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Studies on Process and Physical Parameters for the Production of L Methionine from Newly Isolated *Bacillus cereus* Strains

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ABSTRACT

Studies on process and physical parameters for the production of methionine employing *Bacillus cereus* strains recovered from the soil was carried out. The effect of medium/fermenter volume ratio, inoculum size, agitation time and initial pH, on the effect of growth and methionine production were studied. The maximum growth and methionine production was obtained with 30 mL medium volume, 10% inoculum size, initial pH of 7.5 and 170 rpm agitation rate.

Key words: Effect of medium/fermenter ratio, methionine, *Bacillus cereus,* culture medium

INTRODUCTION

L-methionine belongs to the essential amino acids which human and animal metabolisms are not capable to produce. Most natural feeds as wheat or maize protein, soya bean and fish meal are deficient in methionine, lysine and threonine. For pig and poultry breeding, specific breeding plans exist with L-methionine as essential and sulphur containing amino acid. The impact of L-methionine on animal nutrition and the consequences of its absence as nutritive feed additive have been investigated very well. It has been observed for poultry that the stability of egg shells decreases just as the milk production in cow does (Noftsger *et al*., 2003; Keshavarz, 2003). Several researchers have reported the production of methionine from bacteria. In 1995, the demand for methionine amounted to 300000 tons per year (Leuchtenberge, 1996). The general and cheapest process to obtain L-methionine is the chemical synthesis using acroleine, methyl mercaptan and hydrocyanic acid.

The discovery of glutamic acid producing bacteria by Kinoshita *et al*. (1957) 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. (Okamoto *et al*., 2000; Fan *et al*., 1988; Okamoto and Ikeda, 2000; Herman, 2003; Sharma and Gomes, 2001; Dagley *et al*., 1950; Kumagai, 2000; Kimura *et al*., 2002; Pfefferle *et al*., 2003; Shah *et al*., 2002a; Shah *et al*., 2002b; Khan *et al*., 2006; Rehman *et al*., 2011). Attempts have been made to overproduce biologically active L-methionine using fermentation (Roy *et al*., 1989; Kase and Nakayama, 1975; Mondal *et al*., 1994; Kumar *et al*., 2003). This present investigation was aimed at contributing in this regard by studying some physical parameters which may play a significant role in enhancing the production of L-methionine.

MATERIALS AND METHODS

Microorganisms: *Bacillus cereus* DS13, *Bacillus cereus* AS9 and *Bacillus cereus* RS16 were previously isolated from different soil ecovars in Owerri, Nigeria. It was maintained on nutrient agar (Oxoid) slants at 4°C. The taxonomic identification was been done by the methods recommended by Bergey *et al.* (1974), Finegold and Baron (1990) and Barrow and Feltham (1993). Molecular characterization conducted at Macrogen Inc. Europe confirmed isolates as different strains of *Bacillus cereus*

Growth and cultivation: The medium for seed culture consist of (g L^{-1}): Peptone: 10.0, yeast extract: 10.0, NaCl: 5.0, water: 1 L and pH was adjusted to 7.2 with 1 N NaOH. The medium was sterilized at 121°C for 15 min. Two loopfuls of a 24 h slant of each culture used to inoculate a 250 mL flask containing 50 mL of the seed medium. The flasks were incubated for 16-18 h on a rotary shaker at 120 rpm and 30°C.

Fermentation: The basal medium used for fermentation composed of KH_2PO_4: 0.5 g, K_2HPO_4: 0.5 g, $(NH_4)_2 SO_4$: 20 g, $MgSO_4.7H_2O$: 0.001 g, $FeSO_4.7H_2O$: 0.001 g, $MnSO_4.4H_2O$: 0.001 g, glucose 100 g, water: I L and pH was adjusted to 7.2 with 1 N NaOH. The medium was sterilized at 115°C for 10 min. A 2 mL volume (ca 10^4 cells mL^{-1}) of each seed culture was used to inoculate triplicate Erlenmeyer flasks containing 20 mL of the fermentation medium. After 72 h of incubation on the same rotary shaker at 150 rpm and 30°C, growth and methionine produced were determined form the broth culture. Four uninoculated flask were used as control.

Determination of growth of the isolate: Growth of each isolate was determined turbidimetrically using the culture broth in spectronic 21 spectrophotometer (Camspec).

Analytical methods: Quantitative determination of L-methionine in the culture broth without purification was carried out by the modified calorimetric method of Greenstein and Winitz (1961). A 5 mL volume of each culture broth was centrifuged at 5,000 xg for 20 min and the cell free supernatant was assayed for L-methionine. One milliliter of 5 N NaOH was added to each tube followed by the addition of 0.1 mL 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, 2 mL 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 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.

Study of physical parameters: To investigate the influence of aeration on growth and methionine production, different volumes of basal medium (20, 25, 30, 40 and 50 mL) were inoculated with each organism and fermentation carried out. The optimum pH for growth and methionine production was studied by adjusting the initial pH of the medium to different levels (6, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0). The effect of inoculum size was investigated by varying five levels of inoculum size (1,2,3,4 and 5% (v/w)) while the optimum agitation speed was determined by studying the effect of different speed (50, 80, 110, 140, 170, 200) on growth and production. The fermentation was carried out for 96 h.

Effect of medium/fermenter volume ratio on growth and methionine production: The effect of aeration in the shake flask was measured by the volume of medium in the flask. Oxygen, an important nutrient, is usually supplied to flask through vigorous shaking on a rotary or reciprocal shaker. The effect of culture volume ratio on growth and methionine production by *Bacillus cereus* DS13, *Bacillus cereus* AS9 and *Bacillus cereus* RS16 is presented in Fig. 1, the results showed that growth and methionine production increased with increasing volume ratio for each of the isolates. Maximum growth and methionine production was observed in the flask containing 30 mL broth volume for *Bacillus cereus* DS13 and *Bacillus cereus* RS16 while maximum methionine production was observed in the flask containing 40 mL volume broth for *Bacillus cereus* AS9. Further increase in volume ratio caused a decreased in both growth and methionine production The highest methionine concentration of 1.63 mg mL^{-1} was produced by *Bacillus cereus* RS16.

Under conditions of insufficient oxygen, large amount of succinic and lactic acid are accumulated, while excess of oxygen increases the amount of a keto glutaric acid.

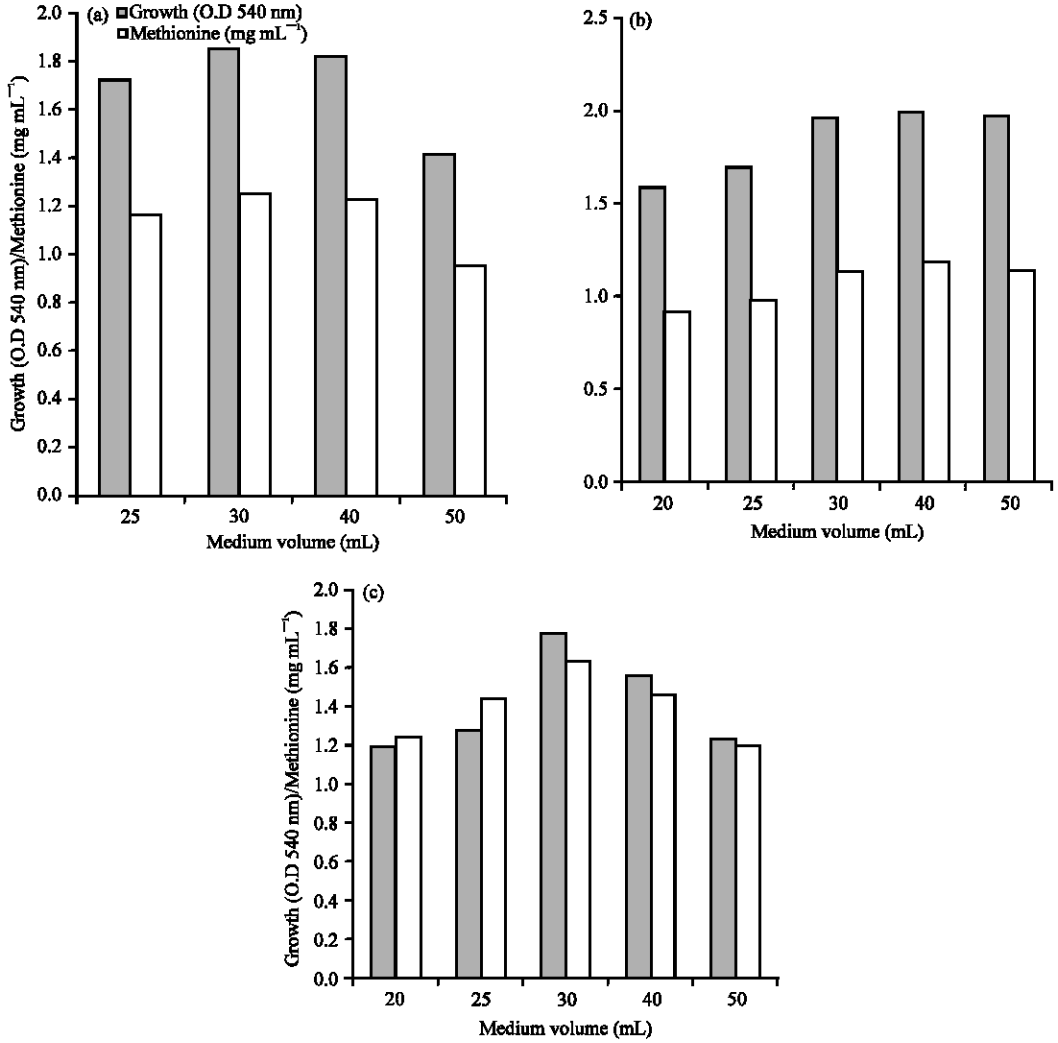

Fig. 1(a-c): Effect of medium volume/fermenter ratio on growth and production of methionine by isolates, (a) *Bacillus cereus* DS13 (b) *Bacillus cereus* AS9 and (c) *Bacillus cereus* RS16

Hallaert *et al.* (1987), Inbar *et al.* (1985) and Liu (1986) pointed out that both over abundance and meager aeration are undesirable in amino acid fermentation. They stated that the former inhibits cell growth while the latter hinders maximum production of amino acids.

The phenomenon proposed by Hallaert *et al.* (1987), Inbar *et al.* (1985) and Liu (1986) may have been responsible for the trend observed in our studies. There are still no reports on the effect of medium/fermenter volume on the fermentative production of methionine. Ganguly and Banik, (2010) also reported maximum L-glutamic acid production by mutant of *Micrococcus glutamicus* in the flask containing 30 mL of fermentation broth.

Effects of agitation on growth and production of methionine: The effect of agitation on growth and methionine production was examined. Inoculum vessel containing liquid medium was agitated to provide homogeneity. Agitation rates have been shown to affect metabolite production in various strains of bacteria. (Pourrat *et al.*, 1988; Darah and Ibrahim, 1996; Mehrotra *et al.*, 1999; Mabrouk *et al.*, 1999). The effect of agitation on L-methionine fermentation in rotatory shaker incubator is shown in Fig. 2.

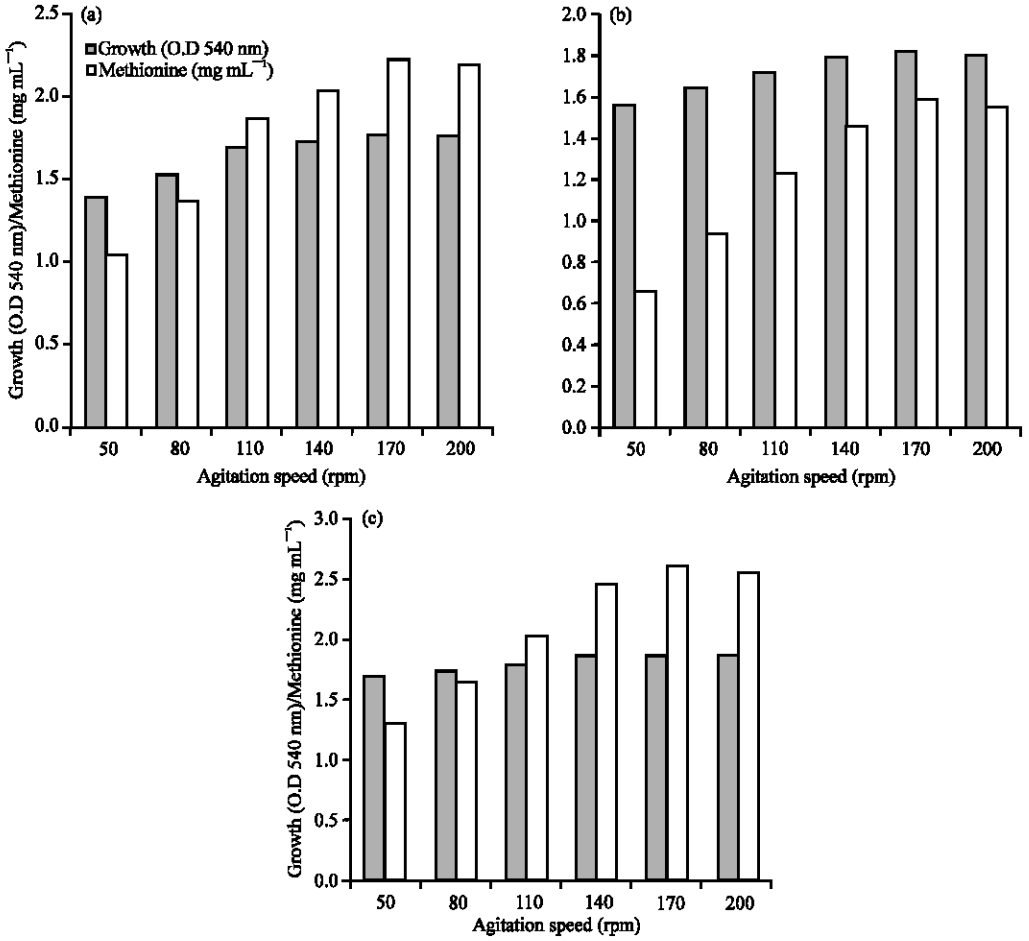

Fig. 2(a-c): Effect of agitation speed on growth and methionine production by isolates, (a) *Bacillus cereus* DS13, (b) *Bacillus cereus* AS9 and (c) *Bacillus cereus* RS16

As agitation increased from 50 to 170 rpm, L-methionine production increased rapidly and then leveled off, maximum yield was observed at 170 rpm for each isolate. Nutrient uptake by bacteria will also be increased (Beg and Gupta, 2003).

Shafee *et al.* (2005) pointed out that mixing is especially important because oxygen is a very low solubility nutrient. The oxygen transfer capability of flask can limit the amount of biomass that can be grown in the flask. When premature oxygen limitations are imposed on growth, changes in physiological state occur which results in reduced inoculum effectiveness.

Lee *et al.* (2002) suggested that agitation rates above 200 rpm will lead to denaturation of enzymes with attendant low production of metabolites. Darah and Ibrahim (1996) pointed out that excessive agitation could lead to cell lysis and increased cell permeability due to abrasion by shear forces.

Effect of inoculum size: The effect of inoculum size (1-5 mL) on growth and methionine production was also examined. The amount of inoculum inoculated into the fermentation broth also affects metabolite production. The effect of inoculum size on L-methionine production was examined (Fig. 3). The results showed that at 10% inoculum size, their was optimal growth and

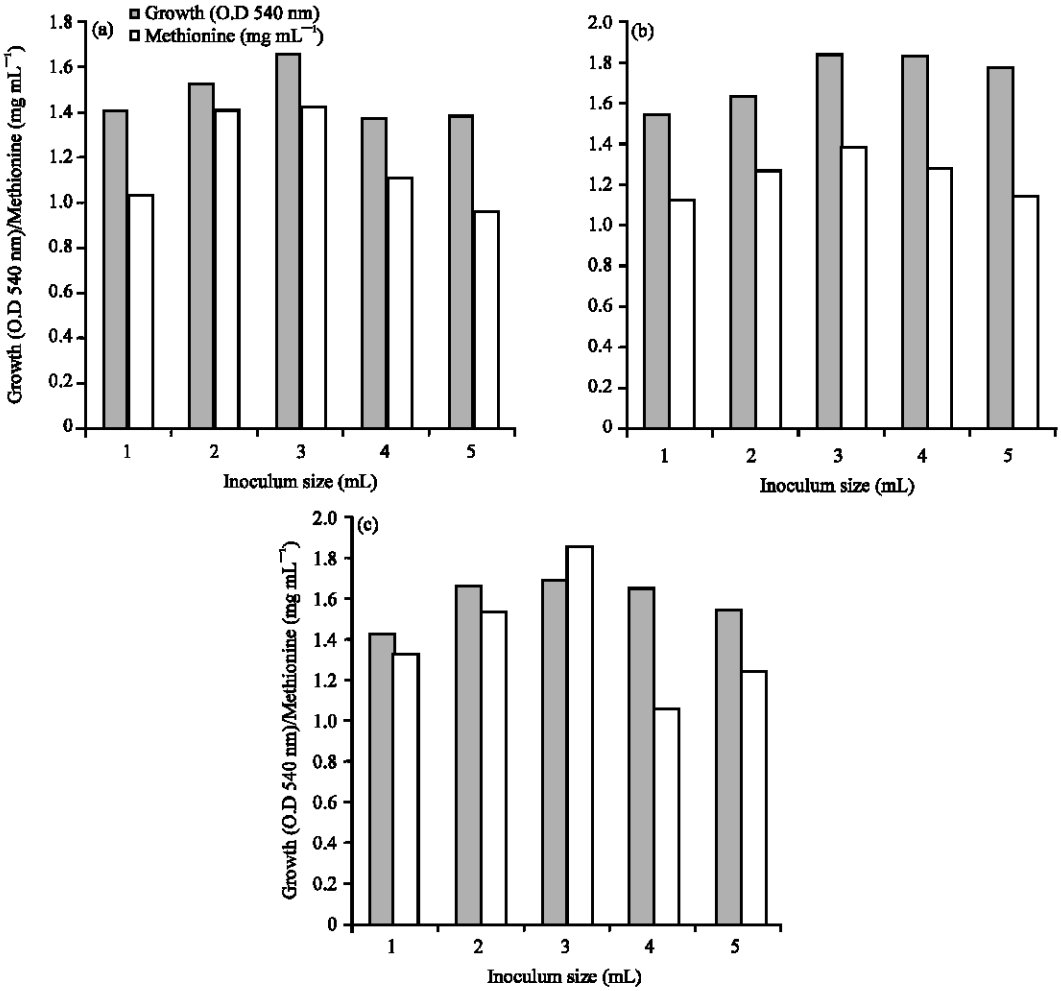

Fig. 3(a-c): Effect of inoculum size on growth and methionine production by isolates, (a) *Bacillus cereus* DS13, (b) *Bacillus cereus* AS9 and (c) *Bacillus cereus* RS16

methionine production for *Bacillus cereus* DS13 and *Bacillus cereus* RS16. Above 10%, their was decrease in growth and methionine accumulation in the medium. *Bacillus cereus* AS9 showed optimal growth and methionine yield at 7.5% inoculum size. *Bacillus cereus* RS16 produced the highest methionine yield of 1.86 mg mL^{-1} while *Bacillus cereus* AS9 produced the lowest yield of 1.14 mg mL^{-1}.

Kinoshita *et al.* (1961) reported maximum L-lysine production by *Micrococcus glutamicus* using 10% inoculum size. Ekwealor and Obeta (2005) obtained maximum L-lysine by *Bacillus megaterium* SP14 using 10% inoculum size. However, the size of inoculum to be used will depend on the cell mass and the composition of the seed medium to be transferred.

Hallaert *et al.* (1987) reported that inoculum size has a marked effect in fermentation. He opined that small inoculum size causes an increase in growth period because a very low inoculum density may give insufficient biomass. In addition, Rahman *et al.* (2005) suggested that higher inoculum size results in reduced dissolved oxygen and increased competition towards nutrient.

Effect of initial pH: Growth is usually sensitive to variations in pH. Furthermore, the process of growth changes the pH of the medium. The effects of pH of external medium on L-methionine production, due to activity of different biosynthetic enzymes and also due to its effect on export

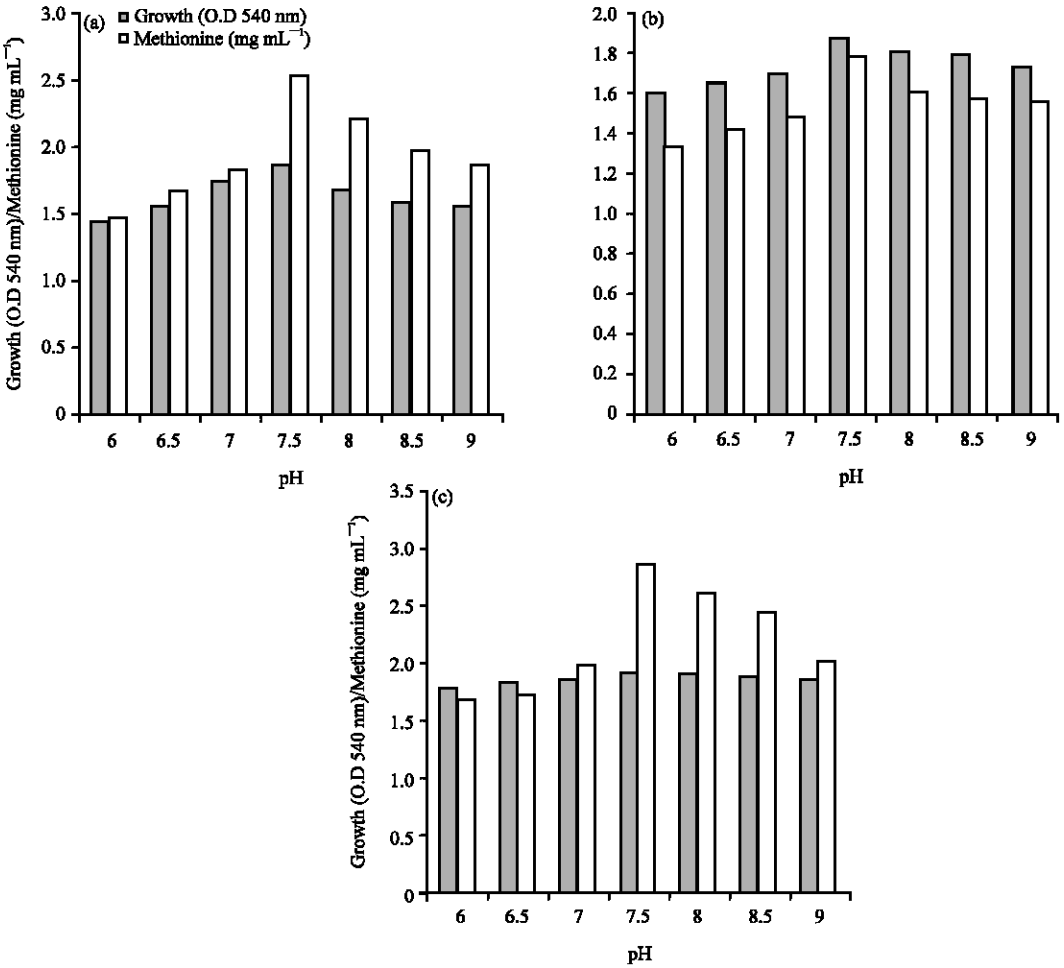

Fig. 4(a-c): Effect of pH on growth and methionine production by isolates, (a) *Bacillus cereus* DS13, (b) *Bacillus cereus* AS9 and (c) *Bacillus cereus* RS16

from the cell pH dependence, is an important feature of the export system (Broer *et al.*, 1993; Naz *et al.*, 2001). Kelle *et al.* (1996) studied the effect of pH on L-lysine transport by *Corynebacterium glutamicum* and found that pH value governed the transport activity and the specific L-lysine export rate. The results of our studies revealed that optimum growth and production for *Bacillus cereus* DS13, *Bacillus cereus* AS9 and *Bacillus cereus* RS16 was at pH 7.5. (Fig. 4). pH values higher than 7.5 was shown to have decreased growth and methionine yield.

Maximum yield of 2.86 mg mL^{-1} was observed in *Bacillus cereus* RS16 while *Bacillus cereus* AS9 produced the lowest yield of 1.34 mg mL^{-1}. Banik and Majumdar (1975) reported maximum production of 4.5 g L^{-1} of L-methionine in a medium with an optimum pH of 7.0. Liu (1986) found that L-lysine synthesis decreased at pH lower than 6.5, but found no difference between 6.5 and 8.0. Broer and Kramer (1991) recommended initial pH 7.5 for L-lysine production by *Corynebacterium glutamicum*. Nakayama *et al.* (1978) reported a pH range of (7.0-8.0) for glutamic acid production.

CONCLUSION

The results shows that most bacterial species are capable of producing methionine and that methionine producing organisms may be fairly well distributed in nature. This present work has also shown that environmental factors affect methionine production.

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