

# Optimization of Fermentation Conditions of *Bacillus thuringiensis* EC1 for Enhanced Methionine Production

V. N. Anakwenze\*, C. C. Ezemba, I. A. Ekwealor

Department of Applied Microbiology and Brewing, Nnamdi Azikwe University, Awka, Nigeria  
Email: \*[ynanakwenze@yahoo.com](mailto:ynanakwenze@yahoo.com)

Received 12 March 2014; revised 12 April 2014; accepted 30 April 2014

Copyright © 2014 by authors and Scientific Research Publishing Inc.  
This work is licensed under the Creative Commons Attribution International License (CC BY).  
<http://creativecommons.org/licenses/by/4.0/>



Open Access

---

## Abstract

*Bacillus thuringiensis* EC1, isolated from the fermented oil bean seed, *Pentachletra macrophila* Benthon, produced a methionine yield of 1.89 mg/ml. The influence of cultural conditions on methionine accumulation by *B. thuringiensis* EC1 showed that a 20% medium/fermenter volume ratio and a 5% inoculum size increased methionine yield. The carbon of choice was maltose and at 8% level stimulated methionine production. Among the nitrogen sources studied, ammonium sulphate was found to be the best and at 1% concentration produced a methionine yield of 2.56 mg/ml. All growth-promoting substances and their mixtures enhanced methionine accumulation by *B. thuringiensis* EC1. The effect of Vitamins on methionine production showed that riboflavin and thiamine HCl at 1.0 µg/ml yielded 2.49 mg/ml and 2.80 mg/ml methionine respectively. The influence of bivalent metals on methionine accumulation indicated that Zn<sup>2+</sup> at all concentration stimulated methionine production. Mg<sup>2+</sup> and Ba<sup>2+</sup> at 0.1 µg/ml and 10.0 µg/ml respectively improved methionine yield. Optimizing the cultural conditions of *B. thuringiensis* EC1 in submerged medium gave a methionine yield of 3.18 mg/ml.

## Keywords

Bacillus, Methionine, Optimization, Submerged Medium, Maltose

---

## 1. Introduction

Methionine, an essential amino acid was first isolated by Mueller [1]. High levels of methionine can be found in nuts, meat and some other plant foods. However, in humans and animals, this amino acid cannot be produced

\*Corresponding author.

and is therefore an important nutrient additive in their feeding. The impact of L-methionine on animal nutrition and the consequences of its absence as a nutritive feed additive have been investigated. It was observed for poultry that the stability of shells decreases just as the milk production in cow does [2].

Currently, methionine is produced either by chemical synthesis or hydrolyzing proteins. These processes are expensive. Chemical synthesis produces a mixture of D- and L-methionine [3] whereas hydrolysis of proteins leads to a complex mixture from which methionine must be separated. Biologically active L-methionine can be produced either by enzymatic synthesis (bioconversion of precursors), or by submerged fermentation using microorganisms [3]. Because fermentation processes have been able to inexpensively provide many other amino acids, there is a significant interest in developing a microbial process for commercial production of methionine [4] [5].

A number of microorganisms capable of producing amino acids have been isolated and amino acids such as lysine, threonine, isoleucine and histidine have been produced successfully by fermentation [6] [7]. Attempts have been made to overproduce biologically active L-methionine using fermentation [8]-[10].

This study was conducted to examine the influence of cultural conditions on methionine accumulation by *Bacillus thuringiensis* EC1.

## 2. Materials and Methods

### 2.1. Microorganism

*Bacillus thuringiensis* EC1, isolated from fermented oil bean seeds, *Pentachletra macrophila* Benth and identified based on 16S rRNA sequencing at MacroGen Incop., Republic of Korea was used. The organism was maintained on Nutrient Agar (Lab M) slant at 4°C.

#### Fermentation in Submerged Medium

Fermentation was carried out following the method described by Ozulu *et al.* [11]. A loopful of a 24 h culture of *Bacillus thuringiensis* EC1 was inoculated into 1 ml of a sterile seed medium (peptone, 10.0 g; yeast extract, 10.0 g; NaCl, 5.0 g; H<sub>2</sub>O, 1 L; pH 7.2) in a test tube and incubated at 30°C on a VWR DS2-500-2 orbital shaker at 160 rpm for 24 h. A 100 ml Erlenmeyer flask containing 20 ml of the sterile fermentation medium {KH<sub>2</sub>PO<sub>4</sub>, 0.05 g; K<sub>2</sub>HPO<sub>4</sub>, 0.05 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.001 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.001 g; CaCO<sub>3</sub>, 20.0 g; (basal medium), glucose, 20.0 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10.0 g; H<sub>2</sub>O, 1 L; pH 7.2} was inoculated with 1 ml (ca.  $3.15 \times 10^6$  cell/ml) of the seed medium. The flasks prepared in duplicates were incubated at 30°C on a shaker (160 rpm) and the broth culture assayed for methionine accumulation after 72 h. Uninoculated flasks served as control.

#### Methionine Assay

Methionine was assayed for, following the method described by Greenstein and Wintz [12]. The broth culture was centrifuged at  $1500 \times g$  for 15 min and 5 ml of the supernatant in a test tube was added 1 ml of 5 N NaOH and 0.1 ml of 10% sodium nitroprusside solution. The tube was thoroughly shaken and the mixture allowed to stand for 10 min. Then 2 ml of 3% aqueous solution of glycine was added to the reaction mixture with frequent shaking over a period of 10 min. After an additional 10 min interval, 2 ml of concentrated orthophosphoric acid was added drop wise to the mixture and the test tube properly shaken. Colour development was allowed to proceed for 5 min and colour intensity measured at 540 nm in a spectrophotometer (PerkinElmer Lambda 35 UV-VIS). The methionine yield was extrapolated from a standard methionine curve.

### 2.2. Optimization of Fermentation Conditions for Methionine Production

#### 2.2.1. Effect of Medium/Fermenter Volume Ratio and Inoculum Size on Methionine Production

The effects of medium/fermenter volume ratio (volume of the culture medium: volume of fermentation flask) and inoculum size on methionine production by *Bacillus thuringiensis* EC1 were examined. Erlenmeyer flasks (100 ml) with different volumes (20, 25, 30 ml) of the sterile fermentation medium were each inoculated with 1 ml of seed inoculum. Each 1 ml contains a loopful (ca.  $3.15 \times 10^6$  cell/ml) or two loopfuls (ca.  $7.56 \times 10^8$  cell/ml). The flasks were incubated and methionine accumulation determined as previously described.

#### 2.2.2. Effect of Carbon Source

Different carbon sources (Glucose, Maltose, Mannitol, Sucrose, Lactose) were studied for their effects on me-

thionine accumulation by *B. thuringiensis* EC1. A 100 ml Erlenmeyer flask containing 20 ml of the basal medium, carbon source 20.0 g,  $(\text{NH}_4)_2\text{SO}_4$ , 10.0 g, pH 7.2 was inoculated with 1 ml of a 24 h culture of the seed inoculum. Fermentation processes and methionine determination were as earlier described. The carbon source with the highest methionine yield was used for further studies.

Effect of varying concentrations (20, 40, 60, 80, 100 g/l) of maltose was also investigated.

### 2.2.3. Effect of Nitrogen Source

Effect of different nitrogen sources (ammonium sulphate, ammonium chloride and urea) on methionine production was examined. A 100 ml Erlenmeyer flask containing 20 ml of the basal medium, maltose 20.0 g, nitrogen source 10.0 g, pH 7.2 was inoculated with 1 ml of a 24 h culture of the seed inoculum. Fermentation was carried out as previously described and methionine accumulation determined from the broth culture. The nitrogen source with the highest methionine yield was used for further studies.

Effect of varying concentrations (10, 20, 40, 60, 80 g/l) of ammonium sulphate was also studied.

### 2.2.4. Effect of Growth Stimulators

The influence of 0.1% (w/v) yeast extract, peptone, casein and their mixtures on methionine yield by *B. thuringiensis* EC1 was examined. Methionine was determined as previously described.

### 2.2.5. Influence of Vitamins

Fermentation process was conducted to determine the influence of varying concentrations (0.10, 1.0, 5.0, 10.0  $\mu\text{g/ml}$ ) of riboflavin, pyridoxine, nicotinic acid and thiamine HCl on methionine production by *B. thuringiensis* EC1.

### 2.2.6. Influence of Bivalent Metals

Fermentation process was carried out to investigate the influence of varying concentrations (0.10, 1.0, 5.0, 10.0  $\mu\text{g/ml}$ ) of  $\text{CaCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{BaCl}_2$  and MgO on methionine accumulation in the broth culture of *B. thuringiensis* EC1.

### 2.2.7. Time Course Experiment for Growth, pH, Sugar Utilization and Methionine Production

Time course experiment for growth, pH, sugar utilization and methionine production by *B. thuringiensis* EC1 was studied. The fermentation medium consists of basal medium, maltose, 80 g;  $(\text{NH}_4)_2\text{SO}_4$ , 10 g; yeast extract, 0.05% (w/v); peptone, 0.05% (w/v); riboflavin, 1.0  $\mu\text{g/ml}$ ; MgO, 0.10  $\mu\text{g/ml}$ ;  $\text{H}_2\text{O}$  1 L; pH 7.2. Fermentation process was carried out as previously described and growth determined turbidimetrically with a spectrophotometer at 660 nm. Methionine concentration in the broth culture was determined as previously described and sugar utilization estimated following the method described by Miller [13].

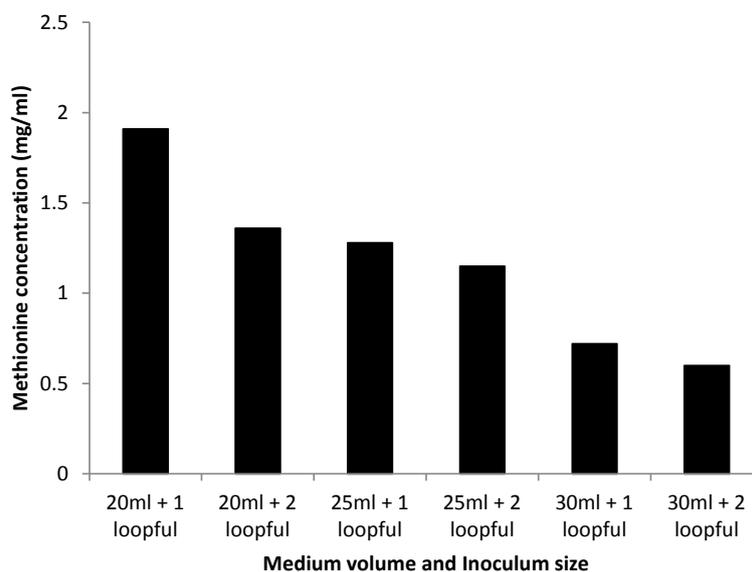
## 3. Results and Discussions

The methionine producer, *Bacillus thuringiensis* EC1, isolated from fermented food product was found to accumulate 1.89 mg/ml methionine in submerged medium. The isolation of *Bacillus* organisms as methionine producers agrees with the works of other investigators [14]. However, *Bacillus* species are not yet known to be very active producers as species of *Corynebacterium*, *Brevibacterium* and *Arthrobacter* [8] [15].

There is need for adequate aeration in microbial production of metabolites [16]. The results obtained with regard to oxygen tension (Figure 1) showed that as the liquid volume increased above 20 ml, methionine production decreased. Therefore, 20% is the optimum medium/fermenter volume ratio for methionine accumulation in submerged culture of *B. thuringiensis* EC1. This finding is supported by the work of Pham *et al.* [4].

The size of the inoculum in a fermentation process influences the metabolite production [10] [17]. As presented in Figure 1, inoculum size of 5% enhanced methionine production by *B. thuringiensis* EC1. High inoculum size results in too much biomass and depletes product formation [18] and also reduces dissolved oxygen, and increases competition towards nutrient [19]. Very low inoculum size may lead to insufficient biomass and reduced metabolite formation [17]. Therefore, at a suitable inoculum size, the nutrient and oxygen levels support sufficient growth of organism and therefore enhance metabolite production [17].

The effect of carbon sources on methionine production indicates that maltose is the carbon of choice. At 8%



**Figure 1.** Effect of medium/fermenter volume ratio and inoculum size on methionine production.

level, it stimulated a high methionine yield of 2.49 mg/ml (**Figure 2**). There is significant positive correlation ( $r = 0.896$ ,  $P = 0.039$ ) between maltose concentration and methionine concentration, thus as maltose concentration increases, methionine production also increases. This observation agrees with the work of Banik and Majumdar [20], who also reported maltose as the best carbon source for methionine production. Camila *et al.* [21], also observed an improved extracellular protease production by *Bacillus* spp. when maltose was the carbon source in the culture medium.

The effect of nitrogen source on methionine production shows that ammonium sulphate gave the highest methionine yield. Statistical analysis of the result shows that there is significant negative correlation ( $r = -0.965$ ,  $P = 0.008$ ) between ammonium sulphate concentration and methionine concentration, thus as ammonium sulphate concentration increases, methionine production decreases and vice versa. This according to Pham *et al.* [4] may be due to the sulphur content in the ammonium sulphate. Since methionine is a sulphur-containing amino acid, it is expected that more synthesis could occur as the elemental sulphur is supplied to the medium. The use of ammonium sulphate in methionine production have been reported by Kase and Nakayama [8], Anike and Okafor [22]. Pham *et al.*, [4] reported that methionine production is a function of nitrogen concentration. As shown in **Figure 2**, methionine production decreased with increase in concentration of ammonium sulphate. This decrease as suggested by Pham *et al.* [4], may be attributed to the osmotic pressure exerted by high nitrogen concentration, which have adverse effect on the growth and methionine accumulation by *B. thuringiensis* EC1. Maximum methionine yield was observed at 1% ammonium sulphate (**Figure 2**) concentration.

All growth promoting substances and their mixture enhanced methionine yield by *B. thuringiensis* EC1 (**Figure 3**). The stimulatory effects of these growth promoters or their mixtures have been reported by many researchers. Carlos and Meirre [23] obtained a maximum thermostable amylase production by *Bacillus* spp. with a mixture of yeast extract and peptone. Moringa *et al.* [24], noted an increase in methionine production by *Pseudomonas* spp. FMS18 with the addition of yeast extract into the culture medium. Phadataré *et al.* [25], reported an enhanced protease production in *Conidiobolus coronatus* in the presence of yeast extract, peptone and tryptone. Ekwealor and Obeta [26], observed an improved lysine production with the addition of casein in the culture broth of *Bacillus megaterium*.

Vitamins effects on methionine accumulation by *B. thuringiensis* EC1 (**Figure 4**), showed that riboflavin and thiamine HCl at 1.0  $\mu\text{g/ml}$  enhanced methionine production. There is significant difference ( $F = 4.522$  (ANOVA),  $P = 0.024$ ) in the different concentrations of vitamins in relation to methionine production. The increase in methionine yield observed in this study is in line with the findings of the other workers. Tani *et al.* [27], reported a high methionine yield when 200  $\mu\text{g/l}$  thiamine was added to the fermentation medium in methylotrophic yeast, *Candida biodinii*. Sen and Chatterjee [28], noted that thiamine HCl stimulated lysine accumula-

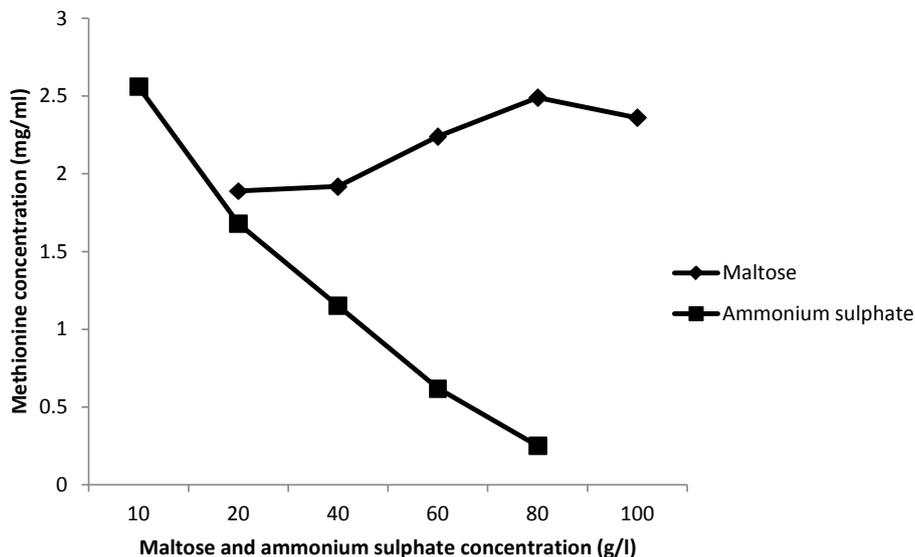


Figure 2. Effect of maltose and ammonium sulphate on methionine production.

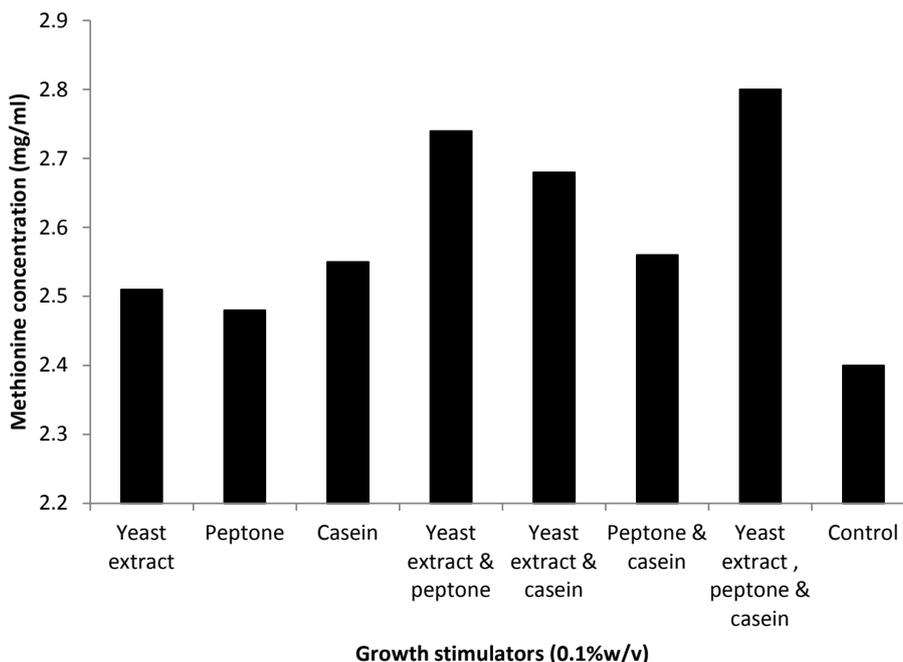


Figure 3. Influence of growth-promoting factors on methionine accumulation.

tion in *Micrococcus varians* 2fa. Ekwealor and Obeta [29], reported improved lysine yields in *Bacillus megaterium* SP 86 and *Bacillus megaterium* SP 76 with the addition of 10 µg/ml and 100 µg/ml of folic acid and riboflavin respectively.

Metal ions are known to play vital roles in fermentation. Most of them serve as cofactors for various enzymes or probably act as activators of enzymes involved in the synthetic steps of metabolites [3] [30]. The influence of bivalent metals on methionine production by *B. thuringiensis* EC1 (Figure 5), showed that Zn<sup>2+</sup>, Ba<sup>2+</sup> and Mg<sup>2+</sup> increased methionine yield. Zn<sup>2+</sup> at all concentrations stimulated methionine production. This result is supported by the works of Ekwealor and Obeta [29], who reported an improved lysine production in a broth culture of *Bacillus megaterium* supplemented with Zn<sup>2+</sup>. Sigel [31], also reported the role of zinc ion in the synthesis of industrially and medically significant microbial secondary metabolites. Mg<sup>2+</sup> at 0.1 µg/ml increased methionine ac-

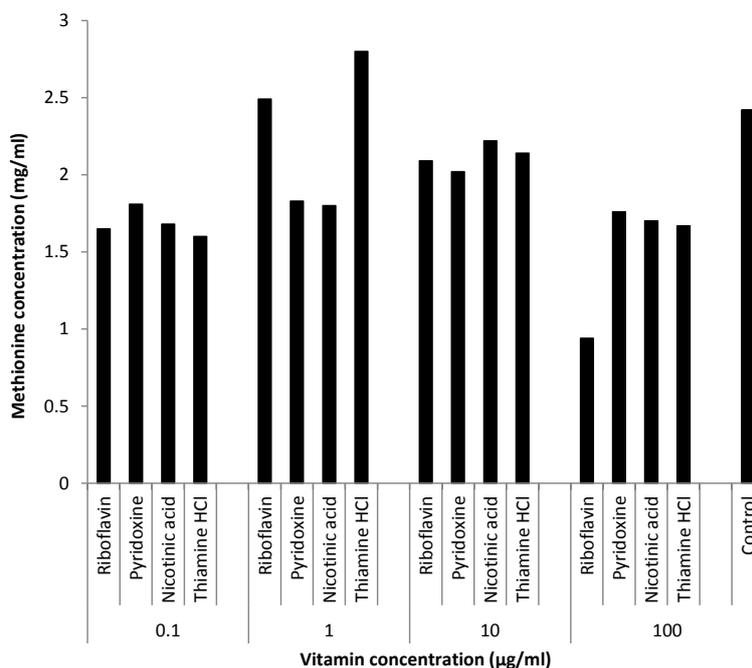


Figure 4. Influence of vitamin on methionine production.

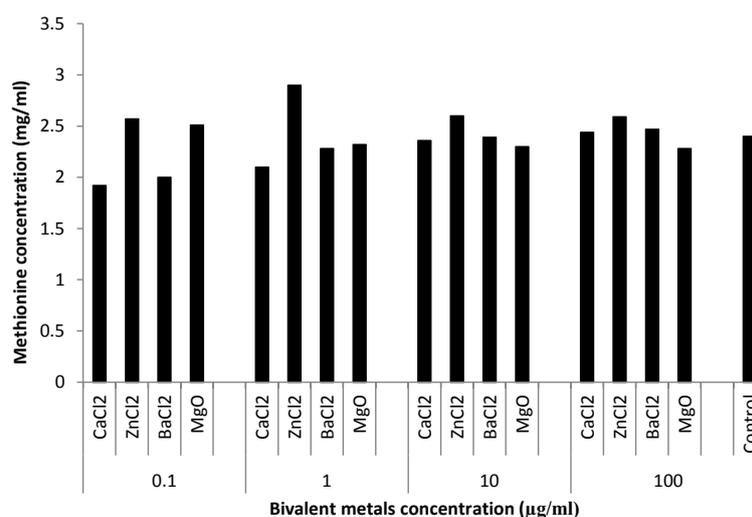


Figure 5. Influence of bivalent metals on methionine production.

cumulation by *B. thuringiensis* EC1 (Figure 5). The stimulatory effect of  $Mg^{2+}$  observed in this study is supported by the findings of El-Sayed [32], who reported an increase in activity of L-methioninase by *Aspergillus flavipes* when magnesium ion was added to the fermentation medium. Similarly, Saxena and Singh [33], observed an enhanced amylase production by magnesium ion in a solid-state fermentation of agro-industrial wastes using *Bacillus* spp.

$Ba^{2+}$  at 10 µg/ml improved methionine accumulation by *B. thuringiensis* EC1 (Figure 5), indicating the possibility of this metallic ion playing a significant role in methionine production.

Time course experiment for growth, pH, sugar utilization and methionine accumulation by *B. thuringiensis* (Figure 6) showed a maximum methionine yield of 3.18 mg·ml after 120 h, at a pH of 6.9 and residual sugar, 39%. The relationship between sugar consumption and methionine production (Figure 6) is similar to that observed by Murgov and Zaitseva [34] in their study on lysine production by *Brevibacterium flavum* 178. Ekwealor and Obeta [26] obtained a similar relationship in their work on lysine accumulation by *B. megaterium*.

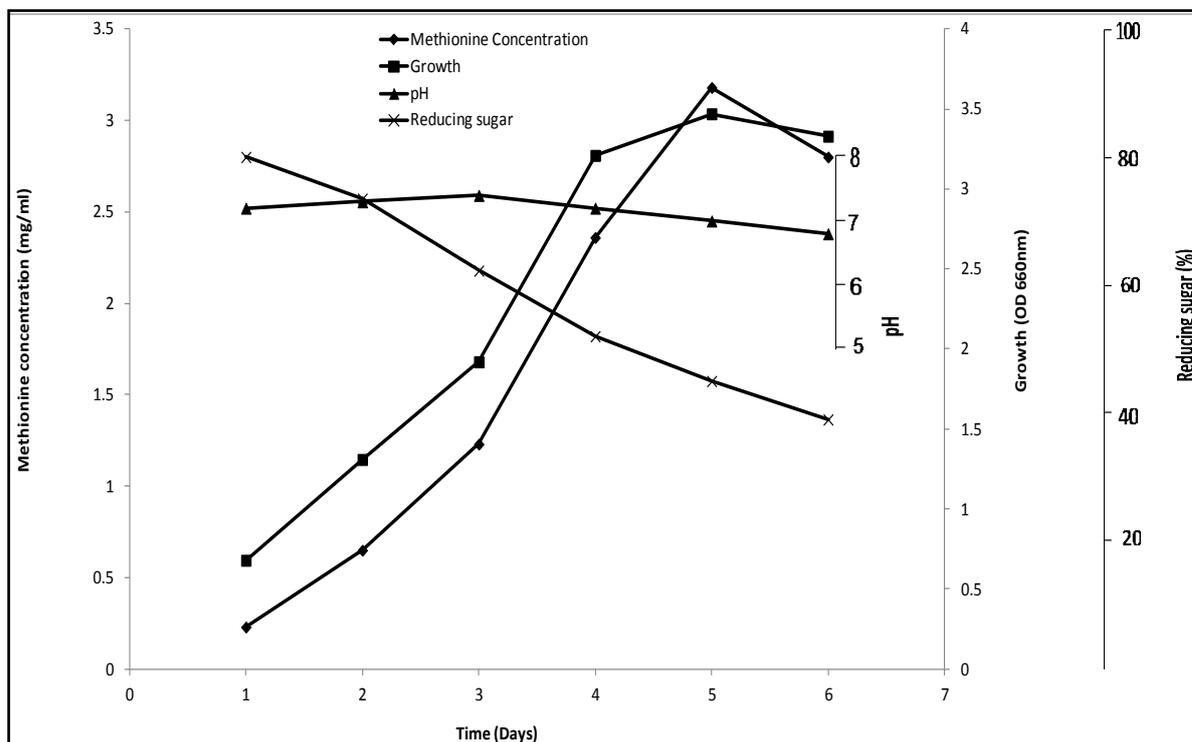


Figure 6. Time course experiment for growth, pH, sugar utilization and methionine accumulation.

#### 4. Conclusion

A 20% medium to fermenter volume ratio containing maltose, ammonium sulphate, yeast extract, peptone, riboflavin and magnesium oxide was observed to increase methionine accumulation by *B. thuringensis* EC1. Therefore, this study has shown that methionine yields by *B. thuringensis* EC1 in submerged medium can be stimulated by improving the cultural conditions. It is also possible that strain improvement will likely bring about further increase in methionine accumulation.

#### Acknowledgements

We are very grateful to Tertiary Education Trust (TET) Fund, Nnamdi Azikiwe University, Awka, Nigeria, for funding this research and to Macrogen Incop., Republic of Korea for their timely help in the identification of the bacterial isolate used for this study.

#### References

- [1] Mueller, J.H. (1922) A New Sulphur-Containing Amino Acid Isolated from Casein. *Proceedings of the Society of Experimental Biology and Medicine*, **19**, 161. <http://dx.doi.org/10.3181/00379727-19-75>
- [2] Noftsker, S., St-Pierre, N.R. and Sylvester, J.T. (2005) Determination of Rumen Degradability and Ruminant Effects of Three Sources of Methionine in Lactating Cows. *Journal of Dairy Science*, **88**, 223-237. [http://dx.doi.org/10.3168/jds.S0022-0302\(05\)72680-1](http://dx.doi.org/10.3168/jds.S0022-0302(05)72680-1)
- [3] Kumar, D. and Gomes, K. (2005) Methionine Production by Fermentation. *Biotechnology Advances*, **23**, 41-61. <http://dx.doi.org/10.1016/j.biotechadv.2004.08.005>
- [4] 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.
- [5] Umerie, S.C., Ekwealor, I.A. and Nawabo, I.O. (2000) Lysine Production from Various Carbohydrates and Seed Meals. *Bioresource Technology*, **75**, 249-252. [http://dx.doi.org/10.1016/S0960-8524\(00\)00052-3](http://dx.doi.org/10.1016/S0960-8524(00)00052-3)
- [6] Okamoto, K. and Ikeda, M. (2000) Development of Industrially Stable Process for L-Threonine Fermentation by an L-Methionine Auxotrophic Mutant of *Escherichia coli*. *Journal of Bioscience and Bioengineering*, **89**, 87-89.

- [http://dx.doi.org/10.1016/S1389-1723\(00\)88057-3](http://dx.doi.org/10.1016/S1389-1723(00)88057-3)
- [7] Hermann, T. (2003) Industrial Production of Amino Acids by *Coryneform* Bacteria. *Journal of Biotechnology*, **104**, 155-172. [http://dx.doi.org/10.1016/S0168-1656\(03\)00149-4](http://dx.doi.org/10.1016/S0168-1656(03)00149-4)
- [8] Kase, H. and Nakayama, K. (1975) L-Methionine Production by Methionine Analog-Resistant Mutants of *Corynebacterium glutamicum*. *Agricultural and Biological Chemistry*, **39**, 153-160. <http://dx.doi.org/10.1271/bbb1961.39.153>
- [9] Nakayama, K., Araki, K. and Kase, H. (1978) Microbial Production of Essential Amino Acid with *Corynebacterium glutamicum* Mutants. *Advances in Experimental Medicine and Biology*, **105**, 649-661. [http://dx.doi.org/10.1007/978-1-4684-3366-1\\_31](http://dx.doi.org/10.1007/978-1-4684-3366-1_31)
- [10] Dike, K.S. and Ekwealor, I.A. (2012) Studies on Process and Physical Parameters for the Production of L Methionine from Newly Isolated *Bacillus cereus* Strains. *Asian Journal of Biological Science*, **5**, 96-104. <http://dx.doi.org/10.3923/ajbs.2012.96.104>
- [11] Ozulu, U.S., Nwanah, O.U., Ekwealor, C.C., Dike, S.K., Nwipko, C.L. and Ekwealor, I.A. (2012) A New Approach to Screening for Methionine-Producing Bacteria. *British Microbiology Research Journal*, **2**, 36-39.
- [12] Greenstein, J.P. and Wintz, M. (1961) Methionine. Chemistry of the Amino Acid. John Wiley & Sons, Hoboken, 2125-2155.
- [13] Miller, G.L. (1959) Use of Dinitrosalicylic Acid Reagent for Detection of Reducing Sugars. *Analytical Chemistry*, **31**, 427-431. <http://dx.doi.org/10.1021/ac60147a030>
- [14] Mondal, S., Das, Y.B. and Chatterjee, S.P. (1996) Methionine Production by Microorganisms. *Folia Microbiologica*, **41**, 465-472. <http://dx.doi.org/10.1007/BF02814659>
- [15] Mondal, S. and Chatterjee, S.P. (1994) Enhancement of Methionine Production by Methionine Analogue Resistant Mutants of *Brevibacterium heali*. *Acta Biotechnologica*, **14**, 199-204. <http://dx.doi.org/10.1002/abio.370140214>
- [16] Khongsay, N., Laopaiboon, L., Jaisil, P. and Laopaiboon, P. (2012) Optimization of Agitation and Aeration for Very High Gravity Ethanol Fermentation from Sweet Sorghum Juice by *Saccharomyces cerevisiae* Using an Orthogonal Array Design. *Energy*, **5**, 561-576. <http://dx.doi.org/10.3390/en5030561>
- [17] Bisht, S.D., Yadav, S.K. and Darmwal, N.S. (2012) Enhanced Production of Extracellular Alkaline Lipase by an Improved Strain of *Pseudomonas aeruginosa* MTCC 10,055. *American Journal of Applied Science*, **9**, 158-167. <http://dx.doi.org/10.3844/ajassp.2012.158.167>
- [18] Shah, A.H., Hameed, A., Ahmad, S. and Majid, K.G. (2002) Optimization of Culture Conditions for L-Lysine Fermentation by *Corynebacterium glutamicum*. *Journal of Biological Science*, **2**, 151-156. <http://dx.doi.org/10.3923/jbs.2002.151.156>
- [19] Rahman, R.N., Geok, P.L., Basri, M. and Salleh, A.B. (2005) Physical Factors Affecting the Production of Organic Solvent-Tolerant Protease by *Pseudomonas aeruginosa* Strain K. *Bioresource Technology*, **96**, 429-436. <http://dx.doi.org/10.1016/j.biortech.2004.06.012>
- [20] Banik, A.K. and Majumdar, S.K. (1974) Studies on Methionine Fermentation. Part I. Selection of Mutants of *Micrococcus glutamicus* and Optimum Conditions for Methionine Production. *Indian Journal of Experimental Biology*, **12**, 363-365.
- [21] Camila, R.S., Andréia, B.D. and Meire, L.L.M. (2007) Effect of the Culture Conditions on the Production of an Extracellular Protease by Thermophilic *Bacillus* sp. and Some Properties of the Enzymatic Activity. *Brazilian Journal of Microbiology*, **38**, 253-258.
- [22] Anike, N. and Okafor, N. (2008) Secretion of Methionine by Microorganisms Associated with Cassava Fermentation. *African Journal of Food, Agriculture, Nutrition and Development*, **8**, 77-90. <http://dx.doi.org/10.4314/ajfand.v8i1.19181>
- [23] Carlos, E.T. and Meire, L.L.M. (2000) Culture Conditions for the Production of Thermostable Amylase by *Bacillus* sp. *Brazilian Journal of Microbiology*, **31**, 298-302.
- [24] Morinaga, Y., Tani, Y. and Yamada, H. (1984) Homocysteine Trans-Methylation in Methanol Utilizing Bacteria and Its Application to L-Methionine Production. *Agricultural and Biological Chemistry*, **48**, 143-148. <http://dx.doi.org/10.1271/bbb1961.48.143>
- [25] Phadatare, S.U., Deshpande, V.V. and Srinivasan, M.C. (1993) High Activity Alkaline Protease from *Conidiobolus caronatu* (NCL 86.8.20): Enzyme Production and Compatibility with Commercial Detergents. *Enzyme Microbial Technology*, **15**, 72-76. [http://dx.doi.org/10.1016/0141-0229\(93\)90119-M](http://dx.doi.org/10.1016/0141-0229(93)90119-M)
- [26] Ekwealor, I.A. and Obeta, J.A.N. (2005) Studies on Lysine Production by *Bacillus megaterium*. *African Journal of Biotechnology*, **4**, 633-638. <http://dx.doi.org/10.5897/AJB2005.000-3115>
- [27] Tani, Y., Lim, W.J. and Yang, H.C. (1988) Isolation of L-Methionine Enriched Mutants of a Methylophilic Yeast, *Candida boidinii* No 2201. *Journal of Fermentation Technology*, **66**, 153-158.

[http://dx.doi.org/10.1016/0385-6380\(88\)90041-6](http://dx.doi.org/10.1016/0385-6380(88)90041-6)

- [28] Sen, S.K. and Chatterjee, S.P. (1989) Influence of B-Vitamins and Trace Elements on Lysine Production by *Micrococcus varians* 2fa. *Acta Biotechnologica*, **9**, 63-67. <http://dx.doi.org/10.1002/abio.370090111>
- [29] Ekwealor, I.A. and Obeta, J.A.N. (2007) Effect of Vitamins and Bivalent Metals on Lysine Yield in *Bacillus megaterium*. *African Journal of Biotechnology*, **6**, 1348-1351.
- [30] Hughes, M.N. and Poole, P.K. (1989) *Metals and Microorganisms*. Chapman and Hall, Boca Raton, 1-37.
- [31] Sigel, I. (1983) *Metal Ions in Biological Systems: Zinc and Its Role, Biology and Nutrition*. Marcel Dekker Inc., New York.
- [32] El-Sayed, A.S.A. (2009) L-Methioninase Production by *Aspergillus flavipes* under Solid-State Fermentation. *Journal of Basic Microbiology*, **49**, 331-341. <http://dx.doi.org/10.1002/jobm.200800318>
- [33] Saxena, R. and Singh, R. (2011) Amylase Production by Solid-State Fermentation of Agro-Industrial Wastes Using *Bacillus* sp. *Brazilian Journal of Microbiology*, **42**, 1334-1342. <http://dx.doi.org/10.1590/S1517-83822011000400014>
- [34] Murgov, I.D. and Zaitseva, Z.M. (1973) Optimization of the Composition of Nutrient Medium for the Biosynthesis of L-Lysine by *Brevibacterium flavum* 178 Using Mathematical Planning of the Experiment. *Applied Microbiology*, **9**, 845-851.