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Optimization of Nutritional Parameters for the Production of L-Methionine from Newly Isolated *Bacillus cereus* Strain

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Authors' contributions

This work was carried out in collaboration between all authors. Author IAE designed the study, while author KSD performed the laboratory work in collaboration with author IAE. Authors KSD and CEO managed the literature searches, performed the statistical analysis, wrote the first and revised draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study evaluated nutritional parameters for optimum methionine production
Study Design: Methionine was assayed using the colorimetric method of Greenstein and Wintz (1961).
Place and Duration of Study: Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria, between April 2010 and November 2011.
Methodology: *Bacillus cereus* RS 16 was previously isolated from a soil ecovar in Owerri, Nigera. It was maintained on nutrient agar (Oxoid) slants at 4ºC. *Bacillus cereus* 16 was confirmed by 16Sr RNA conducted at Macrogen Incoporated Korea. Methionine production was carried out in a submerged medium. A 10% (v/v) seed culture was used to inoculate a 100ml Erlenmeyer flask containing 30 ml of fermentation medium in a rotatory shaking incubator at 170 rpm and 30ºC. Growth and methionine accumulation was determined from the broth. Nutritional parameters were studied to determine optimum methionine production
Results: Glucose and ammonium chloride were the best carbon and nitrogen source for L-methionine production. Maximum methionine (4.55mg/ml) were obtained with 80.0g glucose, 20.0g ammonium chloride, 20.0g calcium carbonate, 0.1.g DL-ornithine

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monohydrate, 0.1g peptone, 0.5g potassium dihydrogen phosphate, 0.5g potassium hydrogen phosphate, 0.01g magnesium sulphate heptahydrate, 0.001g manganese sulphate tetrahydrate, 0.001g ferrous sulphate heptahydrate at 72h fermentation period. There was a remarkable increase of methionine level to 4.55 mg/ml after optimization compared to methionine level of 1.92 mg/ml before optimization

Conclusion: This present investigation has determined optimum parameters for maximum production of L- methionine by the newly isolated mesophilic bacterium *Bacillus cereus* strain RS 16. This information has enabled formulation of media composition for maximum methionine production by this organism.

Keywords: Methionine; Bacillus cereus; fermentation; effect; growth; correlation.

1. INTRODUCTION

Methionine is required in small amounts in the diet of humans and other mammals for normal growth and body metabolism. In humans, some hereditary diseases like cystathioninuria and homocystinuria are caused by defective metabolism of methionine. [1]. Patients suffering from these diseases may exhibit one or more symptoms such as mental retardation, seizures, thrombocytopenia, clubfoot, skeletal abnormalities, lens dislocation, and hearing defects [2]. Dietary deficiency of methionine has been linked to such ailments as toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson's disease, liver deterioration, and impaired growth [3].

Methionine is essential for the absorption, transportation and availability of selenium and zinc in cellular functions. Methionine is used to produce creatinine required by the brain. 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 [4]. A shortage of methionine in poultry diets, reduces feed efficiency, constrains growth and in extreme cases results in nutritional deficiency. Supplementation with isolated amino acids increases feed conversion efficiency, thus lowering feed costs per unit gain of the animal. Amino acid supplementation is widely used in livestock production

The general and cheapest process to obtain L-methionine is the chemical synthesis using acroleine, methyl mercaptan and hydrocyanic acid [5]. Another method to obtain L-methionine is the extraction from protein hydrolysates [6]. The discovery of glutamic acid producing bacteria by [7], eventually led to fermentation processes for producing various other amino acids. Since then a number of microorganisms capable of producing amino acids have been isolated and the production of amino acids has become an important aspect of industrial microbiology. Amino acids such as lysine, threonine, isoleucine and histidine have been produced successfully by fermentation. [5,8,9,10,11], attempts have been made to overproduce biologically active L-methionine using fermentation [12,13,14,15,16,17].

In our previous work, we reported the production of L- methionine by methionine yielding strains of *Bacillus cereus* isolated from different soil ecovars in Owerri, Nigeria [13].

In this present work, we have examined the effect of nutritional parameters on growth and methionine production by this methionine producing *Bacillus cereus* RS16 strain, a study that may contribute in determining suitable conditions for achieving maximum methionine production.

2. MATERIALS AND METHODS

2.1 Microorganism

Bacillus cereus RS 16 was previously isolated from a soil ecovar in Owerri, Nigera. It was maintained on nutrient agar (Oxoid) slants at 4ºC.The taxonomic identification was done by the methods recommended by [18] and [19]. *Bacillus cereus* 16 was confirmed by 16Sr RNA conducted at Macrogen Incoporated Korea

2.1.1 Growth and cultivation

The medium for seed culture consist of (g/L: peptone, 10.0; yeast extract, 10.0; NaCl, 5.0; water, 1 litre, pH was adjusted to 7.5 with 1N NaOH. The medium was sterilized at 121ºC for 15 minutes. Two loopfuls of a 24h slant of the culture used to inoculate a 100ml flask containing 30ml of the seed medium. The flasks were incubated for 16-18h on a rotary shaker at 170 rpm and 30ºC. The basal medium used for fermentation composed of KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$ 10.0g; $CaCO_3$, 5.0g; $MgS0_4.7H_2O$ 0.001g; $FeS0_4.7H_2O$ 0.01 g; glucose 20.0g; Water I litre; pH was adjusted to 7.2 with 1N Na0H.The medium was sterilized at 115ºC for 10 mins. A 2ml volume (ca 2.8 X 10^8 cells/ml) of the seed culture was used to inoculate triplicate Erlenmeyer flaks containing 30ml of the fermentation medium. After 72 h of incubation on the same rotary shaker at 170 rpm and 30ºC, growth and methionine produced were determined from the broth culture. Four uninoculated flask were used as control. Growth of the isolate was determined turbidimetrically using the culture broth in spectronic 21 spectrophotometer (Camspec).

2.1.2 Analytical methods

Quantitative determination of L-methionine in the culture broth without purification was carried out by the modified calorimetric method of [20]. A 5ml volume of the culture broth was centrifuged at 5,000xg for 20 minutes and the cell free supernatant was assayed for L-methionine.1 ml of 5N NaOH was added to a test tube followed by the addition of 0.1ml of 10% sodium nitroprusside solution with through 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 540nm in a spectrometer. 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.

Carbon sources chosen for the study were glucose, sucrose, maltose and mannitol. Sources of nitrogen include NH_4NO_3, KNO_3, $(NH_4)_2SO_4$, NH_4Cl, $MgNO_3$ and $NH_4H_2PO_4$. The effect of different concentration of carbon and nitrogen sources, $CaCO_3$, amino acids and growth promoting factors (yeast extract, peptone, casein, soya bean, a mixture of peptone and yeast extract) were also monitored. The time course for methionine production was studied by growing *Bacillus cereus* RS 16 in a basal medium containing K_2HP0_4, 0.5g; K_2HPO_4, 0.5g; NH_4Cl_2, 20.0g; $MnS0_4.4H_2O$, 0.001g; $MgS0_4.7H_2O$, 0.001g; $CaCO_3$, 20.0g; $FeSO_4.7H_2O$, 0.001 g; peptone. 0.1g; glucose, 80.0g, distilled water, 1 litre; pH 7.5. Growth, methionine concentration and glucose utilization were determined every 24h for a

fermentation period of 120h. All experiments were conducted in triplicates with uninoculated flasks as control.

2.1.2.1 Test for reducing sugar

The reducing sugar (glucose) in the time-course fermentation broth was estimated by the modified method described by [21]. A 1ml volume of dinitrosalicylic acid was added to 1ml of the supernatant in a test tube and the mixture heated in boiling water for 10 minutes. The test tube was cooled rapidly under tap water. 1ml of 4% potassium sodium tartarate was added and the volume was adjusted to 12 ml with distilled water. A blank containing 1litre of distilled water and 1 ml of dinitrosalicylic acid was similarly prepared. The optical density of the sample was read against the blank in a spectrophotometer at 540nm. The concentration of the reducing sugar in the supernatant was estimated from a standard glucose curve.

2.3 Statistical Analysis

The experimental work was carried out in triplicates and data obtained from the experiments were subjected to correlation analysis. The SPSS® 14.0 package was used for all analysis.

3. RESULTS AND DISCUSSION

3.1 Effect of Carbon Sources

The effect of sucrose, maltose and mannitol on growth and methionine production was compared with that of glucose. Glucose in the basal medium was replaced by equivalent concentration of these carbon sources. Among the substrates tested (Fig. 1), glucose proved to be the best carbon source for methionine production. This is in line with the works of several researchers who reported glucose as carbon source for the production of L-Methionine [17,22,23].

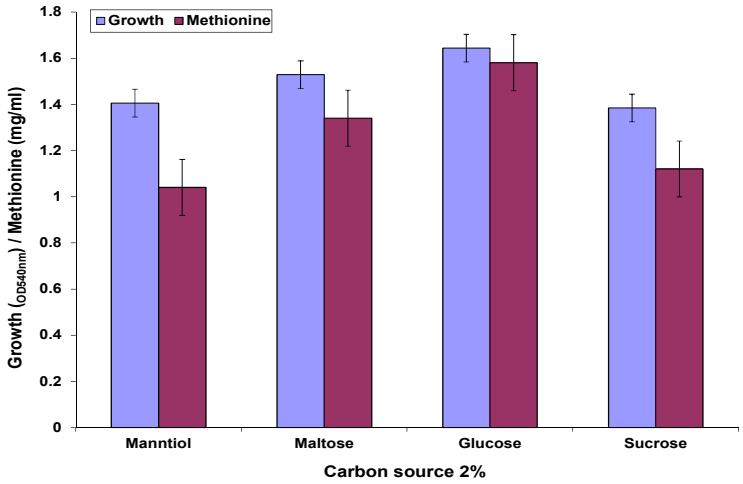

Fig. 1. Effect of different carbon sources on growth and methionine production

Medium: $(NH_4)_2SO_4$, 10.0g; K_2HPO_4,0.05g; KH_2PO_4, 0.05g; $MgSO_4.7H_2O$, 0.01g; $MnSO_4.4H_2O$, 0.001 g; $FeSO_4.7H_2O$, 0.001 g; $CaCO_3$, 10.0g; Carbon source, 20 g; Distilled water, 1L; pH 7.5; Fermentation time 72h; Temperature 30ºC

Result of the effect of different concentrations of glucose (Fig. 2) show that methionine production was a function of the initial sugar concentration in the fermentation medium. An increase in glucose concentration exceeding 8.0% resulted in poor methionine yield.

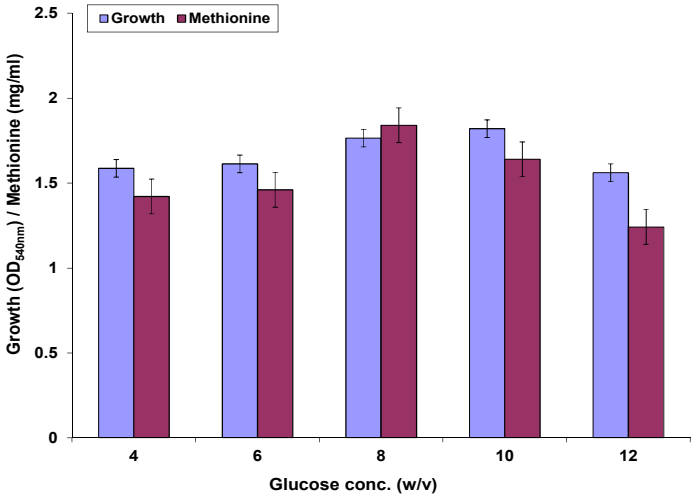

Fig. 2. Effect of different concentration of glucose on growth and methionine production

Medium: $(NH_4)_2S0_4$, 10.0 g; K_2HPO_4, 0.05 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$,0.01 g; $MnSO_4.4H_2O$, 0.001 g; $FeSO_4.7H_2O$, 0.001 g; $CaCO_3$, 10.0 g; Glucose, 40-120 g; Distilled water, 1L; pH 7.5; Fermentation time 72h; Temperature 30ºC.

Correlation studies show a weak positive correlation between glucose concentration and growth of the organism (r=.213) and a weak negative correlation between glucose concentration and methionine production (r=-.125). All correlations were not significant at 0.05 confidence level (Table 1).

Table 1. Correlation of the effect of different glucose concentration on growth and methionine production

		Glucose concentration	Growth in OD of *Bacillus cereus* RS16	Methionine production of *Bacillus cereus* RS16
Glucose concentration	Pearson Correlation	1	.213	-.125
	Sig. (2-tailed)		.731	.842
	N	5	5	5
Growth in OD of *Bacillus cereus* RS16	Pearson Correlation	.213	1	.856
	Sig. (2-tailed)	.731		.064
	N	5	5	5
Methionine production of *Bacillus cereus* RS16	Pearson Correlation	-.125	.856	1
	Sig. (2-tailed)	.842	.064	
	N	5	5	5

**Correlation is not significant at 0.05 level (2-tailed)*

3.2 Effect of Nitrogen Source

In order to investigate the effect of ammonium sulphate, diammonium hydrogen phosphate, ammonium dihydrogen phosphate, potassium nitrate and magnessuim nitrate on growth and methionine accumulation, ammonium chloride in the basal medium was replaced by equimolar concentration of the various nitrogen sources. The results presented in Fig. 3, shows that medium with ammonium chloride was the best for methionine production.

The relationship between varying concentration of nitrogen and methionine accumulation (Fig. 4) suggest that methionine production could be a function of the initial nitrogen concentration present in the medium. This view is supported by the experimental observations of [24] on the extracellular accumulation of nitrogen compounds in the culture medium of various microorganisms. They noted a close relationship between the initial inorganic nitrogen compounds added to the medium and the nitrogenous materials formed, and showed that the extracellular nitrogen material was not simply a product of autolysis or senescence. As observed beyond nitrogen concentration of 2%, growth and methionine production decreased. [25] also reported 2% nitrogen source for methionine production. This decrease as suggested by [26] may be attributed to the osmotic pressure exerted by high nitrogen concentration, which have an adverse effect on growth and methionine accumulation in *Bacillus* cereus RS 16.

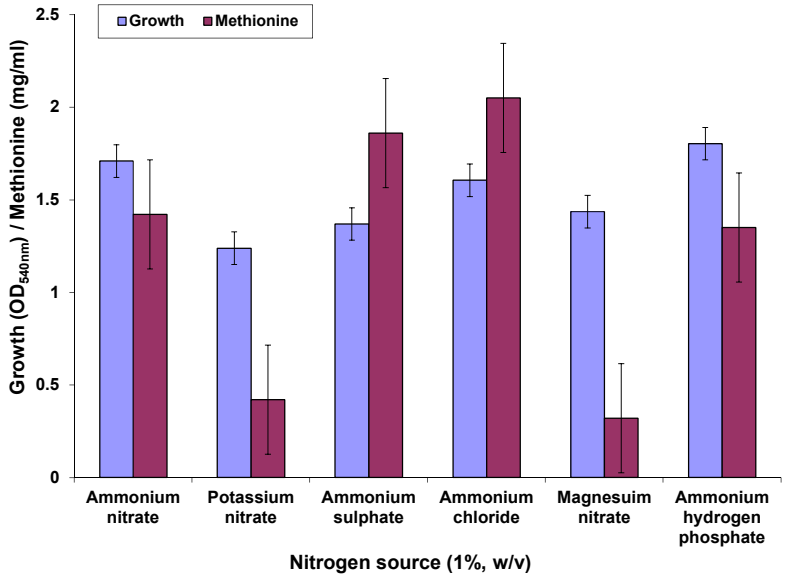

Fig. 3. Effect of nitrogen sources on growth and methionine production

Medium: Glucose, 80.0 g; K_2HPO_4, 0.05 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.01g; $MnS0_4.4H_2O$, 0.001 g; $FeSO_4.7H_2O$, 0.001 g; $CaCO_3$, 10.0 g; Nitrogen source, 10.0 g; Distilled water, 1L; pH 7.5; Fermentation time 72 h; Temperature 30ºC

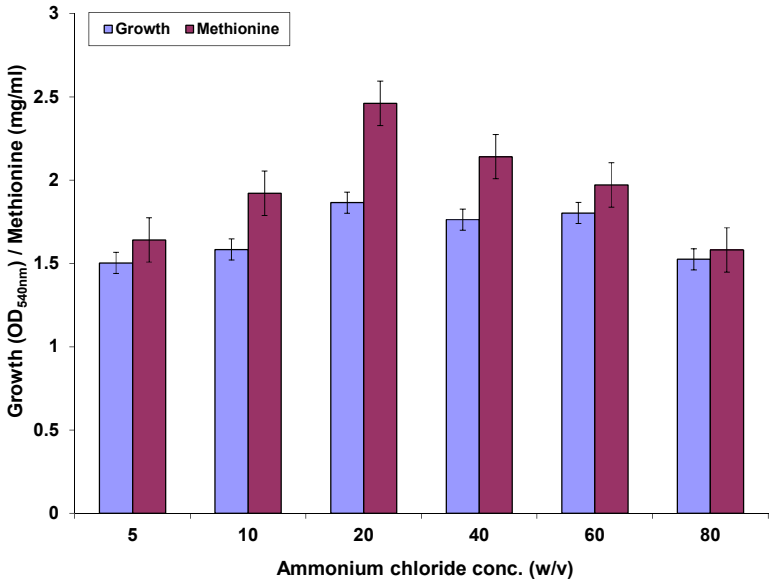

Fig. 4. Effect of different concentration of Ammonium chloride on growth and methionine production

Medium: Glucose, 80.0 g; K_2HPO_4, 0.05 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.01 g; $MnSO_4.4H_2O$, 0.001 g; $FeS0_4.7H_20$, 0.001 g; $CaCO_3$, 10.0 g; NH_4Cl, (5-80.0 g); Distilled water, 1L; pH 7.5; Fermentation time 72 h; Temperature 30ºC.

As observed from Table 2, All correlations were not significant at 0.05 confidence level; the correlation between ammonium chloride and growth of the organisms was r=.152 and between ammonium chloride and methionine was r= - .132.

Table 2. Correlation of the effect of concentration of NH_4Cl_2 on growth and methionine production

		NH_4Cl source conc. for RS16	**Growth in OD of *Bacillus cereus* RS16**	**Methionine production by *Bacillus cereus* RS16**
Nitrogen source conc. For *Bacillus cereus* RS16	Pearson Correlation	1	.152	-.132
	Sig. (2-tailed)		.773	.803
	N	6	6	6
Growth in OD of *Bacillus cereus* RS16	Pearson Correlation	.152	1	.700
	Sig. (2-tailed)	.773		.014
	N	6	6	6
Methionine production by *Bacillus cereus* RS16	Pearson Correlation	-.132	.700	1
	Sig. (2-tailed)	.803	.014	
	N	6	6	6

**Correlation is not significant at 0.05 level (2-tailed).*

3.3 Effect of $CaCO_3$

In fermentation process, the pH of the broth decreases due to accumulation of pyruvic acid, lactic acid, gluconic acid etc. As a result, the growth of the bacterium ceases with concomitant decrease in product yield. Calcium carbonate neutralizes pH of fermentation broth thus shortening the fermentation time. The effect of 1 to 4% calcium carbonate was evaluated. Optimum yield was obtained at 2% calcium carbonate. Without the addition of $CaCO_3$ the final pH was very low and the methionine concentration decreased considerably (Fig. 5). Thus pH is one of the most important factors affecting microbial propagation. As nutrients are consumed and converted into products during fermentation process, the pH changes drastically in the absence of suitable control mechanism. In order to maintain optimal pH, reagents like calcium carbonate must be added to the culture medium at the beginning of the fermentation [7]. [27] reported that though the pH of the fermenter automatically controlled by ammonia water yet small amount of $CaCO_3$ must be added. According to the authors, this eliminates the lag phase of the cell growth thereby shortening fermentation time.

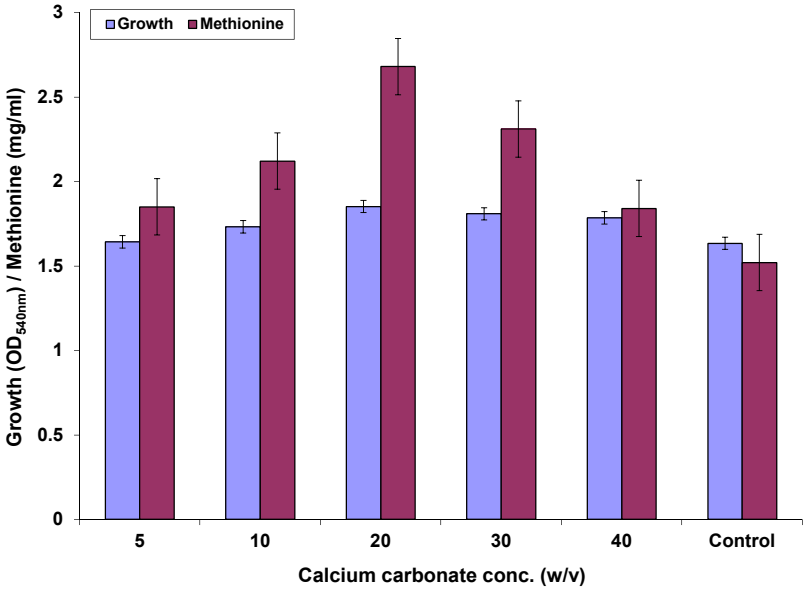

Fig. 5. Effect of different concentration of $CaCO_3$ on growth and methionine production

Medium: Temperature 30ºC glucose, 80.0 g; K_2HPO_4, 0.05 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_20$, 0.01 g; $MnS0_4.4H_2O$, 0.001 g; $FeSO_4.7H_20$, 0.001 g; NH_4Cl, 20.0 g; $CaCO_3$, 5-40.0 g; Distilled water, 1L; pH 7.5; Fermentation time 72 h; Control: No calcium carbonate added.

All correlations were not significant at 0.05 confidence level; the correlation between calcium carbonate concentrations and growth of the organism was r=.764 and between calcium carbonate and methionine production was r=.361 (Table 3).

Table 3. Correlation of the effect of concentration of $CaCO_3$ on growth and methionine production

		$CaCO_3$ concentration	**Growth of *Bacillus cereus* RS16**	**Methionine production by *Bacillus cereus* RS16**
$CaCO_3$ concentration	Pearson Correlation	1	.764	.361
	Sig. (2-tailed)		.077	.483
	N	6	6	6
Growth of *Bacillus cereus* RS16	Pearson Correlation	.764	1	.844
	Sig. (2-tailed)	.077		.035
	N	6	6	6
Methionine production by *Bacillus cereus* RS16	Pearson Correlation	.361	.844	1
	Sig. (2-tailed)	.483	.035	
	N	6	6	6

**Correlation is not significant at 0.05 level (2-tailed).*

3.4 Effect of Growth Factor

In this study, the influence of 0.1% (w/v) growth promoting substances such as peptone, yeast extract, soya bean and casein on methionine production was examined. Fermentation was carried out for 72h on rotary shaker at 170 rpm and 30ºC. Growth and methionine production were determined as previously described. The result in Fig. 6 indicates that all growth factors stimulated growth. Yeast extract and peptone enhanced methionine accumulation at concentration of 1mg/ml. The stimulatory effect of yeast extract reported in this work agrees with the works of [28], who studied the effect of concentration of yeast extract on methionine production in *Pseudomonas spp* FM518 and reported an increase of 0.8mg/l when 7.5mg/ml of yeast extract was added. [29], reported improved methionine production in an obligate methylotroph strain OM 33 when 5mg/ml of yeast extract was added.

Table 4 show that all correlations were not significant at 0.05 confidence level; the correlation between growth promoting factors and growth of the organism was r=.328 and between growth promoting factors and methionine production was r=-.797.

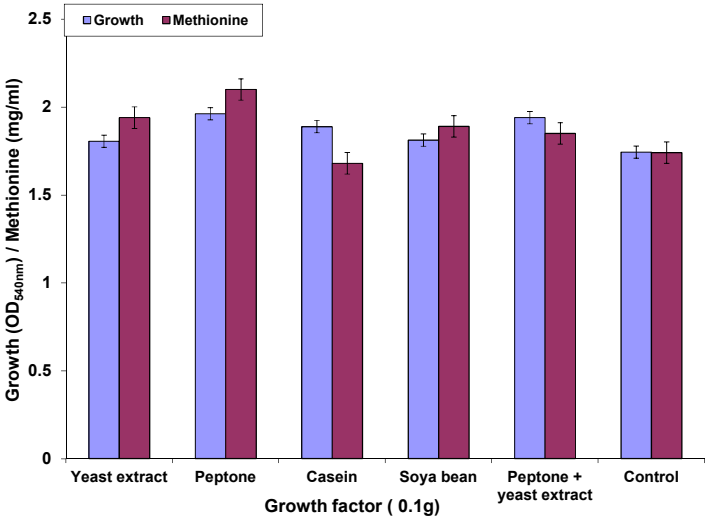

Fig. 6. Influence of growth promoting factors on growth and methionine production
Medium: Glucose, 80.0 g; K_2HPO_4, 0.05 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.01 g;$MnS0_4.4H_20$, 0.001 g; $FeSO_4.7H_2O$, 0.001 g; $CaCO_3$, 20.0 g; NH_4Cl, 20; growth factor,0.1g; Distilled water, 1L; pH 7.5; Fermentation time 72 h; Temperature 30ºC; Control: No growth factor added.

Table 4. Correlation of the effect of growth promoting factors on growth and methionine production

		Growth factor	Growth in OD of *Bacillus cereus* RS16	Methionine production by *Bacillus cereus* RS16
Growth factor	Pearson correlation	1	.328	-.797
	Sig. (2-tailed)		.526	.058
	N	6	6	6
Growth in OD of *Bacillus cereus* RS16	Pearson Correlation	.328	1	-.509
	Sig. (2-tailed)	.526		.303
	N	6	6	6
Methionine production by *Bacillus cereus* RS16	Pearson Correlation	-.797	-.509	1
	Sig. (2-tailed)	.058	.303	
	N	6	6	6

**Correlation is not significant at 0.05 level (2 tailed)*

3.5 Effect of Amino Acid

The result of the effect of amino acid on growth and methionine production show that methionine was enhanced by the addition of L-asparagine, DL-serine, L-glutamic acid, DL-ornithine monohydrate, DL- isoleucine, L-phenyalanine and L-lysine enhanced methionine accumulation. DL- ornithine monohydrate accumulated maximum methionine concentration of 3.22 mg/ml (Fig. 7). This is in line with the works of [29]. They reported an increase in methionine production in an obligate methylotroph strain OM33 by the addition of L-lysine

and L-serine. [30] observed a similar increase in lysine production by *Micrococcus glutamicus*, with the addition of 400 µg/ml serine.

All correlations were not significant at 0.05 confidence level; the correlation between amino acid and growth of the organism was r=-.41 and between amino acid and methionine production was r=-.110 (Table 5).

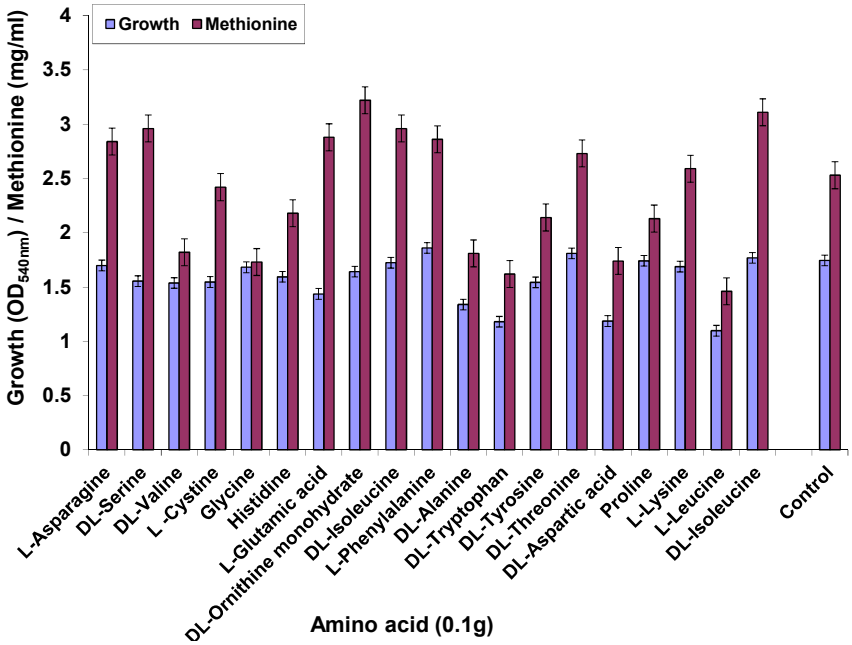

Fig. 7. Effect of amino acid on growth and methionine production

Medium: Glucose, 80.0g; K_2HPO_4, 0.05g; KH_2PO_4, 0.05g; $MgSO_4.7H_2O$, 0.01g; $MnS0_4.4H_2O$, 0.001g; $FeSO_4.7H_2O$, 0.001g; $CaCO_3$, 20.0g; NH_4Cl, 20; amino acid 0.1g; Distilled water, 1L; pH 7.5, Fermentation time 72h; Temperature 30ºC; Control: No amino acid added

Table 5. Correlation of the effect of amino acid on growth and methionine production

		Amino acids	Growth in OD of *Bacillus cereus* RS16	Methionine production of *Bacillus cereus* RS16
Amino acids	Pearson Correlation	1	-.041	-.110
	Sig. (2-tailed)		.860	.634
	N	21	21	21
Growth in OD of *Bacillus cereus* RS16	Pearson Correlation	-.041	1	.687
	Sig. (2-tailed)	.860		.000
	N	21	22	22
Methionine production of *Bacillus cereus* RS16	Pearson Correlation	-.110	.687	1
	Sig. (2-tailed)	.634	.000	
	N	21	22	22

**Correlation is not significant at 0.05 level (2 tailed)*

3.6 Time Course of Fermentation for Methionine Production

As observed from the result presented in Fig. (8), growth and methionine concentration was accompanied by utilization of sugar in the medium. Maximum concentration of 4.55mg/ml of methionine was recorded after 96h. Glucose in the culture broth was reduced to 16% after 96h fermentation. [25] reported a similar relationship between sugar consumption and methionine production by bacteria. According to [31] decline in methionine production in *Bacillus cereus* RS 16 after 4 days could be attributed to the age of the bacteria, depletion of sugar content and decreased available nitrogen in the fermentation medium. [32] reports that microbial production of metabolites usually starts after a lag phase of one day and reaches maximum at the onset of stationary phase or late.

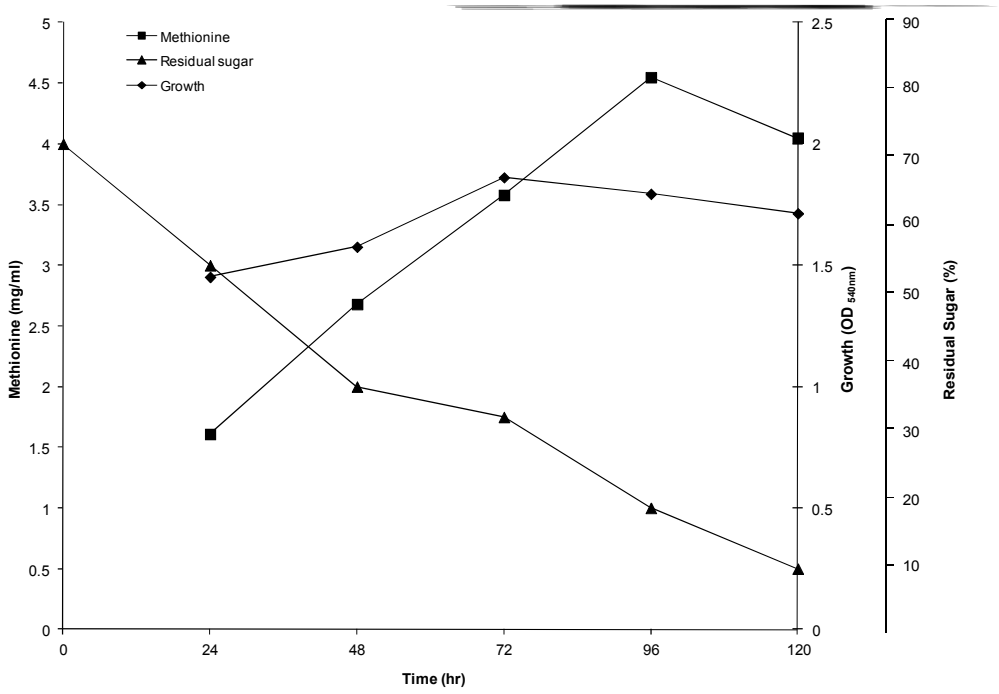

Fig. 8. Time course of fermentation for methionine production

Medium: Glucose, 80g/l; NH_4Cl, 20g/l; K_2HPO_4, 0.5g; K_2HPO_4, 0.5g; $MnSO_4.4H_2O$, 0.001g; $MgS0_4.7H_2O$, 0.001g; $CaCO_3$, 20.0g; $FeSO_4.7H_2O$ 0.001g; DL-ornithine monohydrate 0.1g, volume of medium, 30ml/100ml flask. pH 7.5; Temperature 30ºC;

4. CONCLUSION

In view of the results obtained, it can be concluded that methionine production by *Bacillus cereus* RS 16 depend markedly on the composition of the culture medium, carbon, nitrogen content, inducer compounds and other nutritional parameters. Results show that methionine was maximally produced with, 0.5% (w/v) K_2HPO_4, 0.5% (w/v) KH_2PO_4, 0.001% (w/v) $MnSO_4.4H_2O$, 0.001% (w/v) $MgSO_4.7H_2O$, 8% (w/v) glucose, 2% (w/v) NH_4Cl, 2% (w/v) $CaCO_3$, 0.1% (w/v) peptone and 0.1% (w/v) DL-ornithine monohydrate.

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COMPETING INTERESTS

Authors have declared that no competing interest exists.

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