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# Optimization of fermentation and nutritional parameters for the production of L-Methionine by *C.glutamicum* using agriculture products

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**Abstract**

Methionine is an essential amino acid required in the diet of humans and mammals. Presently methionine is being produced by chemical and enzymatic methods. Chemical methods require hazardous chemicals and enzymatic method requires expensive enzymes. After the discovery of glutamic acid producing bacteria by Kinoshita et al. a number of microorganisms capable of producing amino acids have been separated and attempts have been made to over produce L-methionine by fermentation. But none of the methionine production process has become commercialized so far. In the present work, the effect of fermentation parameters such as temperature, pH, volume ratio of medium/fermenter, inoculum size and agitation (rpm) and nutritional parameters such as carbon source, nitrogen source and calcium carbonate on growth and methionine production by *C.glutamicum* using agricultural products were examined. After 96 hours of fermentation, Maximum biomass and methionine accumulation were 4.16 g/l and 4.6 g/l respectively with temperature $30^0$C, pH 7.0, volume ratio of medium to fermenter 30 ml/ml, inoculums size 3 ml, agitation 170 rpm, plantain starch hydrolysate as carbon source 20 g/l, groundnut as nitrogen source 10 g/l and $CaCO_3$ 20 g/l.

**Key words**: L-Methionine, *C.glutamicum*, Optimization and Production

## INTRODUCTION

Methionine is an essential sulfur containing amino acid that is required in the diet of humans and mammals for normal growth and function of body metabolism. Sulfur containing amino acids first had been detected in 1847 at Liebig's laboratory by Fleitmann [1], where he discovered heat instability of proteins in strong alkali solutions. Later, Osborne [2] determined in highly purified proteins two sulfur containing amino acids and one of them as correctly attributed to cysteine and the other was first isolated from casein and described later by Muller [3]. Three years later, Barger and Coyne [4] identified chemical formula as methylthiol-α-aminobutyric acid and suggested and agreeing with Dr. Mueller, the shorter name as methionine.
Dietary deficiency of methionine leads to diseases such as toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson's disease, liver deterioration, and impaired growth [5]. In humans, some genetic diseases like cystathioninuria and homocystinuria are caused by defective metabolism of methionine [6]. 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 [7]. The L form of methionine is used extensively in medical field for a variety of therapeutic purposes. The shortage of methionine in poultry feed reduces feed conversion efficiency and limits the growth.

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. The discovery of glutamic acid producing bacteria by [8], 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 [9,10,11,12,13], attempts have been made to overproduce biologically active L-methionine using fermentation [14,15,16,17,18,19]. But, no methionine production has been commercialized due to feedback inhibition and repression.

The history of species *Corynebacterium* as amino acid producer started in the 1950s when Dr. Kinoshita was the first to discover that *Corynebacterium glutamicum* is a superior amino acid producer [20, 21, 22]. Since the then a number of microorganisms capable of producing various amino acids have been isolated and the production of amino acids has become important aspect of industrial microbiology. Currently, amino acids such as L-glutamic acid, L-lysine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine are being produced by *Corynebacteria* in terms of high production rate and economical value.

Methionine can be found in high levels in some plant foods such as nuts, meat and others [23]. However, some Plant proteins are also deficit in methionine. Therefore, additional supplement in diet will be needed to meet nutritional requirements in both humans and animals [6]. Submerged fermentation is useful to produce biologically active L-Methionine. Fermentation processes have been

able to inexpensively produce many other amino acids using agriculture products. The importance of using agriculture media is the low cost, rich in nutrients and free of toxins. It has been reported that India has a large production of corn, millet, plantain, rice, wheat, potato, cowpea, pigeon pea and ground nut. Due to huge production of these agricultural products in India, methionine production is likely to be more economical.

In the present work, we have examined the effect of fermentation and nutritional parameters on growth and methionine accumulation by *C. glutamicum* (MTCC 2745) using agriculture products and this will be helpful to find out the suitable fermentation conditions and nutrient composition required for maximal production of L-Methionine in aerobic submerged fermentation.

## MATERIALS AND METHODS

### Microorganism

*C.glutamicum* MTCC 2745 powder was obtained from the microbial type collection centre, Chandigarh, India. It was maintained on nutrient agar slants and stored at refrigeration temperature 4°C.

### Inoculum Preparation

The medium used for seed culture consists of the following composition: peptone, 10.0 g/l; yeast extract, 10.0 g/l; NaCl, 5.0 g/l; water, 1 litre, pH was adjusted to 7.2 with 1N NaOH and sterilized at $121^0$C for 15 mins. Two loopfuls of 24 hour slant culture was used to inoculate a 100 ml flask containing 30 ml of seed medium. The flasks were incubated on a rotary shaker at 170 rpm and $30^0$C for 16-18 hr.

### Carbon sources: Preparation of starches

Agriculture products utilized here for the preparation of starches are corn, millet, plantain, rice, wheat and potato. Starches were prepared according to the method portrayed by [24]. Potato and plantain samples were first peeled, washed and cut into little pieces before being homogenized with water in Moulinex blender. Corn, millet, rice and wheat were drenched for 48 hours to soften the seeds and then homogenized with water. Every homogenate blended with excess water was tied in cheese cloth and placed on tripod stand overnight, to take into account extraction of starch into a clean plastic bowl. The supernatant was emptied and the sedimented starch dried at $50^0$C for 48 hours. The resultant chips were grounded into powder and utilized as starches.

### Saccharification of starch

Saccharification of starches took after the method illustrated by [25]. A 500 ml flask containing a mixture of 30 g of starch and 100 ml of water was heated for 15 min at $95^0$C in a water bath to gelatinize starch. The beaker was covered with aluminium foil after adding 1 ml of α-amylase and again heated in water bath for 10 min at $95^0$C to impact liquefaction. After cooling liquefied starch to $60^0$C, 1 ml amyloglucosidase enzyme was added before replacing the beaker in the water bath at $60^0$C for 48 hr for saccharification to takes place.

### Nitrogen sources: Preparation of defatted proteins

The proteins utilized here from agricultural products as nitrogen sources are cowpea, pigeon pea and groundnut. For preparation of defatted proteins took after the strategy explained by [25]. Cowpea, pigeon pea and groundnut were crushed in a blender and then some division of homogenized proteins was defatted by Soxhlet extraction method using diethyl ether. The meals obtained after extraction were oven dried at 34-$35^0$C for 20 hr and afterward ground into fine powder.

### Shake Flask Experiments

Fermentation experiments were conducted based on the method described by [26]. The medium used for fermentation composed of the following composition: Plantain (starchy material as carbon source, 20 g/l; Groundnut (defatted protein as nitrogen source), 10 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH was adjusted to 7.2 with 1N NaOH. Thirty ml of medium in 100 ml Erlenmeyer flask was sterilized in an autoclave at $115^0$C for 10 minutes, cooled and then inoculated with 3 ml of seed inoculums. After 72 hr of incubation on rotary shaker at 170 rpm and $30^0$C, growth and methionine accumulation were determined from culture broth. Duplicate flasks were used and uninoculated flasks served as control.

### Analytical Methods

### Identification of Methionine

A 5ml volume of the culture broth was centrifuged at 5,000xg for 20 minutes and the cell free supernatant was identified for methionine using Ninhydrin test and paper chromatography. **Ninhydrin test:** To 1 ml amino acid solution (supernatant) add 5 drops of 0.2% ninhydrin solution in acetone, boil over a water bath for 2 minutes and allow to cool to observe the formation of blue colour.

**Paper Chromatography:** Supernatant was applied 1.5cm above one edge of Whatman No.1 filter paper. 1 µl standard methionine solution (0.1mg/ml) was applied along side with the supernatant and the chromatogram was developed in a solvent mixture of n-butanol, acetic acid and water (4:1:1) for 18 hours. The chromatogram was air dried at room temperature, sprayed with 0.15% ninhydrin solution in butanol and dried again before heating at 60°c for 5 minutes in an oven. The $R_f$ value of the ninhydrin-positive spot (bluish-violet) of the supernatant that corresponded with the $R_f$ value of the standard methionine solution was taken to indicate presence of methionine in the broth culture. The value of $R_f$ =0.91 cm which corroborates $R_f$ = 0.89 from literature for L-methionine.

### Estimation of Methionine

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.

**Estimation of 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 1 litre 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.

**Biomass Estimation**

The biomass taken from shake flask experiments was centrifuged at 5,000xg for 20 min to bring into pellet form. The biomass was washed twice with sterile distilled water and dried at 65°C and then weighed.

## RESULTS AND DISCUSSION

**Effect of temperature on growth and methionine production**

The impact of varying temperatures on growth and methionine production was observed. By varying temperatures at desired levels and keeping all other variables held steady. The preculture was inoculated into shake flask at sought temperature levels. These were incubated for 72 hrs on an orbital shaker. Results from Fig.1 proved that methionine production was function of temperature. As temperature increases methionine generation also increases up to $30^0$C (2.16 g/l methionine concentration), past which methionine concentration diminished. So, temperature of $30^0$C was selected as optimum for maximum production of methionine. This is mainly due to the fact that high temperatures are lethal to microorganisms thereby decreases fermentation rate. At high temperatures enzymes move too fast and denaturation takes place. Ideal temperature of $30^0$C acquired here in accordance with the works of few researchers [27, 28, 29] for maximum methionine production.

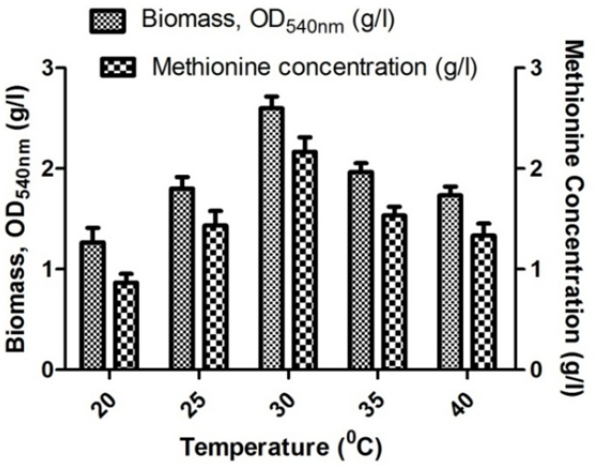


Fig.1 Effect of temperature ($^0$C) on growth and methionine production at plantain (starch hydrolysate) 10 g/l in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 6.0; time 72 hr.

**Effect of pH on growth and methionine production**

Most microbes grow best around neutral pH values (6.5 - 7.0), some microorganisms produce acid as they grow. This acid is excreted and brings down the pH or the surrounding environment. This eventually brings bacterial growth to a halt unless something else in the environment neutralizes the bacterial acid.

The effect of differing pH on methionine generation by *C.glutamicum* was inspected. These pH values were balanced with 1 N HCl and 2 N NaOH by using pH digital meter. The preculture was inoculated into shake flask at required pH levels. These were incubated for 72 hrs on an orbital shaker. Results demonstrated that methionine production was a function of pH as appeared in Fig 2. The optimum pH value observed as 7 under which methionine generation was 2.13 g/L. The resultant pH of 7.0 acquired here in reasonably good agreement with works of [30, 31].

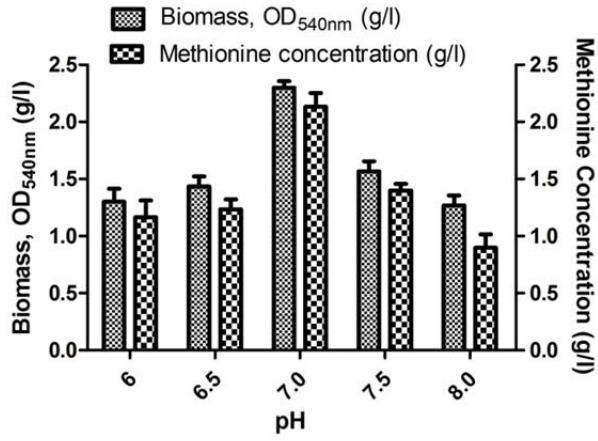


Fig. 2 Effect of pH on growth and methionine production at plantain (starch hydrolysate) 10 g/l in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; time 72 hr; temperature $30^0$C.

**Effect of volume ratio of medium to fermenter on growth and methionine production**

The impact of aeration in the shake flask was measured by volume of medium in the flask. Oxygen is an important nutrient in flask usually supplied through air on vigorous shaking in orbital shaker. Fermentation process requires enough aeration for growth and methionine production. The effect of volume ratio of medium to fermenter on growth and methionine production is presented in Fig. 3. Results showed that growth and methionine production increased with increasing volume ratio of medium to fermenter. Maximum growth and methionine production were obtained at 30% volume of medium to fermenter beyond which methionine production diminished. In this way, 30% was chosen as the optimum volume ratio of medium to fermenter for growth and methionine accumulation. The highest methionine concentration of 2.46 g/l was obtained at 30% volume ratio of medium to fermenter. Under conditions of inadequate oxygen, huge amount of succinic and lactic acids are produced, while surplus oxygen increases the amount of keto glutaric acid. [32, 33, 34] reported that both inadequate and surplus oxygen is undesirable in amino acid fermentation. They confirmed that former inhibits cell growth and latter hampers the production of amino acids. Similar results have been obtained by [35], who reported that 30 ml of fermentation broth was optimum for maximum production of L-glutamic acid by *Micrococcus glutamicus.*

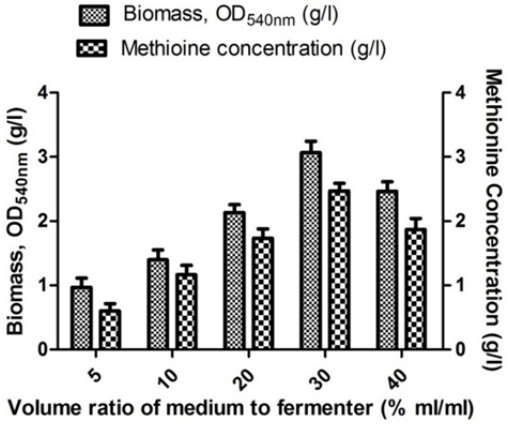


Fig.3 Effect of volume ratio of medium to fermenter on growth and methionine production at plantain (starch hydrolysate) 10 g/l in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

**Effect of agitation speed (rpm) on growth and methionine production**

The influence of differing agitation on growth and methionine production was evaluated. Agitation is very important in fermentation flask since oxygen is low solubility nutrient. Oxygen transfer capabilities in the flask controls the growth and product formation. Inoculum vessel containing liquid medium was agitated to obtain homogeneity. The effect of agitation on L-methionine fermentation was shown in Fig. 4. As agitation speed increased from 50 to 170 rpm, methionine production increased rapidly and afterward leveled off, maximum production of 2.36 g/l methionine obtained at 170 rpm. [36] Suggested that agitation rates above 200 rpm will prompt denaturation of enzymes with low production of metabolites.

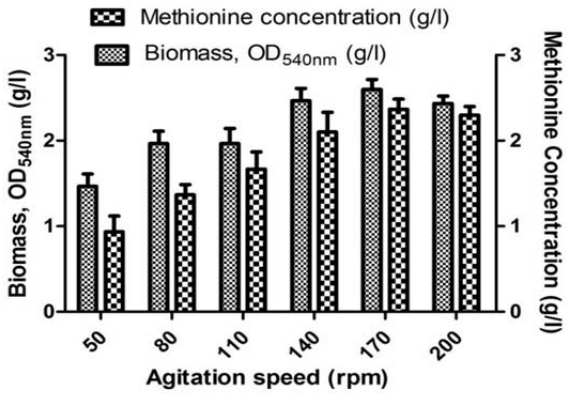


Fig. 4 Effect of agitation speed (rpm) on growth and methionine production at plantain (starch hydrolysate) 10 g/l in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

**Effect of inoculum size on growth and methionine production**

The impact of inoculums size on growth and methionine production was evaluated. The amount of inoculum inoculated into fermentation broth also affects metabolite production. From Fig. 5 Results showed that at 3% inoculums size was the optimal for growth and methionine production (1.33 g/l). Above which growth and methionine production decreased. [32] 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. [37] Suggested that higher inoculum size results in reduced DO and increased competition towards nutrients.

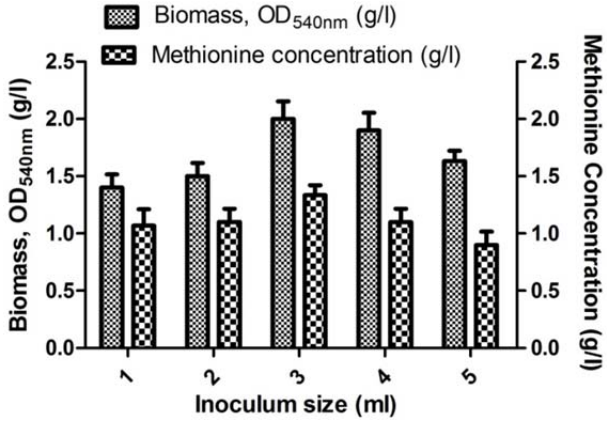


Fig. 5 Effect of inoculum size on growth and methionine production at plantain (starch hydrolysate) 10 g/l in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

**Effect of different starch hydrolysates (carbon source) on growth and methionine accumulation**

The influence of various starch hydrolysates such as corn, millet, plantain, rice, wheat and potato as carbon sources on growth and methionine production were evaluated at 10 g/l. Among the starch hydrolysates tested, plantain was proved to be best starch hydrolysate for growth and methionine production as shown in Fig. 6. This is reasonably in good agreement with the works of [28] who reported plantain as good starch hydrolysate (carbon source) for methionine production.

The effect of different concentrations of plantain starchy hydrolysates on methionine production shown in Fig. 7 Results showed that methionine production was a function of initial plantain concentration (glucose concentration) in the fermentation medium. Methionine concentration increased up to 20 g/l plantain concentration (100 g/l glucose), beyond which methionine concentration decreased due to the inhibition of microbial growth at higher substrate concentrations.

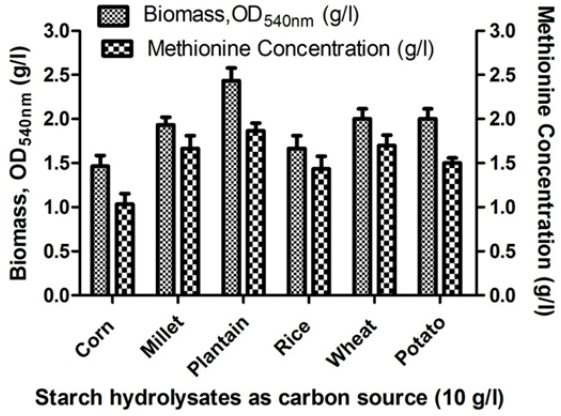


Fig. 6 Effect of different starch hydrolysates (carbon source) on growth and methionine production at 10 g/l in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

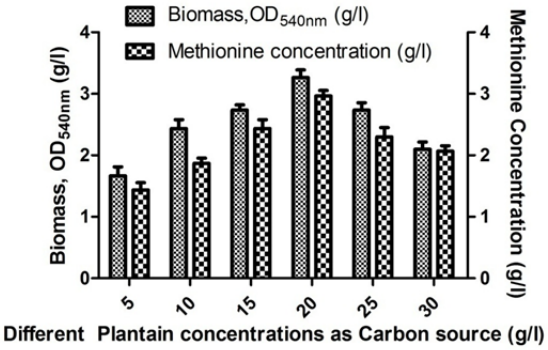


Fig. 7 Effect of different concentrations of plantain on growth and methionine production in the medium containing groundnut, 5 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

**Effect of defatted proteins as nitrogen sources on growth and methionine production**

In order to investigate the effect of various defatted proteins as nitrogen sources namely cowpea, pigeon pea and groundnut were studied for production of growth and methionine accumulation at 5 g/l. Results presented in Fig.8 showed that ground nut was the best nitrogen source for the production of methionine.

From Fig.9 Results confirmed that methionine production was function of initial nitrogen concentration, methionine concentration increased up to 10 g/l, beyond which methionine concentration reduced due to the osmotic pressure exerted by nitrogen source, which has an adverse effect on growth and methionine production in *C.glutamicum*, as suggested by [38].

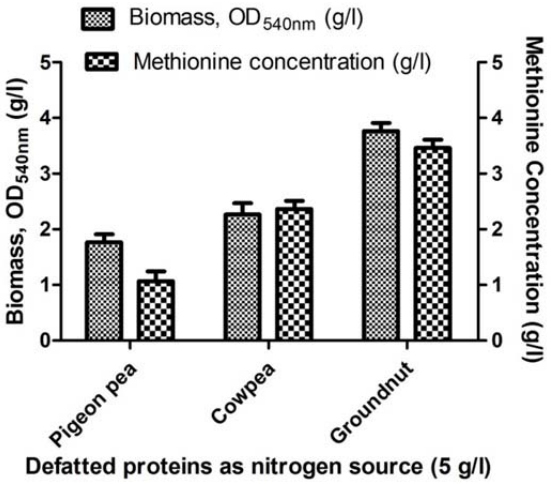


Fig. 8 Effect of defatted proteins as nitrogen sources on growth and methionine production at nitrogen source of 5 g/l in the medium containing plantain, 20 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

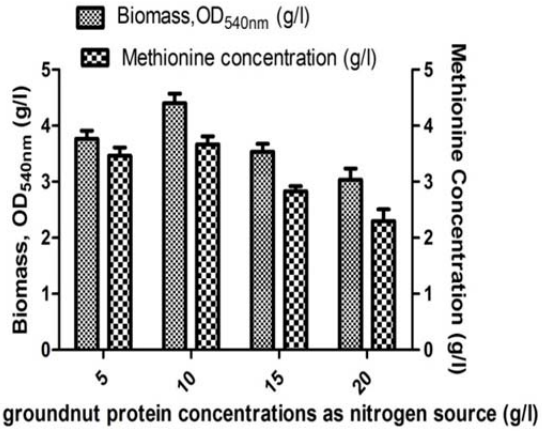


Fig. 9 Effect of different concentrations of groundnut on growth and methionine production in the medium containing plantain, 20 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 5 g/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

**Effect of $CaCO_3$ on growth and methionine production**

During fermentation process, pH of fermentation broth decreases due to formation of pyruvic acid, lactic acid and gluconic acid etc. As a result, growth of cells decreases with simultaneous decrease in methionine yield. Calcium carbonate neutralizes the pH of fermentation broth by eliminating lag phase of cell growth thereby shortening fermentation time. 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 [8]. Effect of different concentrations of $CaCO_3$ was assessed on growth and methionine production; results showed that maximum methionine concentration obtained at 20 g/l $CaCO_3$ as shown in Fig.10. Growth and methionine accumulation were determined as previously described.

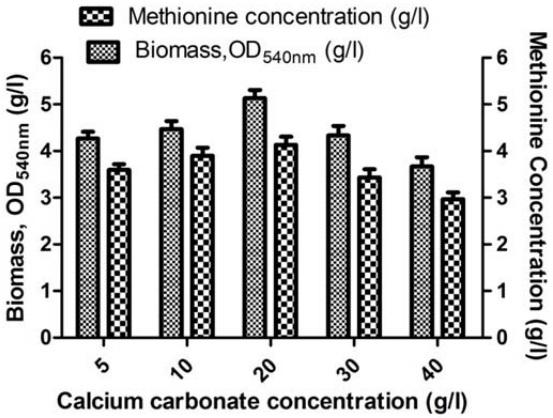


Fig. 10 Effect of different concentrations of $CaCO_3$ on growth and methionine production in the medium containing Plantain, 20 g/l; groundnut 10 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; water 1 liter; pH 7.2; time 72 hr; temperature $30^0$C.

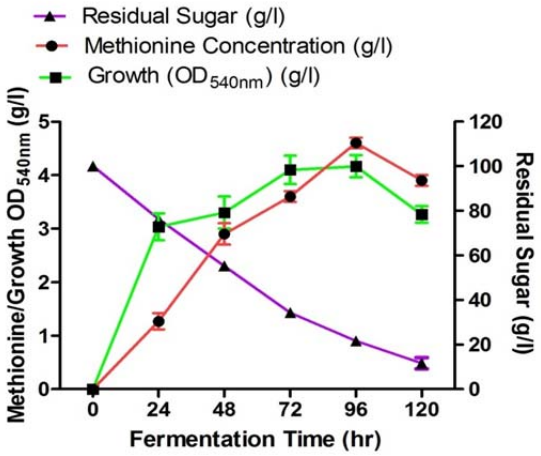


Fig. 11 Time course of fermentation for methionine production in the medium containing plantain, 20 g/l; grounnut 10 g/l; $KH_2PO_4$, 0.5 g/l; $K_2HPO_4$ 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; water 1 liter; pH 7.2; temperature $30^0$C.

**Time course of fermentation for methionine production**

In view of the results obtained from Fig.11, growth and methionine production were accompanied by function of plantain concentration (glucose concentration) in the medium. After 96 hrs of fermentation, glucose in the fermentation broth was reduced to 10 g/l. Maximum concentration of methionine was obtained 4.6 g/l at 96 hrs. The relationship between methionine production and sugar consumption was in good agreement with results reported by [39]. Reduction in methionine production after 96 hrs could be attributed to age of bacteria, depletion of sugar and nitrogen sources and the same has been reported by [24].

## CONCLUSIONS

The fermentation conditions and nutritional parameters for microbial production of methionine by *C.glutamicum* (MTCC 2745) using agriculture products were optimized in laboratory shake flask. Among the agriculture products (corn, millet, plantain, rice, wheat and potato) tested as starch hydrolysates (carbon source) for maximum methionine production, plantain at 20 g/l concentration gave the highest methionine and for nitrogen source groundnut at 10 g/l gave the highest methionine amongst cowpea, pigeon pea and groundnut. Optimum values of fermentation conditions and nutritional parameters for methionine production were temperature $30^0$C, pH 7.0, volume ratio of medium to fermenter 30 ml/ml, inoculums size 3 ml, agitation 170 rpm, plantain starch hydrolysate as carbon source 20 g/l, groundnut as nitrogen source 10 g/l and $CaCO_3$ 20 g/l. At these optimum values biomass and methionine production were 4.16 g/l and 4.6 g/l after 96 hour of fermentation.

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