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Original Research Article

Methionine Production and Optimization Using *Bacillus cereus* Isolated From Soil

J U Mari[1*], Aliyu A[1], Nasiru S[2], A B Muhammad[1], A A Ibrahim[1], H Magaji[3], M Bala[1]

[1]Department of Biochemistry, Faculty of Basic Medical Science Bayero University, Kano
[2]Department of Biochemistry, Faculty of Science Federal University Dutse, Jigawa
[3]Department of IJMB Science, Nuhu Bamalli Polytechnic Zaria, Kaduna

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***Corresponding author:** Jamila Umar Mari
Department of Biochemistry, Faculty of Basic Medical Science Bayero University, Kano

Abstract

Fermentation processes have become a common practice for overproduction of amino acids nowadays as it is cheaper and easier than other processes for commercial production of methionine and other essential amino acids. Methionine is a sulfur-containing essential amino acid required in the diet of humans and other animals for normal growth and functions of the body. It is widely used in feed, pharmaceutical and food industries. The research was focused on fermentative production of Methionine by *Bacillus cereus* isolated from soil. Methionine was produced under optimized conditions (Temperature, pH, Incubation time and agitation rate) using one factor at a time (OFAT). Optimum methionine yield of (4.520 mg/mL) was obtained at temperature of 35^{o}C, pH of 7.5, and agitation rate of 125 rpm and incubation time of 96 h using OFAT. Characterization of methionine produced using *Bacillus cereus* shows similar properties with the commercially produced methionine by using Fourier Transform Infrared Spectroscopy (FTIR) which shows the amino and carbonyl group spectrum at 1640 $^{cm-1}$ and 3257 $^{cm-1}$ respectively. Thin Layer Chromatography (TLC) showed a band of methionine produced by *bacillus cereus* aligned with the commercial methionine.

Keywords: Amino acid, Methionine, *Bacillus cereus*, one factor at a time optimization.

BACKGROUND OF THE STUDY

Currently, the amino acid fermentation industry is so huge that more than 5 million metric tons of amino acids are manufactured annually all over the world, and this number continues to grow (Shin-ichi and Hashimoto, 2016). Industrial production of methionine was first done by Degussa AG (Evonik) in the 1950s. The amount of DL-methionine, produced chemically, reached to around 850,000 tons annually in 2013. Efforts to produce methionine using microbial fermentation have been ongoing for the last three decades. Since the 2000s, extensive metabolic engineering has been conducted on major hosts such as *Corynebacterium glutamicum* and *Escherichia coli* (Jihyun *et al.,* 2016).

These factors (pH, Temperature, Incubation time and Agitation rate) can be optimised to achieve faster bacterial growth and maximum methionine production. The conventional method for achieving this is the one-factor-at a-time (OFAT) technique which is tedious, laborious, time consuming and often ignores the interactive effect between different factors.

The demand of L-Methionine (L-Met) has increased in recent years due to the rapid growth of feed additive market driven by the globally increasing consumption of meat and milk products as a source of protein and other nutrients (Hai-Yan Zhou *et al.,* 2019). However, chemical routes for L-Met synthesis result in serious environmental pollution, requires lot of procedures and equipment, high cost of production and contain toxic substances such as hydrogen cyanide, prophylene and methyl mercaptan (Li *et al.,* 2017). Therefore, a low-cost, environmentally friendly production of pure L-Met with microorganisms based on natural renewable resources is becoming more attractive and imperative (Hai-Yan Zhou *et al.,* 2019). Furthermore, few works were reported on optimization parameters (Temperature, pH, and incubation time and agitation rate) of methionine production. Hence, optimization parameters may be vital in increasing the yield of methionine production by methionine

producing bacteria isolated from soil. The aim of the study is to produce and optimize Methionine using Bacillus cereus isolated from freshly collected soil.

MATERIALS AND METHODS

Chemicals and Reagents

Luria Bertani Agar, Nutrient Agar, Mannitol Agar and Starch Agar were purchased from Titan Biotech Ltd. (India). Ninhydrine, Calcium Carbonate, Glucose and Sodium Chloride were from BDH Laboratory Ltd. Ammonium Sulfate, Citrate, Propanol were from M & B Laboratory. Standard Methionine, Chloroform and Hydrogen Chloride were from Loba Chemie Laboratory Ltd. NaOH was from Molychem Ltd and Acetone from Guanddong Guanghua Chemical Factory Co., Ltd. (China).

Sample Collection

The samples were collected from Jere LGA, Borno State. The soil sample was collected and transferred safely to the laboratory in a clean, sterilized polythene bag to avoid any contamination during collection. The soil sample was stored neatly in a laboratory locker inside an air-tight container for safety.

Isolation of Bacteria from Soil Sample

The soil sample was prepared by serial dilution method. Exactly 9ml of distilled water was taken to 5 different test tubes and 1 mL of the soil liquid suspension was serially transferred to each 9mL distilled water containing test tubes. Precisely, 1 mL of the last two test tubes was used for the preparation of culture medium using a pour plate method. Plates were kept in an incubator at 35°C overnight. The growth of separate colonies was observed. Pure culture was isolated using streak plate technique on the solid prepared agar surface and incubated at 35°C for 24 h.

Morphological and Biochemical Identification

Bacterial isolates were identified by Gram staining, endospore, motility test, to study morphological characteristics. Different biochemical tests including catalase test, starch hydrolysis test, Voges Proskauer (VP) test, citrate test, urease test, indole test was performed to study the physiological characteristics of these amino acid producing bacteria according to Bergy's Manuel of bacteriology (Brenner *et al*., 2015).

Screening Method for Methionine Production

According to Gutcho (1973) with some little modification, the screening media was dissolved in 100ml of distilled water and Autoclave for 30 min at 121°C and 15lbs pressure. 20 mL of the screening medium was taken to four different test tubes and a loopful of 24 h old cultures of bacterial isolates were inoculated into each fermentation medium in test tube and incubated at 37°C for 48 h on a shaker at 125 rpm. Samples were taken after 48 h and centrifuged at 10,000 rpm for 15 min; the supernatant was then examined for methionine production qualitatively and quantitatively using thin layer chromatography (TLC) and spectroscopy respectively.

Screening of Bacterial Isolates for Methionine Production Media Formulation

Fermentation media was formulated to screen isolates for Methionine production as described by Kase and Nakayama (1975), with a little modification (Dike and Ekwealor, 2012) and quantification shown in (Table 1).

Table 1: Composition of the Primary Screening Medium for Methionine Producing Bacteria

Ingredients	Quantity
Glucose	4 g
Ammonium sulfate	0.5 g
Biotin	0.001 g
$CaCO_3$	2 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$MgSO_4$	0.001 g
$FeSO_4$	0.01 g
Water	100 mL

The media was prepared and sterilized at 121ºC and 15lbs pressure for 15-20 min. The initial pH of the fermentation media was kept neutral in screening stage. Screening was done after a fermentation setting of the bacterial growth in a basal media for 48 h in an incubator shaker at 30ºC. Both quantitative and qualitative analyses are done to determine methionine production.

Qualitative and Quantitative Analysis of Methionine

Precisely 5ml of fermented broth was centrifuged at 10,000 rpm for 15 min. Supernatant containing amino acids was separated and subjected to qualitative and quantitative identification of methionine using acid ninhydrin method as described by (Farah, *et al.*, 2012).

In quantitative analysis, ninhydrin reagent was prepared by dissolving 0.2 g of ninhydrin powder in 20 mL Acetone. Exactly 2 mL of each sample (fermented broth) was taken in test tubes and 0.1 mL of acid ninhydrin reagent was added. The tubes were heated for 10 min in 60ºC water bath and were cooled at room temperature. Optical density (absorbance) was recorded at 580 nm and quantity estimated using a standard curve prepared by taking known concentrations of methionine.

The standard curve of methionine was done using (0.2, 0.4, 0.6, 0.8, 1.0) mg/mL concentration, following the same procedure used to identify the quantity of methionine produced by bacterial isolates using acid ninhydrin method.

One Factor at a Time (OFAT) for Methionine Yield

For optimum growth of the bacterial isolates, four parameters i.e., temperature, Incubation time, agitation rate and pH will be considered one factor a time (OFAT). For determination of optimum pH, Luria Bertani (LB) broth was prepared and pH was adjusted at 5.5, 6, 6.5, 7 and 8. Exactly 10 mL of the LB broth was dispensed in 15 bottles (triplicate for each pH) and inoculated with 1 loopful freshly prepared culture of bacterial isolate. After an incubation period of 24 h, absorbance was taken at 580 nm using Jenway 6705UV/V Spectrophotometer.

For determination of optimum temperature, 10 mL LB broth was added in 18 bottles (in triplicate for each temperature), autoclaved and inoculated with 1 loopful of freshly prepared culture of bacterial isolate by overnight growth at room temperature in LB agar. At the best pH from the previous result, the 12 bottles were incubated at 25ºC, 30ºC, 35ºC, 40ºC and 45ºC. After an incubation period of 24 h, their absorbance will be taken at 580 nm using Jenway 6705UV/V Spectrophotometer. The determination of incubation time and optimal temperature goes hand in hand i.e. the optimal condition of methionine production is determined at optimal temperature per incubation time for all bottles. Hence, 30ºC, 35ºC, 40ºC, 45ºC and 50ºC is determined at 24 h, 48 h, 72 h and 96 h. After the proposed incubation period, their absorbance was taken at 580 nm using Jenway 6705UV/V Spectrophotometer. For determination of agitation rate, 10 mL LB broth will be added in 12 bottles (in triplicate for each rpm), autoclaved and inoculated with 1 loopful of freshly prepared culture of bacterial isolate by overnight growth at 37ºC in LB agar. At the optimum pH, temperature and incubation time from the previous result, sterilized bottles will be incubated with LB broth at different agitation rates of 110 rpm, 120 rpm, 125 rpm, 130 rpm and 140 rpm. After an optimized incubation time (96 h), their absorbance was taken at 580 nm using Jenway 6705UV/V Spectrophotometer.

Statistical analysis

The average data and standard deviations were obtained from the triplicate of experiments for each run using Microsoft Excel (Office, 2019). A confidence level of 95% was used in this study. Any p-vales less than 0.05 were considered significant and vice versa.

RESULTS

Results obtained for morphological identification of *Bacillus specie* were presented in table 1. In bacterial isolation, all isolates showed good growth on luria-bertani during the isolation process, 4 organisms designated JS1, JS2, JB1 and JB2. The isolates reacted positively to gram staining, spore formers and rod shaped. JS1, JB1 and JB2 are bacilli in shape under microscopic view, form spores, their cell arrangement is in chain and all isolates were negative for capsule formation. JS2 is cocci under microscopic view, no spores are formed, its cell arrangement is in bunch and it's negative for capsule formation.

Results obtained for the biochemical identification of isolates are presented in Table 2, according to Bergey's manual of systematic bacteriology (Brenner *et al.,* 2015). The bacilli isolates were positive in starch hydrolysis, VP test, Cell diameter (width), citrate test and were not able to grow in 6.5% NaCl. Catalase test was positive for isolates JS1, JS2 and negative for isolates JB1 and JB2. The cocci isolate JS2 does not ferment mannitol, and was negative to citrate and yellow pigment.

Table 1: Morphological Characterization of the Bacteria isolates viewed under microscope

Isolation Code (IC)	Gram Reaction	Morphology	Cell Arrangement	Spore	Capsule formation
JS1	Positive	Bacilli	Chain	Positive	Negative
JS2	Positive	Cocci	Bunch	Negative	Negative
JB1	Positive	Bacilli	Chain	Positive	Negative
JB2	Positive	Bacilli	Chain	Positive	Negative

JS1= Plate 1 Small colony JS2= Plate 2 Small colony JB1= Plate 1 big colon JB2= Plate 2 big colony

Table 2: Biochemical Characterization of Isolates using Bergey's Manual of Determinative Bacteriology

Isolation Code	**JS1**	**JS2**	**JB1**	**JB2**
Starch hydrolysis	Positive	-	Positive	Positive
VP test	Positive	-	Positive	Positive
Cell diameter (width)	Positive	-	Positive	Positive
Citrate	Positive	Negative	Positive	Positive
6.5%NaCl growth	Negative	-	Negative	Negative
Catalase Test	Positive	Positive	Negative	Negative
Mannitol fermentation	-	Negative	-	-
Identified Isolates	***Bacillus cereus***	***Staphylococcus spp.***	***Bacillus cereus***	***Bacillus anthracis***

JS1= Plate 1 Small colony JS2= Plate 2 Small colony JB1= Plate 1 big colon JB2= Plate 2 big colony.

Results obtained for the optimum production of methionine at different parameters (pH, temperature, incubation period and agitation rate) using one factor at a time (OFAT) technique are presented in Figure 1, 2, 3 and 4. These were achieved by varying one factor and keeping other factors constant (Okoroma *et al.*, 2012).

Figure 1 Optimum yield of methionine was achieved at pH of 7.5 (2.53 mg/mL),, increase in methionine yield was observed with increase in pH from 5.5 to 7.5 and declined at pH of 8 keeping other factors constant as presented in.

Figure 2 presented the optimum temperature for methionine production using *Bacillus cereus*, optimum yield of methionine (2.32 mg/mL) was obtained at 35^{o}C, increase in temperature result to increase in methionine yield. However, the yield declined at the temperature of 40^{o}C.

Figure 3 presented the optimum incubation time for methionine production using *Bacillus cereus*, optimum yield of methionine (4.52 mg/mL) was obtained at incubation time of 96 h. There was gradual increase in methionine yield with increasing incubation time from 24 h to 96 h and later declined at 120 h.

Figure 4 presented the optimum agitation rate for methionine production using *Bacillus cereus*, optimum yield of methionine (2.34 mg/mL) was obtained at agitation rate of 125 rpm. Increase in agitation rate results to decrease in methionine yield.

Results for the Fourier transform infrared (FTIR) of Methionine produced using *Bacillus cereus* and the commercial Methionine are presented in Table 3. The spectrum of the produced Methionine revealed the presence of functional groups such as carboxylic group (COOH) at 3257 $^{cm-1}$, amine (-NH2) at 1640 $^{cm-1}$ that are similar with the spectrum of the commercial methionine of carboxylic group and amine respectively.

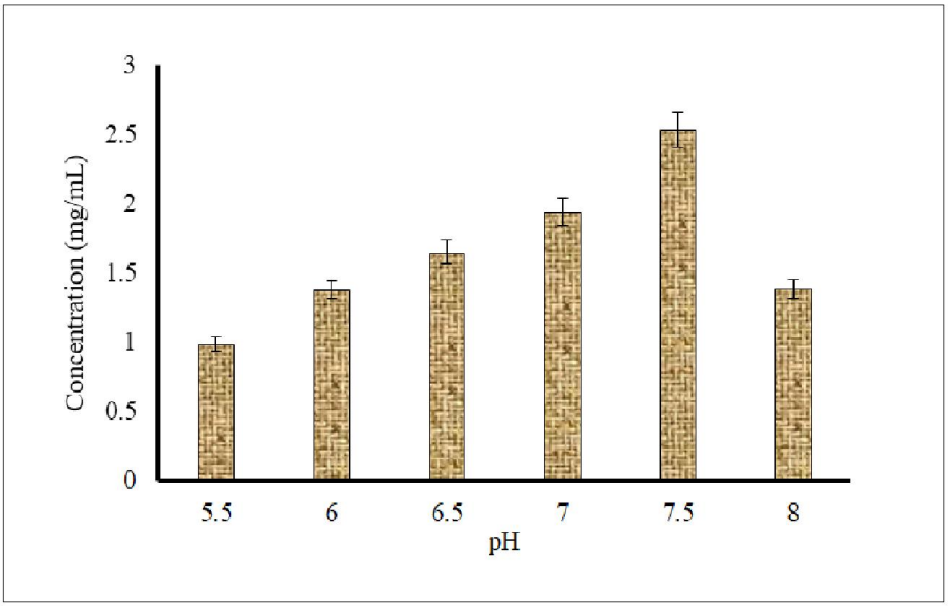

Figure 1: Effect of pH on Methionine production at constant temperature, incubation time and agitation rate (30^{o}C, 48 h and 125 rpm respectively) using one factor at a time (OFAT)

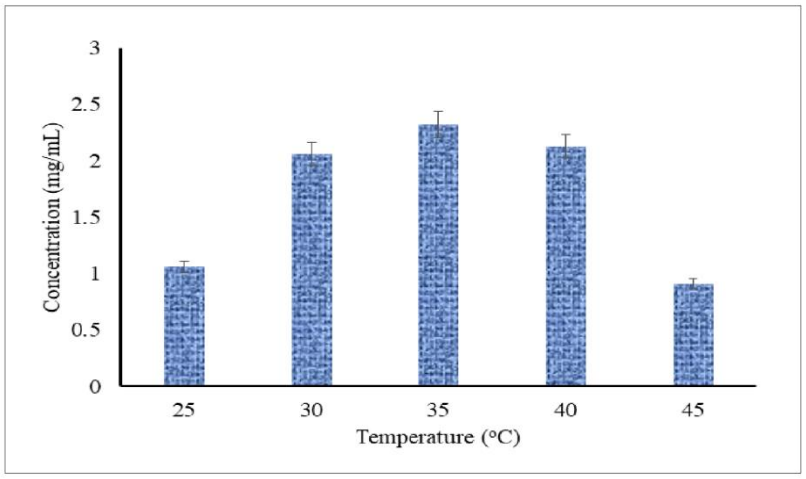

Figure 2: Effect of temperature on Methionine production at constant pH, incubation time and agitation rate (7.5, 48 h and 125 rpm respectively) using one factor at a time (OFAT)

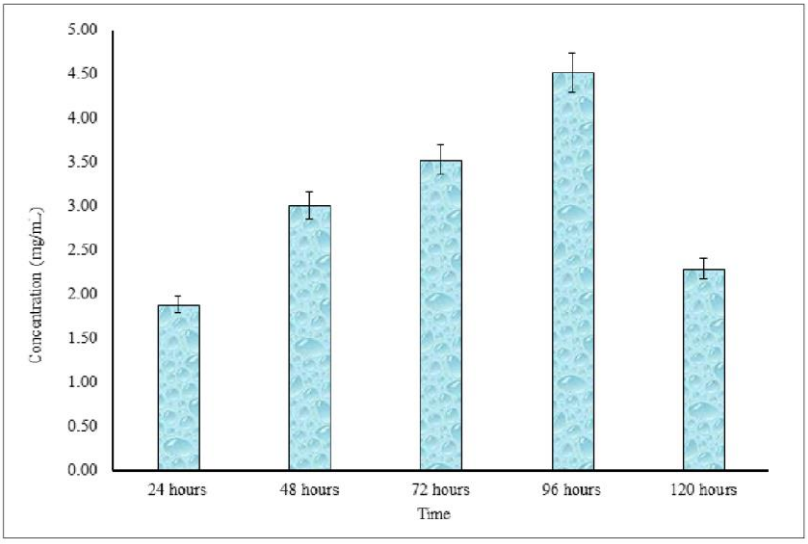

Figure 3: Effect of incubation time on Methionine production at constant pH, temperature and agitation rate (7.5, 35ºC and 125 rpm respectively) using one factor at a time (OFAT)

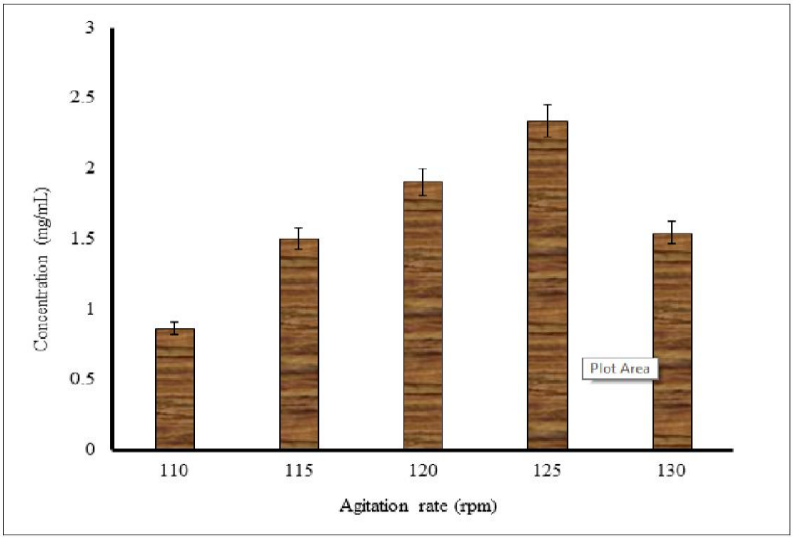

Figure 4: Effect of agitation rate on Methionine production at constant pH, temperature and incubation time (7.5, 35ºC and 96 h respectively) using one factor at a time (OFAT)

Table 3: FTIR result of a) Commercial methionine b) Methionine produced by Bacillus cereus

Functional Group	Methionine Produced Using *Bacillus Cereus* (CM^{-1})	Commercially Produced Methionine (CM^{-1})
OH	3257	3260
NH_2	1640	1640
C=O	2113	2100
SH	2525	2550

Result obtained for methionine characterization using thin layer chromatography was presented in **Figure 5**, showing the single band for standard methionine having the same Rf-value with the corresponding band of the methionine produced by the *Bacillus cereus* on the TLC plate.

Result of the boiling point Characterization presented in figure 6 demonstrated that the boiling point of Methionine produced by *Bacillus cereus* is higher than Standard Methionine.

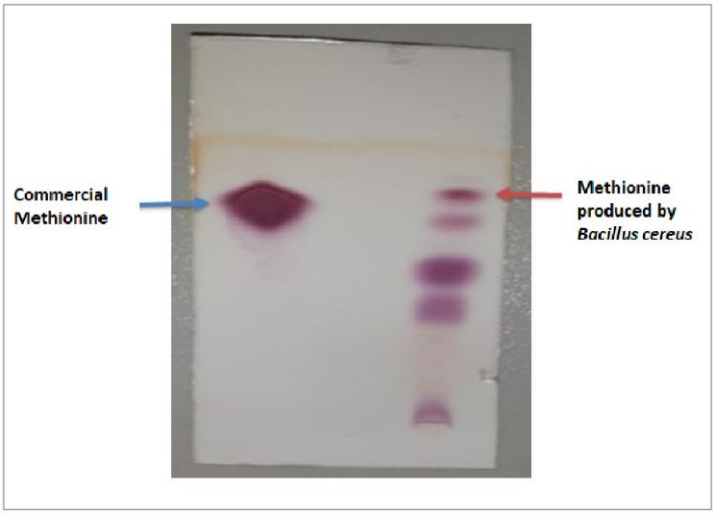

Figure 5: TLC showing the band for standard methionine and band of methionine produced by the Bacillus cereus on the TLC plate.

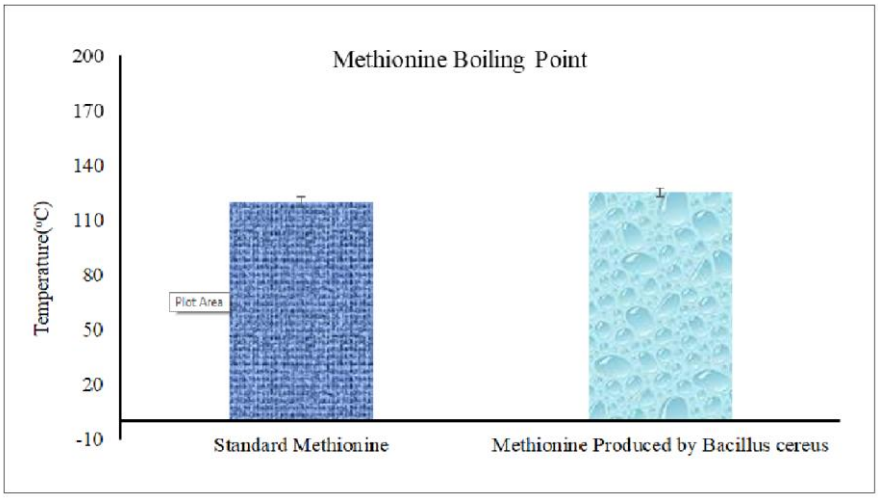

Figure 6: Shows the boiling point of Standard Methionine and Methionine produced by Bacillus cereus

DISCUSSION

In this study, four bacterial isolates were identified according to the morphology and biochemical characterization as shown in table 1 and 2 respectively, *Bacillus cereus* can enhance the production yield of 4 essential amino acids, methionine inclusive. The use *Bacillus* cereus for methionine production by many workers has been observed. Although they are not yet known to be overproducers like species of *Corynebacterium* and *Brevibacterium* (Anakwenze *et al* 2014; Dike and Ekwealor 2012). High methionine yield

was obtained by ***Bacillus cereus***, which is in contrast with the findings of Imran and Wei (2019).

Effect of initial medium pH experiment was carried out ranging from pH 5.5 to pH 8 Maximum methionine yield of 2.53 mg/mL was obtained at pH of 7.5 as shown in figure 1 which is in disagreement with the work of Narayana *et al.,* 2017 that reported maximum yield of methionine of 2.13 g/L at pH 7. Most microbes grow best around neutral pH values (6.5 -7.5), some microorganisms produce acid as they grow. This acid is excreted and brings down the pH or the surrounding environment. This brings bacterial growth to a halt unless something else in the environment neutralizes the bacterial acid. The effect of differing pH on methionine generation by *C.glutamicum* was inspected. These pH values were balanced with HCl and NaOH by using pH digital meter. The isolate was inoculated into shake flask at varied pH levels. These were incubated for 48 h on an orbital shaker. Results showed that methionine production was a function of pH as appeared in the figure mentioned above.

The effect of temperature on methionine production was observed by varying temperatures ranging from 25^{o}C to 45^{o}C and keeping all other variables constant. Maximum yield of 2.32 mg/mL methionine was obtained at 35^{o}C as demonstrated in figure 2**.** As temperature increases methionine generation also increases until maximum yield was obtained at 35^{o}C as the temperature increases, methionine production decreases. This is mainly due to the fact that high temperatures are lethal to microorganisms thereby decreases fermentation rate. At high temperatures enzymes move too fast and denaturation takes place. Ideal temperature of 35^{o}C acquired here is in line with the work of Mohanta *et al.*, (2017), for maximum methionine production and contrary with the work of Narayana *et al.,* (2017), that reported 2.16 g/L at 30^{o}C.

The effect of incubation time on methionine production was observed by varying time ranging from 24 h to 120 h and keeping all other variables constant. Maximum yield of methionine was obtained 4.52 mg/mL after 96 h of fermentation as shown in figure 3 which is in agreement with the result obtained by Dike and Ekwealor (2012): Narayana *et al.,* (2017), which reported 4.55 mg/mL and 4.6 g/L respectively, after 96 h of fermentation. The relationship between methionine production and sugar consumption is in good agreement with results reported by Dike and Ekwealor (2012), Anike and Okafor, (2008). According to Javed *et al.*, (2011), decline in methionine production in *Bacillus cereus* after 4 days could be attributed to the age of the bacteria, depletion of sugar content and decreased available nitrogen in the fermentation medium. Microbial production of metabolites usually starts after a lag phase of one day and reaches maximum at the onset of stationary phase or late.

The effect of agitation on methionine production was observed by varying agitation rate ranging from 110 rpm to 130 rpm and keeping all other variables constant. The influence of differing agitation rate on growth and methionine production was evaluated. Agitation is very important in fermentation flask since oxygen is low solubility nutrient. Oxygen transfer capabilities in the flask controls the growth and product formation. Inoculum vessel containing liquid medium was agitated to obtain homogeneity. The effect of agitation on L-methionine fermentation was shown in figure 4. As agitation speed increased from 110 to 125 rpm, methionine production increased rapidly and declined at 130rpm, maximum production of 2.34 mg/ml methionine obtained at 125 rpm which is in agreement with the work of Shakoori *et al.,* (2011) that reported maximum yield of 10 g/L at 125 rpm and contrary to the work of Narayana *et al.,* (2017) which reported maximum production of 2.36 g/L methionine obtained at 170 rpm. Lee *et al.,* (2012), suggested that agitation rates above 200 rpm will prompt denaturation of enzymes with low production of metabolites.

However in this research, the methionine produced by *Bacillus cereus* was characterized using Fourier transform infrared (FTIR) and the spectrum of the produced methionine revealed the presence of functional groups, amino and carboxyl group spectrum at 1640 $^{cm-1}$ and 3257 $^{cm-1}$ respectively that are similar with the commercially produced methionine (Table 3). Thin layer chromatography (TLC) (figure 5) of the methionine produced by *Bacillus cereus* was determined to show the similarities between the produced methionine and the commercial methionine and were found to be comparable and thus confirming what was produced is methionine. TLC showed a band aligned with the commercial methionine and the presence of 3 other amino acids. Boiling point result presented in Figure (figure 6) illustrated that the boiling point of Methionine produced by Bacillus cereus is higher than Standard Methionine. The difference in boiling point may be caused due to some impurities in the methionine produced, but the two substances were comparable with no significant differences with P-value > 0.05 and what was produced was confirmed to be methionine.

CONCLUSION

This research study revealed methionine production using microorganism isolated from the soil. The microorganism was biochemically and morphologically identified as *bacillus cereus*. Methionine was produced under optimum conditions of (Temperature, pH, incubation time and agitation rate) by preliminary OFAT. High yield of the methionine was produced (4.520 mg/mL) was obtained at temperature of 35^{o}C, pH of 7.5, agitation rate of 125 rpm and incubation time of 96 h using OFAT. The compound produced was characterized by (Boiling point, FTIR and TLC) confirmed the presence of

methionine and other amino acids produced by *Bacillus cereus* isolated from the soil. Methionine producing organisms are well distributed in nature, most bacteria specie are competent of producing methionine and environmental factors affect methionine production.

REFERENCES

- Anakwenze, V. N., Ezemba, C. C., & Ekwealor, I. A. (2014). Optimization of fermentation conditions of Bacillus thuringiensis EC1 for enhanced methionine production. *Advance in Microbiology*. 4: 344-352
- Anike, N., & Okafor, N. (2008). Secretion of methionine by microorganisms associated with Cassava fermentation. *African Journal of Food, Agriculture, Nutrition and Development*, *8*(1), 77-90.
- Brenner, D.J., Krieg, N., Garrity, G., & Staley, J., (2015). The proteobacteria, Part B: the Gamma-proteobacteria. In: Bergey's Manual of Systematic Bacteriology, vol. 2. Springer, pp. 323-376.
- Dike, K. S., & Ekwealor, I. A. (2012). 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.
- Shakoori, F. R., Butt, A. M., Ali, N. M., Zahid, M. T., Rehman, A., & Shakoori, A. R. (2012). Optimization of fermentation media for enhanced amino acids production by bacteria isolated from natural sources. *Pakistan Journal of Zoology*, *44*(4).
- Zhou, H. Y., Wu, W. J., Niu, K., Xu, Y. Y., Liu, Z. Q., & Zheng, Y. G. (2019). Enhanced L-methionine production by genetically engineered Escherichia coli through fermentation optimization. *3 Biotech*, *9*(3), 1-11. https://doi.org/10.1007/s13205-019-1609-8
- Imran, S. Z., & Wei, L. S. (2019). Selected Essential Amino Acid Enhancement by Bacillus cereus from Solid State Fermentation of Soy Pulp Through Carbon Concentration and Fermentation Period. *J Aquac Res Development*, *10*(566), 2.
- Sadia, J., Muhammad, A., Munir, A. S., Haq, N., & Amer, J. (2011). Enhanced citric acid production by Aspergillus niger EB-3 mutant using an inert solid support in molasses medium. *African journal of Biotechnology*, *10*(55), 11784-11791.
- Shim, J., Shin, Y., Lee, I., & Kim, S. Y. (2016). L-methionine production. *Amino Acid Fermentation*, 153-177. *DOI: 10.1007/10_2016_30*
- Lee, I., K. Lee., K. Namgoong., Y.S.Lee., & Enzyme Micribiol., Technol (2012). 30, 798-803
- Li, H., Wang, B. S., Li, Y. R., Zhang, L., Ding, Z. Y., Gu, Z. H., & Shi, G. Y. (2017). Metabolic engineering of Escherichia coli W3110 for the production of l-methionine. *Journal of Industrial Microbiology and Biotechnology*, *44*(1), 75-88. 1. https ://doi.org/10.1007/s1029 5-016-1870-3
- Mohanta, M. K., Islam, M. S., Haque, M., & Saha, A. K. (2016). Isolation and characterization of amino acid producing bacteria from cow dung. *J. Microbiol. Biomed. Res*, *3*(2), 1-8.
- Narayana, V.A., Vesipriya, A., Ventata, N.R., Swami, A.V.N., Sumalatha, B. & Vijaya, L.M., *Res.J. Pharm.Biol.Chem. Sci*., 4: 1489-1497 (2017).
- Okoroma, E. A., Garelick, H., Abiola, O. O., & Purchase, D. (2012). Identification and characterisation of a Bacillus licheniformis strain with profound keratinase activity for degradation of melanised feather. *International Biodeterioration & Biodegradation*, *74*, 54-60.
- Ali, N. M., Shakoori, F. R., & Shakoori, A. R. (2011). Improvement in methionine production by local bacterial isolates. *Pakistan Journal of Zoology*, *43*(3). *pp. 611-614, 2011*.
- Hashimoto, S. I. (2016). Discovery and history of amino acid fermentation. *Amino Acid Fermentation*, 15-34. DOI: 10.1007/10_2016_24