



# The Effect of Inoculum Size and Medium Volume on Methionine Production by *Bacillus* species EZ-13 and ZM-10

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## Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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## ABSTRACT

The effect of inoculum size and medium volume on methionine production by *Bacillus* species EZ-13 and ZM-10 was studied. The methionine-producing bacteria had already been isolated from within Anambra State University (now Chukwuemeka Odumegwu Ojukwu University), Uli campus Anambra State. They were purified and identified as *Bacillus* species EZ-13 and ZM-10 using cultural and biochemical characteristics. Thereafter, they were used to evaluate the influence of inoculum size and medium volume on methionine production by *Bacillus* species EZ-13 and ZM-10 in 250 ml flasks containing 50 ml of sterile fermentation medium. The findings revealed that *Bacillus* species EZ-13 and ZM-10, at inoculum size of 4% v/v, recorded maximum methionine yields of 2.8 mg/ml and 2.1 mg/ml respectively. The methionine concentrations of both *Bacillus* species decreased beyond inoculum size of 4% v/v. The medium volume of 50 ml stimulated maximum methionine yield of 3.0 mg/ml in *Bacillus* species EZ-13 and 2.4 mg/ml in *Bacillus* species ZM-10. The methionine concentrations for both bacteria decreased beyond medium volume of 50 ml. The result of the study showed that varying the inoculum sizes and medium volumes, could influence methionine yield by fermentation.

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## 1. INTRODUCTION

“L-methionine is an essential amino acid that is required in the diet of humans and mammals for normal growth and function of body metabolism. It cannot be synthesized internally but may be added to food and feed materials to improve the protein quality” [1]. “Deficiencies can be overcome by supplementing the diet with methionine and, therefore, methionine is of significant interest” [2]. “As an important amino acid, methionine is widely used in feed, pharmaceutical and food industries” [3,4,5]. “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” [6].

“Methionine deficiency has been linked to development of various diseases and physiological conditions including toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson’s liver deterioration, and impaired growth” [7]. “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” [8].

“Methionine is generally being produced by chemical and enzymatic methods, both are expensive, chemical method requires hazardous chemicals such as acroleine, methyl mercaptan and hydrocyanic acid and enzymatic method requires expensive enzymes. To date, almost all the commercial production of Met relies on chemical synthesis. However, chemical routes for Met synthesis not only produce a racemic mixture of D- and L-forms but also result in serious environmental pollution” [9,10]. “Microbial production of different amino acids has been initiated through the isolation of *Micrococcus glutamicus* by Kinoshita and his colleagues in 1957 for L-glutamic acid production. Since then, several microbial strains were isolated and tested for different amino acid production” [10]. “Very recently, a mutant strain *Lactobacillus plantarum*, wild strains of *Bacillus cereus*, wild strain of *Corynebacterium glutamicum* K, *Bacillus thuringiensis* EC1 are in common use for fermentative production of L-methionine” [11,12,13,14].

“To successfully establish an economically viable, eco-friendly method for microbial production of L-methionine, high yielding strains must be isolated or developed. Wild-type of strains are not capable of accumulating huge amount of L-methionine in the fermentation broth as its biosynthesis is tightly regulated” [15,16].

“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” [5].

“Extensive research has been made in order to improve the fermentation process not only from the point of lowering production costs but also of increasing productivity” [17]. “Improvements have included for example, increased yield of desired metabolites, removal of unwanted co-metabolites, improved utilization of inexpensive carbon and nitrogen sources, or alteration of the morphology to a form better suited for separation of the organisms from the product” [17]. As Nigeria is a developing country, a huge amount of foreign exchange is spent in the importation of methionine for its local use. There is huge potential in the production of methionine locally by microbiological methods.

In an earlier study, methionine-producing *Bacillus* species (*Bacillus* species EZ-13 and ZM-10) were isolated from soils within Chukwuemeka Odumegwu Ojukwu University, Uli campus, Anambra State [18]. The aim of this research was to determine the influence of inoculum size and medium volume on methionine production by *Bacillus* species EZ-13 and ZM-10.

## 2. MATERIALS AND METHODS

### 2.1 Microorganisms

The two bacteria used in this work, were previously isolated from soils within Chukwuemeka Odumegwu Ojukwu University, Uli campus, Anambra State. They were purified and identified as *Bacillus* species EZ-13 and *Bacillus* species ZM-10 using cultural and biochemical characteristics. They were maintained on Nutrient agar slants at 4°C and transferred to new slants after 30 days in order to keep them viable for use in methionine production.

## 2.2 Inoculum Preparation

Two (2) loopfuls of *B. species* EZ-13 and *B. species* ZM-10 (24 h) were inoculated into 250 ml Erlenmeyer flasks containing 50ml of sterile seed medium. The seed medium consisted of peptone, 10.0 g; yeast extract, 10.0 g; NaCl, 5.0 g; water, 1litre; pH adjusted to 7.2. The flasks were incubated for 24 h on a rotary shaker at 120 rpm and 30°C.

## 2.3 Fermentation Medium

The medium used for fermentation consisted of glucose, 20 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 g; K<sub>2</sub>HPO<sub>4</sub>, 0.5 g; KH<sub>2</sub>PO<sub>4</sub>, 0.5 g; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.001 g; FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.01 g; MnSO<sub>4</sub>.4H<sub>2</sub>O, 0.001 g; CaCO<sub>3</sub>, 5 g; water, 1 litre of water; pH was adjusted to 7.2 with 1 N NaOH.

## 2.4 Effect of Inoculum Size on Methionine Production

Erlenmeyer flasks (250 ml) containing 50 ml of the fermentation medium as was described previously, were sterilized at 121 °C for 15 min . After sterilization, the medium was cooled to room temperature and thereafter various inoculum sizes 1 ml (2% v/v), 2 ml (4% v/v), 3 ml (6 % v/v ), 4 ml ( 8% v/v ) and 5ml (10% v/v ) were inoculated into it. An uninoculated flask was used as control. Bacterial growth and methionine production were determined as was previously described. The inoculum size that gave the maximum methionine yield was used for next experiment (effect of medium volume)

## 2.5 Effect of Medium Volume on Growth on Methionine Production

Erlenmeyer flasks containing various volumes (20 – 70 ml) of fermentation medium as was described previously, were sterilized at 121°C for 15 min. After sterilization the flasks were cooled to room temperature and inoculated with best result of inoculum size of the previous experiment of a 24 h seed inoculum of *Bacillus species*. The flasks were placed on a rotary incubator shaker (160 rpm) at 30 °C for 72 h. An uninoculated flask was kept as control. Bacterial growth and methionine production were determined as was previously described.

## 2.6 Quantitative Determination of Methionine

Quantitative determination of L-methionine in the culture broth without purification was carried out

by the modified calorimetric method of [19]. A 5ml volume of the culture broth was centrifuged at 5,000xg for 20 mins and the cell free supernatant was assayed for L-methionine.1 ml of 5 N NaOH was added to each tube followed by the addition of 0.1 ml of 10% sodium nitroprusside solution with thorough mixing. The mixture was allowed to stand for 10 min. Then two milliliters 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, 2ml of concentrated ortho-phosphoric acid was added drop wise to the mixture with shaking. Colour development was allowed to proceed for 5 min and the colour intensity measured at 540 nm in a spectrophotometer. A blank containing distilled water and all other reagent served as the 100% transference standard. Results obtained with the test samples were interpolated on a standard methionine curve.

## 2.7 Determination of Growth of Bacteria

The growth of the bacterial isolates was determined turbidimetrically from the culture broth in a spectrophotometer at 660nm. Samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, they (cuvettes) were placed in the spectrophotometer and the reading for bacteria growth was determined at 660nm.

## 3. RESULTS AND DISCUSSION

The result of the effect of inoculum size on methionine production by *Bacillus species* EZ-13 is shown in Fig. 1. The inoculum size of 4% v/v stimulated maximum methionine yield of 2.8 mg/ml in *Bacillus species* EZ-13. The result of the effect of inoculum size on methionine production by *Bacillus species* ZM-10 is shown in Fig. 2. The inoculum size of 4% v/v stimulated maximum methionine yield of 2.1 mg/ml in *Bacillus species* ZM-10. The results are contrary to report of Anakwenze et al. [14], who observed that inoculum size of 5 % enhanced methionine production by *B. thuringiensis* EC. Ajogwu et al. [20] also, reported that a 5.0 % inoculum size was optimal for methionine yields of 2.04 mg/ml and 2.40 mg/ml produced by *B. pumilus* and *B. amyloliquefaciens* respectively. [21,22,23] reported a 3% inoculum size for optimal growth and metabolite production. Ekwealor and Obeta [24] observed maximum L-lysine production by *Bacillus megaterium* SP14 using 10 % v/v inoculum size. Also, Shah et al.

[25], reported that “maximum lysine production (15.3g/l) for *Corynebacterium glutamicum* was obtained at 10% v/v inoculum size”.

Hallaert et al. [26], reported that “inoculum size has noticeable effect on fermentation. He pointed out that low inoculum size causes an increase in growth period and gives insufficient biomass. As the inoculum level was further increased in the study, the production of the methionine gradually reduced. It is likely that at a higher inoculum level, the bacteria grow rapidly and the nutrient essential for the growth of bacteria were consumed at the initial stages that resulted in the accumulation of other by-products in the fermentation medium”. [22], Suggested that “higher inoculum size results in reduced DO and increased competition towards nutrients”.

The result of the effect of medium volume on methionine production by *Bacillus* species EZ-3 is shown in Fig. 3. The medium volume of 50 ml stimulated maximum methionine yield of 3.0 mg/ml in *Bacillus* species EZ-13. The results of the effect of inoculum size on methionine production by *Bacillus* species ZM-10 is shown in Fig. 4. The medium volume of 50 ml stimulated maximum methionine yield of 2.4 mg/ml in *Bacillus* species ZM-10. The results of the study is supported by Shah et al. [25], who reported “maximum lysine production of 15.05g/l and sugar utilization of 6.7% when 50 ml broth

volume was utilized. As the volume increased, L-lysine production and sugar utilization decreased, as observed with 150ml culture volume only 8.55 g/l L-lysine was produced and 4.85% sugar utilized”. Contrary results have been obtained by Ganguly and Banik [26], who reported that 30 ml of fermentation broth was optimum for maximum production of L-glutamic acid by *Micrococcus glutamicus*. Narayana and Swamy [21] and Dike and Ekwealor [12], reported that “Maximum growth and methionine production were obtained at 30% volume of medium to fermenter beyond which methionine production diminished”. Ajokwa et al. [20], observed that a medium volume of 20 ml was the best for methionine accumulation by the *Bacillus* species. Anakwenze et al. [14], reported that “20% is the optimum medium/fermenter volume ratio for methionine accumulation in submerged culture of *B. thuringiensis* EC1”. Ekwealor and Obeta [24], reported that “lysine production by *Bacillus megaterium* SP14 increased with increasing medium volume ratio up to 25%. Further increase caused a decrease in lysine synthesis”. Under conditions of inadequate oxygen, huge amount of succinic and lactic acids are produced, while surplus oxygen increases the amount of keto glutaric acid. [27,28,29], reported that both “inadequate and surplus oxygen is undesirable in amino acid fermentation. They confirmed that the former inhibits cell growth and latter hampers the production of amino acids”.

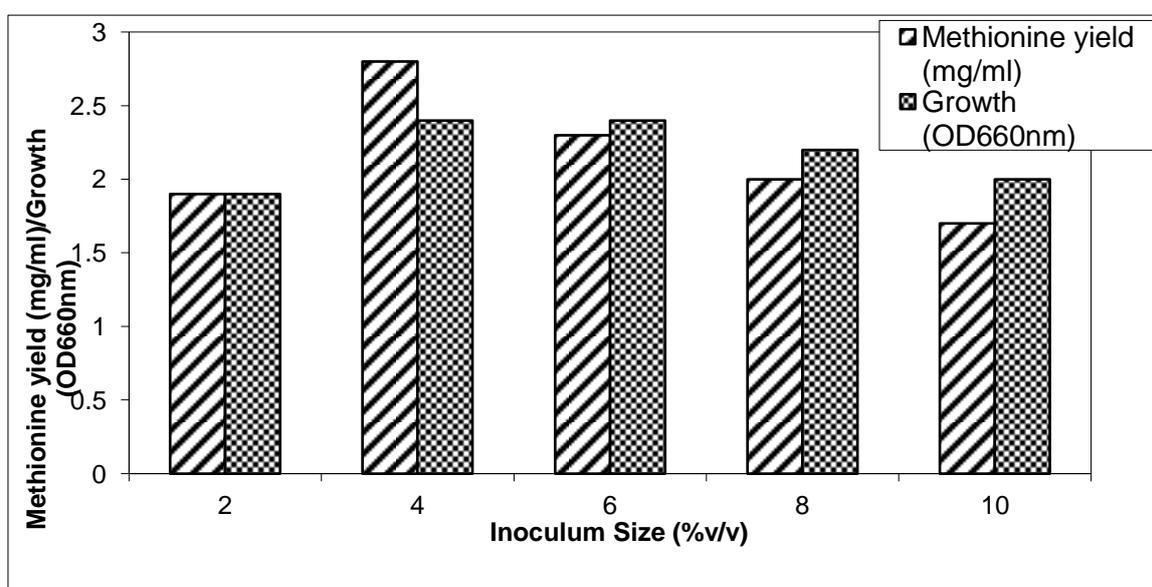


Fig. 1. Effect of Inoculum Size on Methionine Production by *Bacillus* species EZ-13

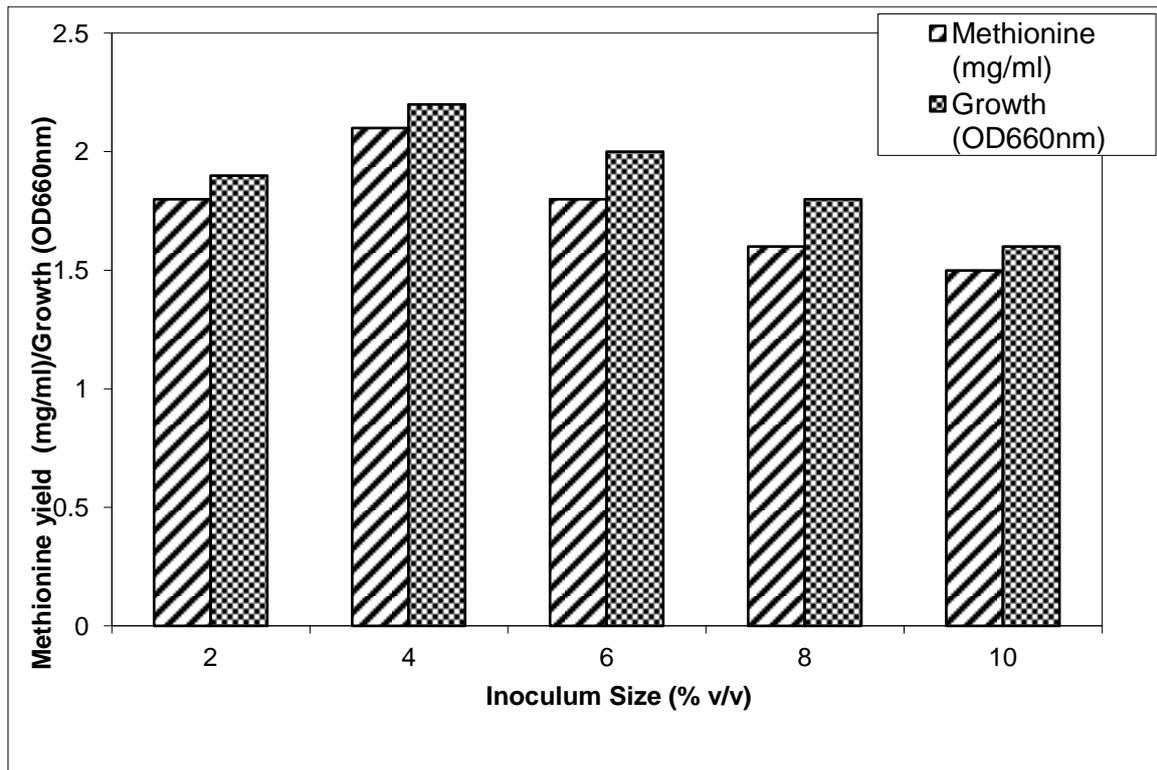


Fig. 2. Effect of Inoculum Size on Methionine Production by *Bacillus* species ZM-10

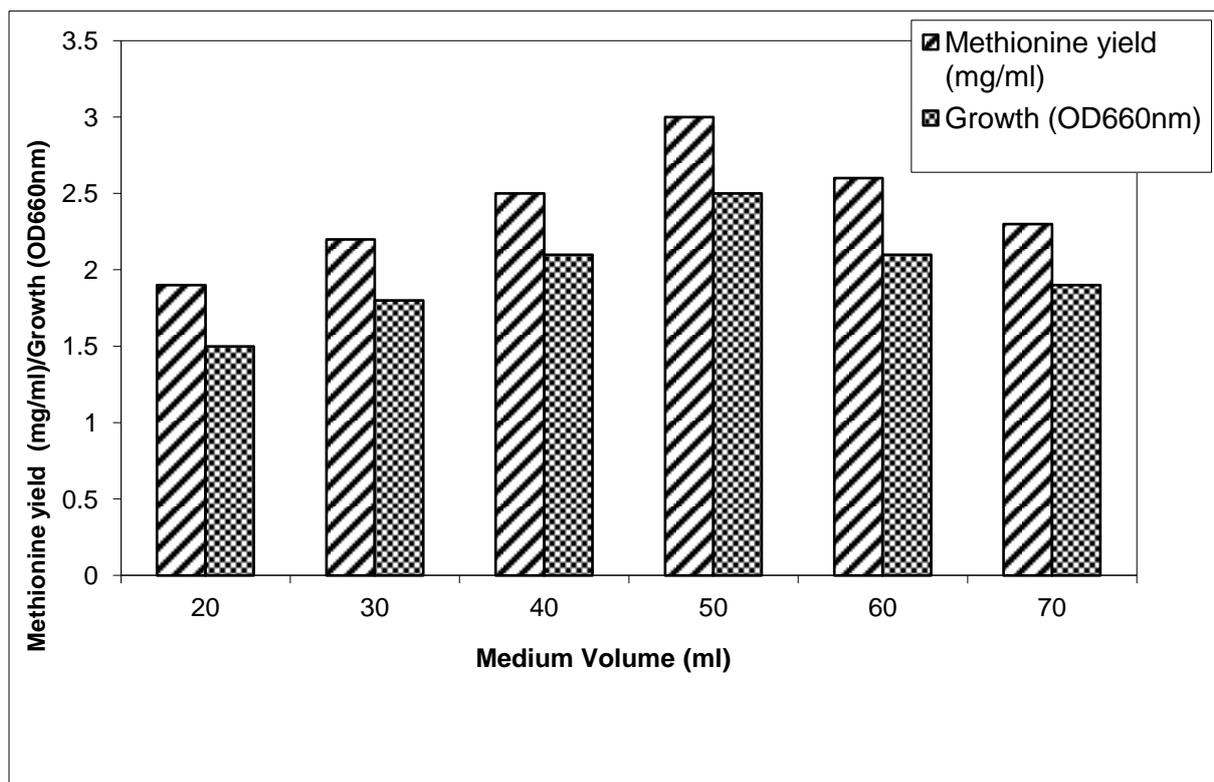


Fig. 3. Effect of Medium Volume on Methionine Production by *Bacillus* species EZ-13

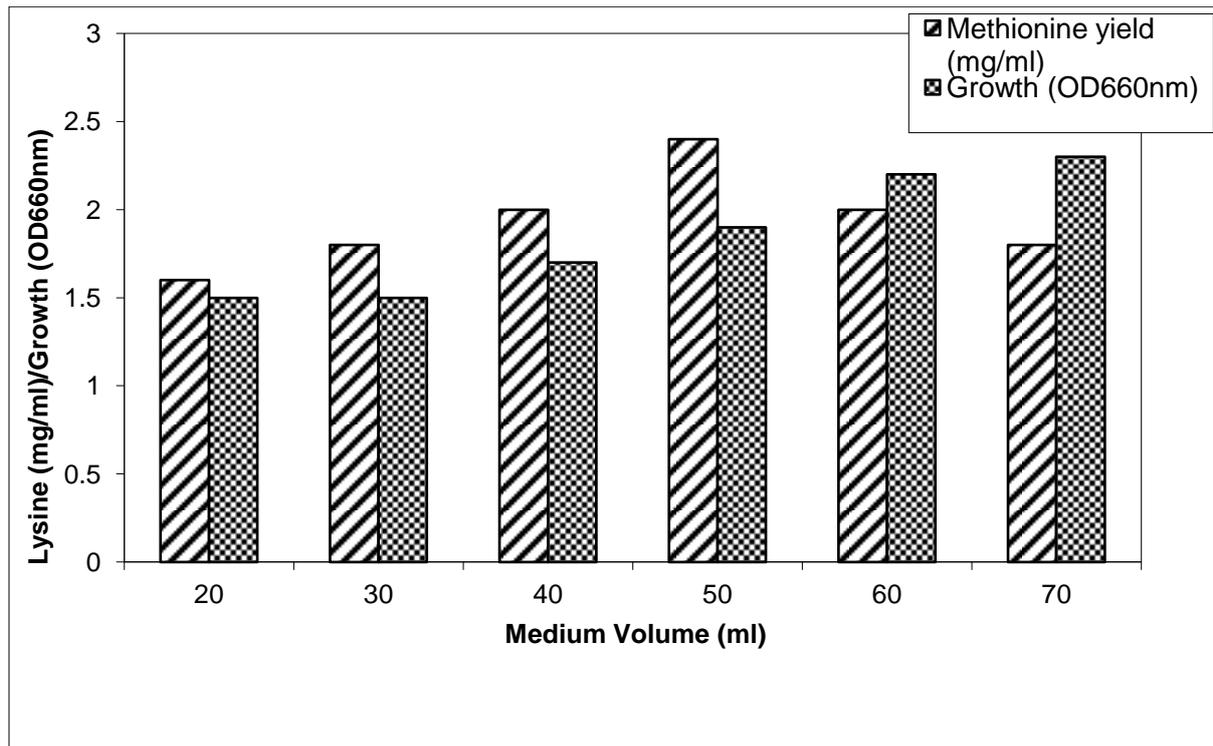


Fig. 4. Effect of Medium Volume on Methionine Production by *Bacillus* species ZM-10

#### 4. CONCLUSION

In the study, an inoculum size of 4% v/v and medium volume of 50 ml stimulated enhanced methionine yield. It is concluded from the research, that methionine yield could be enhanced by improving on the cultural conditions. This development indicates that large scale production of methionine is feasible in Nigeria and this will help to meet with the present-day needs of some of its industries. Further research is needed to study the effect of other conditions on methionine production.

#### COMPETING INTERESTS

Author has declared that no competing interests exist.

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