that United states biofuel industry that converting cereal grains (animal feed) for production of ethanol in biofuel industry[20, 31,5.] Therefore, animal feeds can be can be augmented with specif amino for complete/balance diet [3, 12, 28].

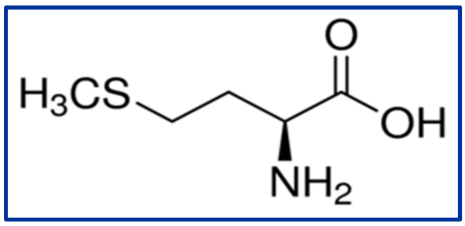

Fig. 1. Structure of L- Methionine

Current study was focused on isolation and identifying methionine producing bacterial cells. It is to reveal a better approach for safe production of methionine using a methionine auxotroph (*Escherichia coli* K-12 4212). This submerged fermentation approach saves time and avoids the hectic method being presently used. With hope that if well developed, this process could lead to the availability of the product locally thereby reducing a heavy dependency on importation or the rigorous potentially harmful alternative methods of methionine production.

2. MATERIALS AND METHODS

2.1 Sample collection and preparation

Poultry waste samples were collected from various spots, depth of 0.1-0.3 cm at Phenoma farm Ngwo, Enugu state, Nigeria. The sample was transported to the laboratory in sterile plastic containers and polythene bags, for microbiological analysis.

2.2 Microorganism used

The culture of the *Escherichia coli* (K-12 4212) a methionine requiring auxotroph was from my supervisor as a gift. Culture of the methionine auxotroph was maintained on Nutrient agar (Oxoid) slants stored at 4 ºC.

2.3 Isolation of Microorganisms

Ten gram of the soil samples was suspended in 100 ml of sterile distilled water contained in 250 ml Erlenmeyer flasks. After agitating the sample using a rotary shaker at 150 rpm for 10 mins, 1ml each of the suspension was serially diluted and Spread plate techniques was employed to introduce 0.1 ml each of 10^{-3} and 10^{-5} dilutions into the Nutrient Agar plates and incubated for 24 hours under ambient temperature (30 ºC), after which the plates were observed for growth. All morphological different colonies were sub cultured and pure cultures were stored on Nutrient Agar slants at 4 ºC. Duplicate plates were prepared for each dilution.

2.4 Preliminary Screening of Isolates for Methionine Production on Solid Medium

The isolates were screened for methionine production using a modified medium[26]. Sterilized plates of minimal agar medium containing glucose, 4.0 g; $(NH4)_2SO_4$, 2.0; K_2HPO_4, 0.05 g; KH_2PO_4, 0.05; $MgSO_4.7H_2O$,

0.1g; $FeSO_4.7H_2O$, 0.01g; $MnSO_4.H_20$, 0.0l g; $CaCO_3$, 0.15 g; Agar, 15.0 g, water, 1 litre; pH, 5.6. The medium (100 ml) which was seeded with 1ml of a 24 h broth culture of the methionine auxotroph, *Escherichia coli* (NCCB1), and then streak inoculated with the isolates. An un-inoculated agar plate served as control.

After 48-72 h incubation at ambient temperature, the plates were examined for growth of the auxotroph. Halo growth of the *E. coli* indicates methionine production by the isolate. The active methionine producers were Gram stained and stored for subsequent screening in submerged medium.

2.5 Screening of Isolates for Methionine Production in Submerged Medium

The preferred isolates were further screened for methionine production in submerged medium[26]; using the same fermentation medium composition without agar, adopting the following procedures;

2.5.1 Seed Inoculum Preparation

The medium for seed culture consist of peptone, 10.0 g; yeast extract, 10.0 g; NaCl, 5.0 g; H_2O, 1 litre. Two 2ml of the medium dispensed in test tube was sterilized at 121 ^oC for 15 mins. Two loops full of 48 h culture of methionine - producing isolates on agar was inoculated into the cooled test tubes and were incubated for 48 h on a Searchtech (HY-2A) test tube shaker at 160 rpm and 30 °C.

2.5.2 Fermentation Experiment

The basal medium composition used was similar to that of the screening on solid medium, but without agar, while the glucose (carbon source) and $(NH_4)_2SO_4$ (nitrogen source) were adjusted to 20.0 g and 10.0 g respectively. The pH was adjusted to 7.2 with 1 N NaOH. Twenty millimetre (20 ml) of the medium in 100 ml Erlenmeyer flask was sterilized in an autoclave at 121 °C for 15 min, 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 prepared and un-inoculated flasks served as control. Methionine accumulation in the broth culture was determined.

2.5.3 Analytical Method for L- Methionine (Methionine assay)

Quantitative determination of methionine in the supernatant without purification was assayed[11]. The broth culture was centrifuged (Searchtech 800D) at 1500 xg for 15 min. To 5 ml of the supernatant, 1ml of 5 N NaOH and 0.1ml of 10% sodium nitroprusside solution were added. The content of the tubes were mixed and was allowed to stand for 10 minutes. To this tube, 5% of glycine solution was added with constant shaking for 10 minutes. Then 2 ml of concentration orthophosphoric acid was added to the tube drop wise. Tubes were allowed to develop color for 5 minutes. The intensity of color was quantified at 540 nm via spectrophotometer. Blank was also introduced containing distilled water and all other reagents. Methionine concentration was calculated from standard curve.

2. RESULTS AND DISCUSSIONS

A total of 10 bacteria isolates were screened and only two microorganisms were recovered as active methionine producers. The degree of the halo growths of the isolates are shown in Table 1, with isolates PW1 and PW3 indicating methionine production. The occurrence of methionine producing bacteria agrees with earlier reports of other researchers[6, 26]. They isolated methionine producing bacteria from native starches and soil. Isolates PW1 and PW3 which were found to be active methionine producers were further used in the subsequent experiments for methionine accumulation in submerged medium.

Table 1. Preliminary screening of isolates for methionine production on solid medium and cultural characteristics on nutrient agar

Isolate	Culture characteristics	Degree of Halo growth of *E. Coli*	Probable Organism
PW1	Creamy, circular, large, rounded, smooth, flat	++	*Bacillus sp.*
PW2	Creamy, circular, small, irregular, smooth, raised	-	*Bacillus sp.*
PW3	Creamy, circular, small, rounded, smooth, flat	++	*Bacillus sp.*
PW4	Milky, circular, large, rounded, smooth, flat	-	*Bacillus sp.*
PW5	Milky, circular, small, rounded, smooth, raised	-	*Bacillus sp.*
PW6	Creamy, circular, small, irregular, smooth, raised	-	*Bacillus sp.*
PW7	Creamy, circular, small, irregular, smooth, raised	-	*Bacillus sp.*
PW8	Milky, circular, large, rounded, smooth, flat	-	*Bacillus sp.*
PW9	Milky, circular, small, irregular, smooth, raised	-	*Bacillus sp.*
PW10	Milky, circular, large, rounded, smooth, flat	-	*Bacillus sp.*

Key: PW = Poultry Waste; - = No methionine production; + = high methionine production; ++ = higher methionine production; +++ = highest methionine production.

From the results obtained in this study, methionine producing microorganisms can be isolated from poultry waste. Table 1 shows methionine produced by the isolates (PW1 and PW3) on solid agar medium, as indicated by the halo growth of *Escherichia coli* (a methionine requiring auxotroph) seeded on the agar medium. Also shown in Table 1 are the cultural morphological characteristics of the isolates.

The active methionine producing isolates were further screened for methionine production in submerged medium; Fig.2 shows the result of methionine production by the isolates in submerged medium. Isolate PW3 gave the best methionine yield of 1.54 mg/ml concentration, and the least production recorded by PW1 1.20 mg/ml.

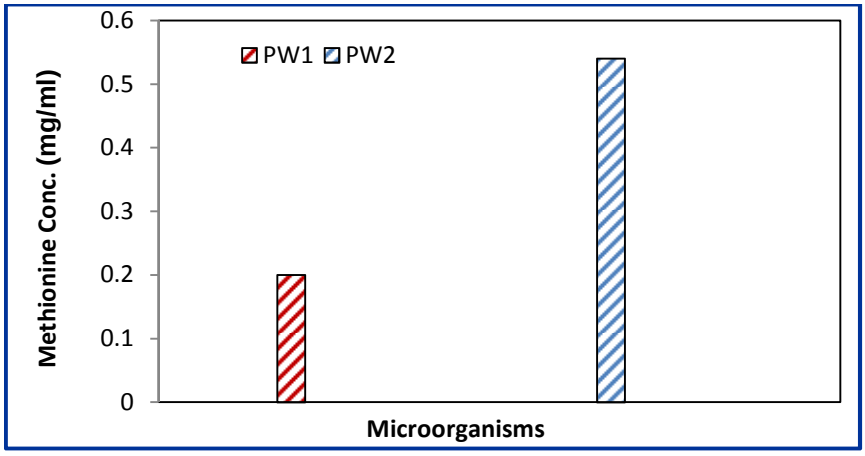

Fig. 2. Screening of isolates for methionine production in submerged medium

The ability of these isolates to produce methionine in submerged fermentation is supported by the works of other researchers[1, 6, 8].They all recorded maximum methionine yield of 1.92, 3.23 and 4.55 mg/ml respectively with different *Bacillus* sp. in submerged fermentation.

Table 2 shows the biochemical tests of the methionine producing isolates. PW1 was found to be *Bacillus* sp and PW3 was also found to be a *Bacillus* sp. There is a specific fermentation period during which microorganisms remain viable and produce maximum quantity of metabolites. After which they start to die

and the quantity of metabolites produced decrease to a significant amount. Maximum methionine production by *Bacillus* sp PW1 and *Bacillus* sp PW3 was observed after a fermentation period of 72 hours. This agrees with the report of other studies that reported maximum methionine production (45 g/l) after 72hours by *Bacillus cereus*[8] and maximum methionine accumulation of 3.48 mg/ml and 1.35 mg/ml by *Lactobacillus plantarum* and Bacillus species respectively after 96hours fermentation[2]. Also, maximum methionine yield of 3.23 and 4.55 mg/ml respectively with two different *Bacillus cereus* strains after 72 hours fermentation were recorded[1, 6]. In this study, the optimum medium volumes were 20 ml for *Bacillus* sp PW1 and *Bacillus* sp PW3. This finding is in line with other works in which a 20% medium/fermenter volume ratio improved methionine yield[1].

Table 2. Gram Stain and Other Biochemical Tests of the Methionine Producing Isolates

Isolate	Gram stain	Motility Test	Citrate Test	Indole Test	Catalase Test	Hemolysis Test	Oxidase Test	Pinicillin Test	Crystal formation	Organism
PW1	+	+	+	-	+	+	+	-	-	*Bacillus Sp.*
PW3	+	+	+	-	+	+	+	-	+	*Bacillus Sp.*

KEY: + = positive; - = negative

4. CONCLUSIONS

The study has clearly revealed that methionine producing microorganisms can be isolated from poultry waste. It has also revealed a better approach to screening for methionine producing organisms using methionine auxotroph. This submerged fermentation approach saves time and avoids the laborious method being presently used. The microbiological process of methionine production if well-developed could lead to the availability of the product locally and this, to some extent, will reduce the importation of the product into the country.

ACKNOWLEGMENTS

The departments of Microbiology Caritas university, Coal City University all in Enugu, Nigeria and Nnamdi Azikiwe University, Awka, Anambra State, Nigeria,

CONFLICT OF INTEREST

All authors declare no conflict of interest regarding this article.

REFERENCES

1. Anakwenze VN, Ezemba CC, and Ekwealor IA. Improved cultural conditions for methionine accumulation in submerged cultivation of *Bacillus cereus* S8. British Microbiology Research Journal, 2014; 4(8): 885 – 895.
2. Anike N and Okafor N. Food Agricultural Nutrition Development. African Journal. 2008;8:77-90
3. Baker DH and HanY. Ideal amino acid profile for chicks during the first three weeks posthatching. Poultry Science, 1994; 73:1441-1447.
4. Campbell KC. Therapeutic Use of d-methionine to reduce the toxicity of platinum-containing anti-tumor compounds and other compounds., 2001. *US Patent* 6:187-817.
5. Chalova VI, Anderson RC,Nisbet DJ and Ricke SC. Biosensors in the animal industry-the need for better nutritional management in the face of rising corn costs and increased ethanol demand. Bioprocess & Bioproducts Technology Trends & Opportunities. Asiatech Publishers Inc*., New Delhi. India*, 2009; Pp 15-53.

6. Dike KS, and Ekwealor IA. Production of L-methionine by *Bacillus cereus* isolated from different soil ecovars in Owerri, south east Nigeria. European Journal of Experimental Biology ., 2012;2(2): 311 – 314.
7. Dike KS, Ekwealor AI and Eziuzor SC. Influence of antibiotics and surfactants additionon growth and methionine productivity by *Bacillus cereus*. Advances in Microbiology, 2013. 3:26-31.
8. Ezemba C.C, Anakwenze VN, Archibong EJ, Anaukwu GC, Obi ZC and Ekwealor CC. Methionine production using native starches and proteins in submerged fermentation by *Bacillus cereus* S8. World Journal of Pharmacy and Pharmaceutical Sciences. 2016; 5(4):2056-2067.
9. Fong CV, Goldgraben GR, Konz J, Walker P And Zank NS. Condensation process for dl-methionine production. AnnArbor Science Publishers.,1981;7:115–194.
10. Funfstuck R, Straube E, Schildbach O, Tietz U."Prevention of reinfection byl-methionine in patients with recurrent urinary tract infection". Medical Microbiology.,1997;92:574–81.
11. Greenstein JP, and Wintz M. Methionine. Chemistry of the amino acid Volume 3. John Wiley & Sons, Hoboken, New York, 1961; pp: 2125 – 2155.
12. HanI K And Lee JH.The role of synthetic amino acids in monogastric animal production. Asian-Australian Journal of Animal Science., 2000;13:543-560.
13. Hermann T. Industrial production of amino acids by Coryneform bacteria. Journal of Biotechnology., 2003;104:155–172.
14. Kalin V, Assena S, Danka O, and Luciano S. Biotechnology in the production of pharmaceutical Industry ingredients: amino acids. Biotechnology and Biotechnology Equipment .,2013;27(2): 3620 – 3626.
15. Kim WK, Froelich, Jr.CA, Patterson PH and Ricke SC. The potential to reduce poultry nitrogen emissions with dietary methionine or methionine analogue supplementation. World's Poultry Science Journal. 2006;62:338-353.
16. Kircher M, and Pfefferle W. The fermentation production of L-lysine as an animal feed additive. Chemosphere .,2001;43: 27 – 31.
17. Kumar J, Bisaria VS, Sreekrishan TR. and Gomes J. Production of methionine by amulti-analogues resistant mutant of *Corynebacteriumlilium*. Process Biochemistry .,2005;38:1165–1171.
18. Kumar D, and Gomes K. Methionine production by fermentation. Biotechnology Advances., 2005;23: 41 – 61.
19. Leuchtenberger W. Amino acids,technical production and uses. Biotechnology.,1996; 6:492.
20. Mayday J. Food, feed, or fuel: ethanol boomer verberates throughout the food system. Meat& Poultry. 2007;53:10-12.
21. Mondal S, Das YB and Chatterjee SP. l methionine production by double auxotrophic mutants of anethionine resistant strain of *Brevibacteriumheali*. Acta Biotechnology.,1994;14:61–66.
22. Noftsger S, St Pierre NR. Supplementation of Methionine and selection of Highly Digestible Rumen Undegradable Protein to Improve Nitrogen Efficiency for Milk Production. Journal of Dairy Science.,2003;86:958–969.
23. Okamoto K, and Ikeda M. Development of industrially stable process for L-threonine fermentation by an L - methionine auxotrophic mutant of *Escherichia coli*. Journal of Bioscience and Bioengineering., 2000;89: 87–89.
24. Okamoto K, Kino K and Ikeda M. Hyper production of L - threonine by an *Escherichia coli* mutant with impaired L - threonine uptake. Biosciences Biotechnology Biochemistry., 2000;61: 1877 – 1882.
25. Odunfa SA, Adeniran SA, Teniola OD, Nordstorm J. Evaluation of lysine and methionine production in some lactobacilli and yeasts from Ogi. International Journal of Food Microbiology ., 2001;63:159–163.
26. Ozulu US, Nwanah OU, Ekwealor CC, Dike SK, Nwikpo CL, and Ekwealor IA. A new approach to screening for methionine-producing bacteria. British Microbiology Research Journal., 2012; 2(1): 36 – 39.
27. Parcell S. Sulfur in human nutrition and applications in medicine. Alternative Medical Reviews .,2002; 7: 22–44.
28. Ricke SC, VanLoo EJ, Johnson MG, O'Bryan CA. Organic Meat Production and Processing. Wiley Scientific journal., 2012; 38:16–24.
29. Rose WC. The nutritive significance of the amino acids. Physiology Review.,1938; 18: 109 – 136.
30. Umerie SC, Ekwealor IA, NawaboI O. Lysine production from various carbohydrates and seedmeals . BioresourceTechnology., 2000;75:249–252.

31.Wisner RN and Baumel CP. Ethanol, exports and livestock: will there be enough corn to supply future needs?. Feed stuffs., 2004;30:20-22.