British Microbiology Research Journal
4(8): 885-895, 2014

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Improved Cultural Conditions for Methionine Accumulation in Submerged Cultivation of *Bacillus cereus* S8

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Authors' contributions

This work was carried out in collaboration between all authors. Author VNA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author CCE managed the literature searches. Author IAE managed the analyses and supervision of the study. All authors read and approved the final manuscript.

Original Research Article

Received 5th December 2013
Accepted 20th February 2014
Published 6th May 2014

ABSTRACT

Aims: To improve the cultural conditions for enhanced methionine production by *Bacillus cereus* S8
Study design: Study of the fermentation process in shake flask culture.
Place and Duration of Study: Department of Applied Microbiology and Brewing, Nnamdi Azikwe University, Awka, Nigeria between 2011 to 2012.
Methodology: The effects of medium/fermenter volume ratio, carbon and nitrogen sources, growth stimulators, vitamins and amino acid on methionine accumulation in the broth culture of *Bacillus cereus* S8 were investigated. The time course for methionine production was also studied.
Results: A 20% medium/fermenter volume ratio improved methionine yield. Glucose and ammonium sulphate at 6.0 and 1.0% respectively stimulated methionine accumulation by *Bacillus cereus* S8. Yeast extract, peptone, DL-leucine and all vitamins studied enhanced methionine production. A methionine yield of 3.23mg/ml was produced after 96h fermentation and at a pH of 6.90.
Conclusion: Improving the cultural conditions of *Bacillus cereus* S8 in submerged medium stimulated methionine increase.

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Keywords: Fermentation; methionine; glucose; ammonium sulphate; submerged medium.

1. INTRODUCTION

Methionine, an alpha-L-amino-gamma-methylthio-n-butyric acid, is nutritionally essential for mammals and fowls [1]. It cannot be synthesized internally but may be added to food and feed materials to improve the protein quality [2]. Plant proteins are deficient in methionine and therefore, an exclusively vegetable diet may fail to meet nutritional requirements [3].

Methionine deficiency has been linked to the development of various diseases and physiological conditions including toxemia, childhood rheumatic fever, muscle paralysis, depression, schizophrenia, Parkinson's disease and impaired growth [4,5]. These deficiencies can be overcome by supplementing the diet with methionine [6].

Methionine can be produced either by chemical or enzymatic synthesis or by submerged fermentation. Chemical synthesis of methionine produces racemic mixture and this is undesirable as it requires some hazardous chemicals such as acrolein, methyl mercaptan, ammonia and cyanide for its production [7]. Submerged fermentation is advantageous in that it produces biologically active L-methionine.

The discovery of glutamic-acid producing bacteria by Kinoshita et al. [8] eventually led to search for other amino acid producing microorganisms [9]. The problem in producing methionine by submerged cultivation is the strict feedback regulation by the wild type strains. For this reason, efforts have been made by several researchers to develop high-yielding strains for producing methionine by fermentation [10,11,12].

The aim of this study was to determine the cultural conditions for the improvement of methionine accumulation by *Bacillus cereus* S8 in submerged fermentation.

2. MATERIALS AND METHODS

2.1 Microorganism

Bacterial isolate from Nigerian soil, *Bacillus cereus* S8, 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 4ºC. After every three months, new cultures were slanted.

2.1.1 Fermentation in submerged medium

Fermentation was carried out following the method described by Ozulu et al. [13]. A loopful of a 24h culture of *B. cereus* S8 was inoculated with 1ml of a sterile seed medium (peptone, 10.0g; yeast extract, 10.0g; NaCl, 5.0g; H_2O, 1L; pH 7.2) in a test tube and incubated at 30ºC on a VWR DS2-500-2 orbital shaker at 160rpm for 24h. A 100ml Erlenmeyer flask containing 20ml of the sterile fermentation medium {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; $CaCO_3$, 20.0g; (basal medium), glucose, 20.0g, $(NH_4)_2SO_4$, 10.0g; H_2O, 1L; pH 7.2}was inoculated into 1ml (ca. 3.15×10^6 cell/ml) of the seed medium. The flasks prepared in duplicates were incubated at 30ºC on a shaker (160rpm) and the broth culture assayed for methionine accumulation after 72h. Uninoculated flasks served as control.

2.1.1.1 Methionine assay

Methionine was assayed for following the method described by Greenstein and Wintz [14]. The broth culture was centrifuged at 1500xg for 15min and 5ml of the supernatant in a test tube 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.

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 cereus* S8 were examined. Erlenmeyer flasks (100ml) with different volumes (20, 25, 30ml) of the fermentation medium was each inoculated with 1ml of seed inoculum. Each 1ml contains a loopful (ca. 3.15×10^6 cell/ml) or two loopful (ca. 7.56×10^8 cell/ml). The flasks were incubated and methionine accumulation was determined.

2.2.2 Effect of carbon source

Carbon sources (Glucose, Maltose, Mannitol, Sucrose, Lactose) were studied for their effects on methionine accumulation by *Bacillus cereus* S8. Fermentation processes and methionine determination were as earlier described. The carbon source that produced high methionine yield was used for further studies. The effect of varying concentrations (20, 40, 60, 80, 100g/l) of the carbon source for methionine accumulation by the organism was also investigated.

2.2.3 Effect of nitrogen source

Different nitrogen sources (ammonium sulphate, ammonium chloride and urea) were examined for their effects on methionine production. The best nitrogen source was used for further studies. The effect of varying concentrations (10, 20, 40, 60, 80g/l) of the nitrogen source for methionine production by the organism was 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 was examined.

2.2.5 Influence of varying concentrations of vitamins

Fermentation process was conducted to determine the influence of varying concentrations (0.10 – 100.0µg/ml) of riboflavin, pyridoxine, nicotinic acid and thiamine HCl on methionine production by *B. cereus* S8.

2.2.6 Influence of amino acid

The influence of 0.01% (w/v) of alanine, DL- aspartic acid, tryptophan, glycine, DL-methionine, DL- threonine and DL- leucine on methioinine yield by *B. cereus* S8 was investigated.

2.2.7 Time course experiment for growth, pH, sugar utilization and methionine production

The fermentation medium used consists of basal medium, glucose, 60g; $(NH_4)_2SO_4$, 10g; yeast extract, 0.05 %(w/v); peptone, 0.05 %(w/v); pyridoxine, 10.0µg/ml; DL- leucine, 0.01% (w/v); H_2O 1L; pH 7.2. Methionine accumulation, pH, growth and sugar utilization were determined from the culture broth of *B. cereus* S8. Growth was determined turbidimetrically with a spectrophotometer at 660nm. The residual sugar (glucose) was estimated following the method described by Miller [15].

3. RESULTS AND DISCUSSION

Bacillus cereus S8 used in this study accumulated a methionine yield of 3.23mg/ml in submerged fermentation. The use of *Bacillus s*pecies as methionine producers have been reported by many researchers [11,16]. However, they are not yet known to be over-producers as species of *Corynebacterium* and *Brevibacterium* [3,17,18].

Adequate aeration of fermentation medium is required for metabolite production in shaken culture [3,19]. As presented in Fig. 1, methionine accumulation (1.55mg/ml) by *B. cereus* S8 was optimum at 20% medium/fermenter volume ratio and further increase resulted in decrease in methionine production.

This finding is in line with the work of Pham et al. [2], in which a 20% medium/fermenter volume ratio improved methionine yield. However, it is important to note that over abundance or meager aeration is undesirable in amino acid fermentation [20,21,22]. The former is said to inhibit cell growth while the latter hinders maximum production of amino acids.

The effect of inoculum size on methionine production Fig. 1 indicates that low inoculum size (one loopful) stimulated high methionine yield. This observation is supported by the works of Pham et al. [2] and Shah et al. [23] but contrasts the view of Hallaert et al. [22]. While Shah et al. [23] suggested that high cell density may produce too much biomass and deplete the substrate or nutrient for product formation, Hallaert et al. [22] opined that small inoculum size causes an increase in growth. They noted that very low density may give insufficient biomass for product formation. The size of inoculum to be used, however, depends on the cell mass and the composition of the seed medium to be transferred.

Carbon sources and their ratio in fermentation media play a significant role in the production of particular metabolite [3]. Among the carbon sources used, glucose gave a methionine yield of 1.52mg/ml, while sucrose, maltose, mannitol and lactose accumulated methionine concentrations of 1.14mg/ml, 0.73mg/ml, 0.66mg/ml and 0.31mg/ml respectively, in the culture broth of *B. cereus* S8.

The use of glucose as the carbon source of choice for methionine production by *B. cereus* S8 is in line with the work of several researchers [24,25,26]. Glucose concentration at 6%

level enhanced methionine production Fig. 2. Statistically, it was observed that there is no significant correlation (r=-0.004, P=0.995) between glucose concentration and methionine production.

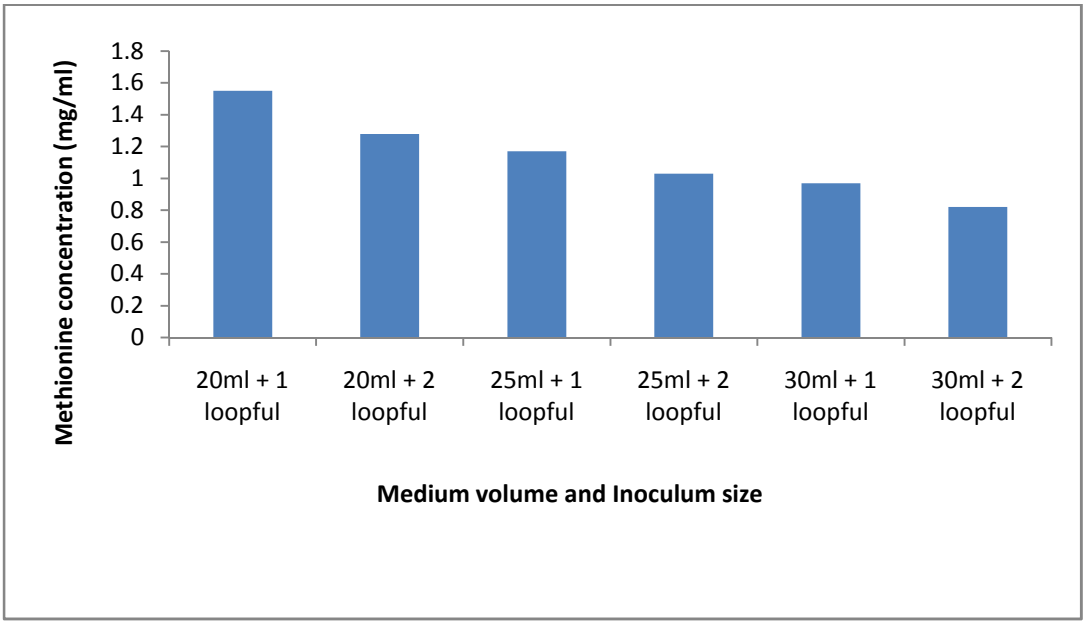

Fig. 1. Effect of medium/fermenter volume ratio and inoculum size on methionine production

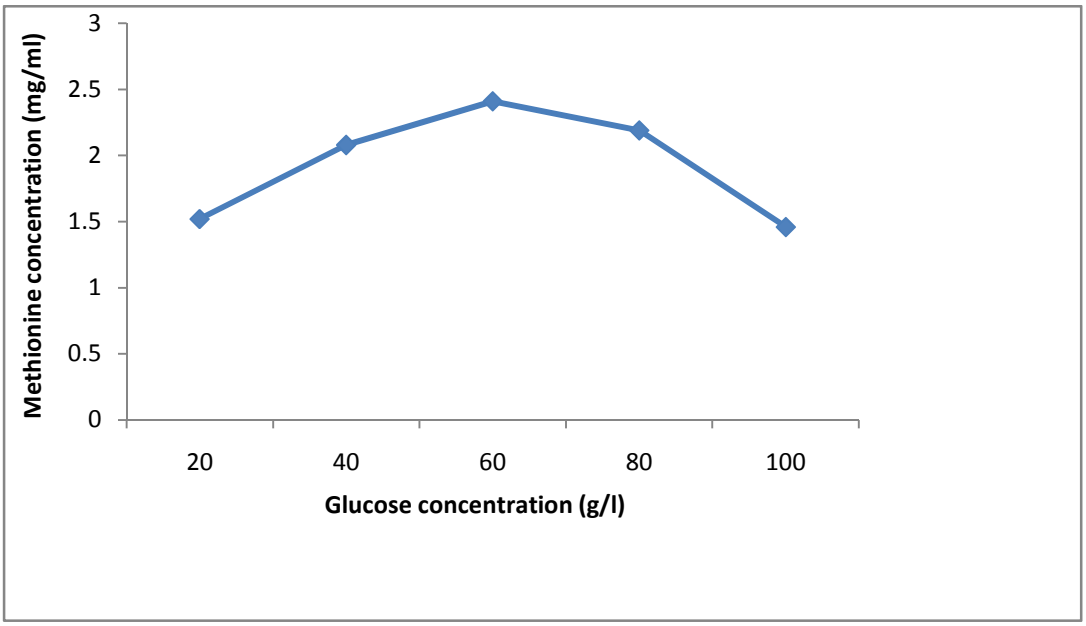

Fig. 2. Effect of glucose concentration on methionine production

Nitrogen sources: ammonium sulphate, ammonium chloride and urea produced methionine yields of 1.61mg/ml, 0.64mg/ml and 0.92mg/ml respectively. The stimulation of methionine by ammonium sulphate in the culture broth of *B. cereus* S8 is supported by the works of Mondal et al. [11], Kumar et al. [25] and Anike and Okafor [27]. They reported maximum production of L-methionine with ammonium sulphate as the nitrogen source.

As presented in Fig. 3, methionine accumulation by *B. cereus* S8 was obtained at 1% level of ammonium sulphate. Statistical analysis of the result showed that there is a significant negative correlation (r=-0.993, P=0.001) between ammonium sulphate concentration and methionine production. Thus, as the ammonium sulphate concentration increases, methionine production decreases and vice versa.

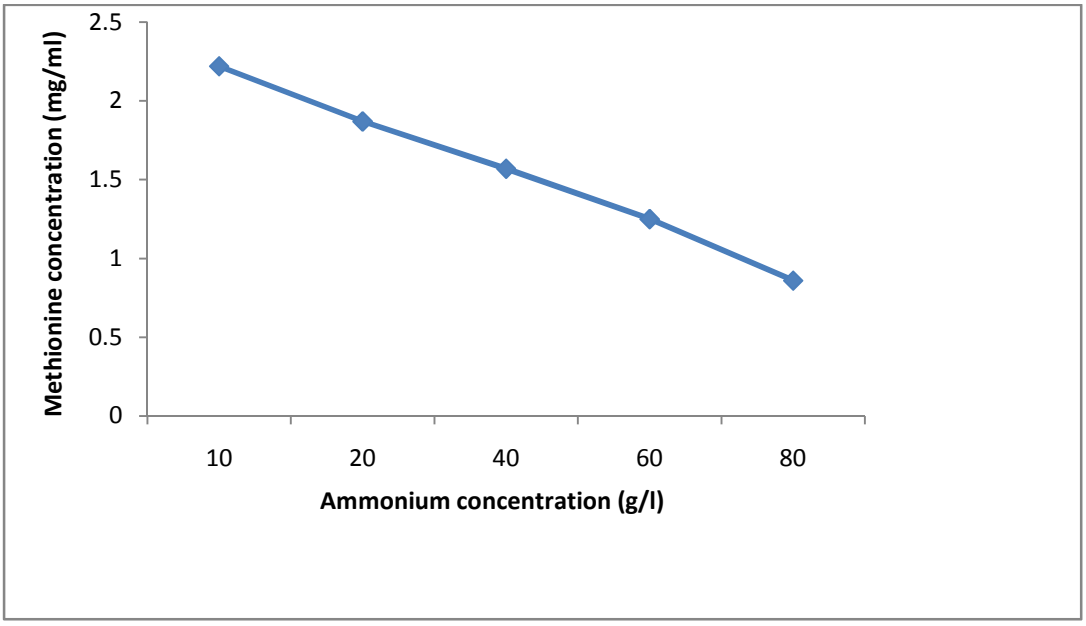

Fig. 3. Effect of ammonium sulphate on methionine production

Several researchers have reported the stimulatory effect of yeast and peptone in metabolite production [28,29]. The growth stimulators except casein improved methionine accumulation by *B. cereus* S8 Fig. 4. The non stimulatory effect of casein may be as a result of the non tolerance of the casein concentration by *B. cereus* S8. This view is supported by the report of Chao and Foster [30]. They noted that at low concentration (0.1mg/ml) of organic extract, glutamic acid formation was optimal but twice this concentration abolished its formation.

All the vitamins studied enhanced methionine production by *B. cereus* S8 Fig. 5 but at different concentrations. Ekwealor and Obeta [31] reported the stimulatory effect of riboflavin on lysine production by *Bacillus megaterium* SP 86. They however, noted that pyridoxine and nicotinic acid did not increase lysine production. This is contrary to our findings in which pyridoxine at 10.0µg/ml produced the highest methionine yield. Strain difference may be responsible for this variation.

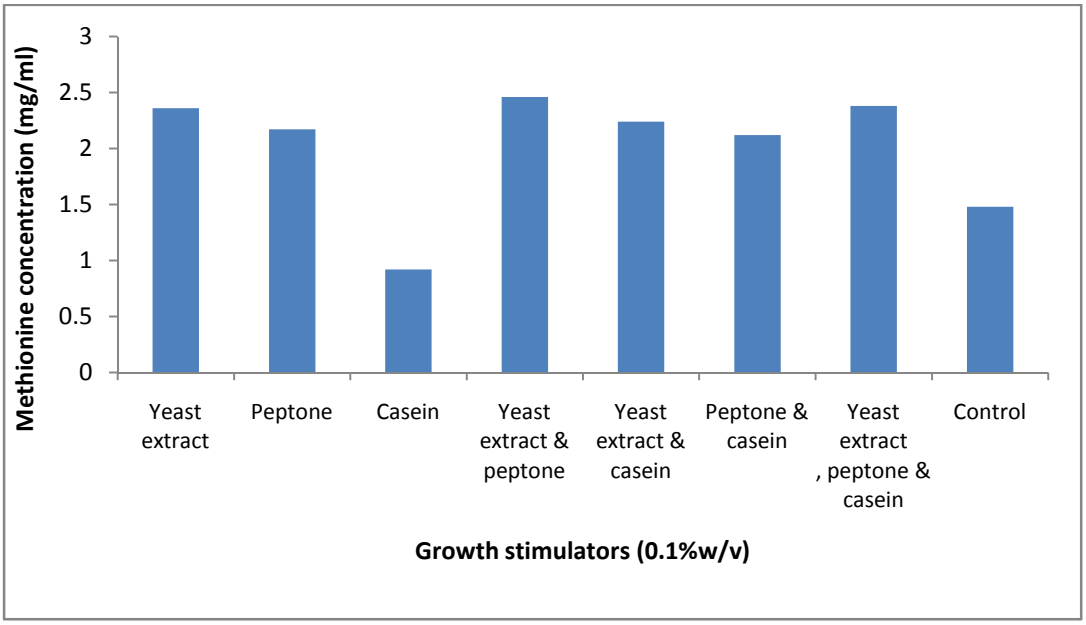

Fig. 4. Effect of growth stimulators on methionine production

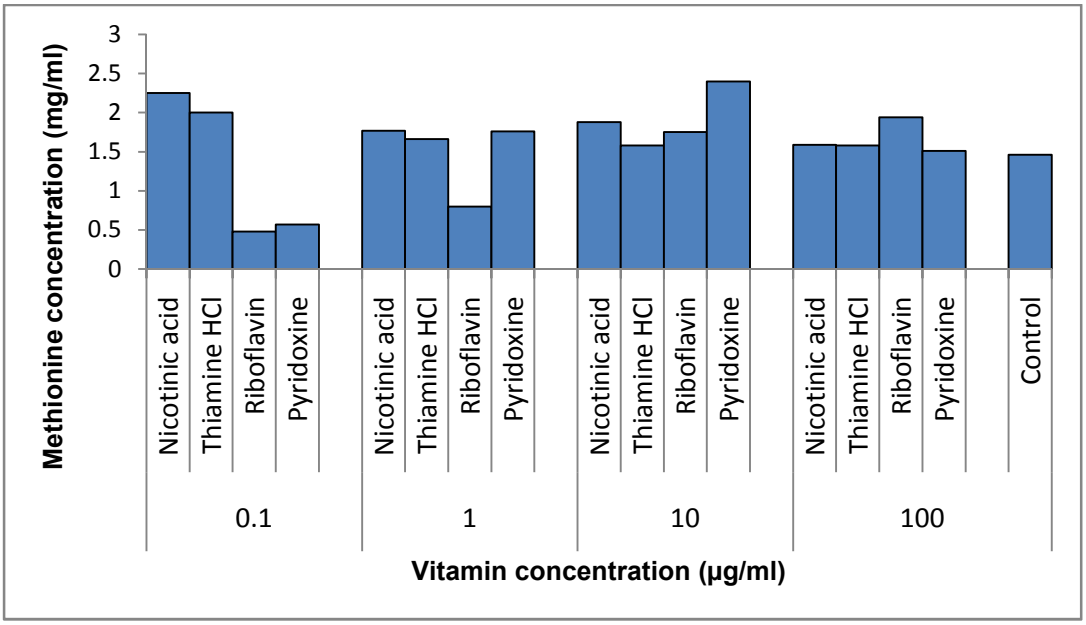

Fig. 5. Influence of vitamin concentration on methionine production

DL-Leucine enhanced methionine accumulation (1.60mg/ml) by *B. cereus* S8 while alanine, DL-aspartic acid, tryptophan, glycine, DL-methionine and DL-threonine inhibited methionine production. The inhibitory effect of the amino acids on methionine production is in line with the findings of Mindlin and Zaitseva [32]. They observed that amino acids cause repression

or inhibition of specific enzymes which direct the biosynthetic pathway for producing lysine. Similar effect may have been responsible for the low methionine yields by *B. cereus* S8.

The cultural conditions for improved methionine accumulation by *B. cereus* S8 are as presented in Fig. 6.

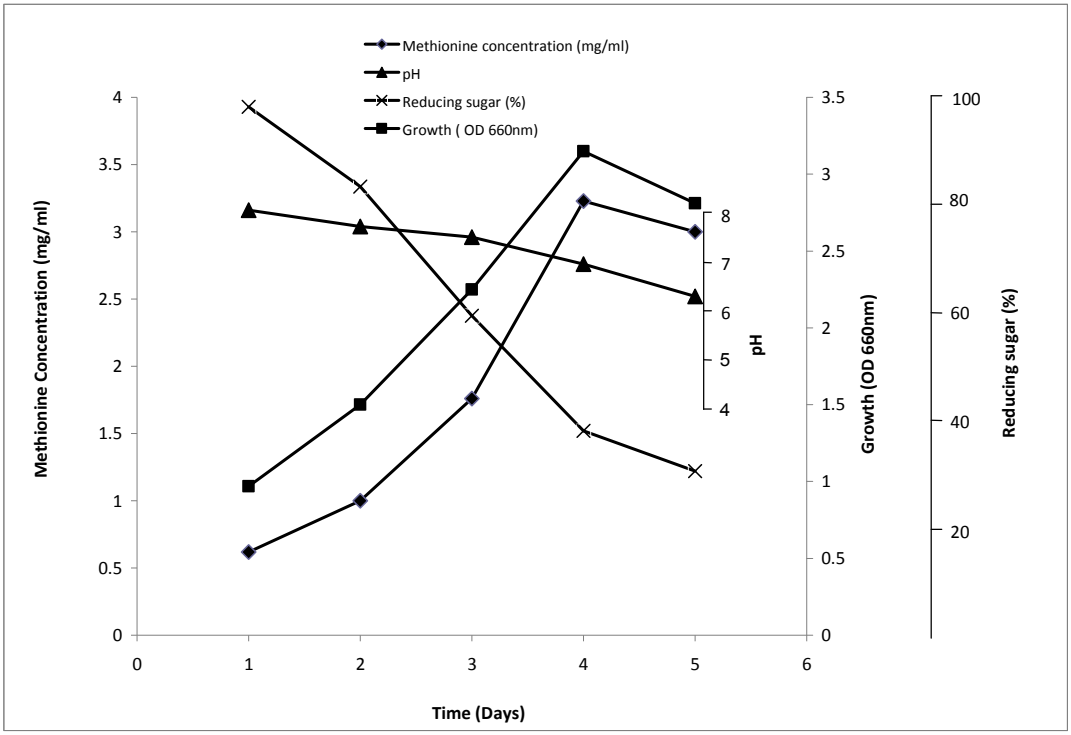

Fig. 6. Time course experiment for growth, pH, sugar utilization and methionine production

Methionine yield of 3.23mg/ml was produced after a fermentation period of 96h and at a pH of 6.90. The residual sugar was 38%. This pattern of methionine production is similar to that observed in the works of Pham et al. [2] on methionine production from various carbohydrates and Ekwealor and Obeta [33] on lysine production by *B. megaterium.*

4. CONCLUSION

A 20% medium to fermenter volume ratio containing glucose, ammonium sulphate, yeast extract, peptone, pyridoxine and leucine was observed to increase methionine accumulation by *B. cereus* S8. Therefore, this study has shown that methionine yields by *B. cereus* S8 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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