Biochemical Changes in the Fermentation Medium During L-methionine Production by *Brevibacterium roseum* SXS2470

**Abstract**: The present investigation was carried out to investigate the biochemical changes in the fermentation broth during submerged L-methionine fermentation using a mutant *Brevibacterium roseum* SXS2470. Dry cell weight rose with the concomitant increase in L-methionine production along with the accumulation of α-ketoglutarate, pyruvate and succinate in the fermentation broth with sharp decrease in residual sugar content. The pH of the medium decreased gradually after 72h. The amino nitrogen increased up to 72h of incubation as an indication of the product formation. Cell nitrogen and ammonical nitrogen also increased gradually throughout the fermentation period. This study was selected to get an idea about the metabolic diversity exhibited during the course of L-methionine production by this mutant.

**Key words**: *Brevibacterium roseum* SXS2470, Dry cell, L-methionine, α-ketoglutarate, succinate, ammonical.

In order to carry out different metabolic activities, a microorganism utilizes a number of nutritional ingredients. These nutrients may be glucose, L-amino acids or other products. Different strains of *Brevibacterium roseum* may utilize different carbon and nitrogen sources. Quantitative uptake of these nutrients provides useful information for monitoring the microbial growth and metabolism. These changes in the cell mass are proportional to glucose concentration. Hence, the knowledge about the glucose concentration can provide a basis for estimation of cell growth. In fermentation, pH change in the fermentation broth is another necessary parameter, which may control biochemical processes such as enzyme functions, protein conformation etc. Thus, by controlling the pH, a cell density and metabolism may be controlled.

Ganguly and Banik (2010) examined some biochemical changes in the fermentation broth during L-glutamic acid production by a biotin auxotroph *Micrococcus glutamicus* AB1007. Ghosh et al. (2012) have conducted a study to examine some biochemical changes in the fermentation broth during citric acid production by *Aspergillus nigri* AB1801.

In this present study, changes in the glucose and nitrogen contents along with pH changes with the advancement of L-methionine production by the mutant *Brevibacterium roseum* SXS2470 were extensively examined.

**Materials and Methods: Microorganism**: *Brevibacterium roseum* SXS2470 developed by induced mutatantion and protoplast fusion, described in the previous chapter was used throughout the study.

**Composition of Growth Medium**: glucose, 20 g; (NH₄)₂SO₄; 1.6 g; NaCl, 2.5g; MgSO₄.7H₂O, 0.25 g; MnSO₄.4H₂O, 0.1 g; K₂HPO₄, 1 g; KH₂PO₄, 1 g; H₂O, 1L and agar, 2 % as a solidifying agent.

**Physical Conditions for Growth**: Volume of medium , 25 ml; initial pH , 7; shaker’s speed , 150 rpm; age of inoculum , 48 h; optimum cell density , 4 X10⁸ cell; temperature , 28°C and period of incubation , 72h.

**Composition of Synthetic L-methionine Production Medium**: It contains Glucose, 100 g/L; (NH₄)₂SO₄, 8 g/L; (in terms of nitrogen) ; K₂HPO₄, 2.2 g/L; MgSO₄.7H₂O, 1.5 g/L; FeSO₄.7H₂O, 0.03 g/L; KH₂PO₄, 2 g/L; CaCO₃, 1.5 g/L; ZnSO₄.7H₂O, 1.6 mg/L; CaCO₃, 1.5 g/L; Na₂MoO₄.2H₂O, 5 mg/L; MnSO₄.4H₂O, 2.5 mg/L; biotin, 80 μg/L; thiamine-HCl, 70 μg/L; Land H₂O, 1 L.

**Estimation of Residual Sugar**: Residual sugar was determined by the DNS method as proposed by Miller (1959).

**Estimation of Total Nitrogen**: Total nitrogen was measured by the micro-Kjeldahl method as described by Allen (1931).
Estimation of Ammonical Nitrogen: Ammonical nitrogen was estimated by micro-Kjeldahl method as described by Allen (1931)10.

Estimation of α-ketoglutarate and Pyruvate: To estimate α-ketoglutarate and pyruvate, the colorimetric method of Friedemann-Haugen was used11.

Estimation of Succinate: Manometric method was applied to determine succinate using succinate dehydrogenase (Umbreit et al., 1951).

Analysis of L-methionine: Descending paper chromatography was employed for detecting L-methionine in the broth and was run for 18h on Whatman No.1 chromatography paper. Solvent system contained: n-butanol: acetic acid: water (2:1:1). The spot was visualized by spraying a solution of 0.2 % ninhydrin in acetone and quantitative estimation of L-methionine in the suspension was done using a colorimetric method12.

Confirmatory Test for L-methionine: Quantitative determination of L-methionine in the fermentation medium without purification was done following the method as described by Greenstein and Wintz (1996)13. 1 ml of 5(N) NaOH, and 0.1 ml of 10% sodium nitroprosside solution, was added to 5 ml supernatant after centrifugation at 5000 rpm for 15 min. The tube was thoroughly shaken and the mixture was allowed to stand for 10 min. 25 ml of 3% aqueous solution of glycerine was added to the reaction mixture with frequent shaking over a period of 10 min. After additional 10 min interval, 2 ml of concentrated orthophosphoric acid was added drop wise to the mixture and the test tube was properly shaken. Color development was developed within 5 min. and color intensity was measured at 540 nm in spectrophotometer (Perkin Elmer Lambda 68 UV VIS). The L-methionine yield was extrapolated from a standard L-methionine curve14.

Recovery of L-methionine from Fermented Broth: An inexpensive down-stream recovery process that is capable of achieving the requisite recovery yield and purity is essential for producing any metabolite. Various levels of down-stream processing are required for the existing amino acid fermentation. The general approach to designing an efficient recovery scheme for bio products has been elucidated by Chisti and Moo-Young (1999)15. The production scheme must contained the various regulatory requirements and consider the end use application of the product. Purification of L-amino acids depends on their physico-chemical properties, particularly solubility and isoelectric point. As the first step of the down-stream recovery process, the cells are separated from the fermentation broth by either centrifugation or filtration. The cell-free broth is then passed through activated charcoal columns for decolorization. L-methionine (isoelectric pH 5.74) can be recovered from the clarified broth by adjusting the pH to 5 with sulfuric acid to convert the amino acid to its cationic form and passing the broth through a bed of Amberlite IR-120 (H+) ion exchange resin at a controlled flow rate. The process is repeated until all the L-methionine is adsorbed. Afterwards, the column is washed with deionized water and eluted with 1(M) NH₄OH to recover the L-methionine. Crystalline L-methionine can be obtained by concentrating under vacuum, treating with absolute alcohol, and drying overnight at 80ºC.

Estimation of Dry Cell Weight (DCW): The cell paste was obtained from the fermentation broth by centrifugation.
and dried at 100°C until constant cell weight was obtained.16

**Statistical Analysis:** All the data were presented as mean ± SEM. Data were analyzed using one way ANOVA followed by Dunnet’s post hoc multiple comparison test using a software Prism 4.0, considering *p<0.05 as significant and **p<0.01 as highly significant.

**Results:** Utilization of glucose increased gradually with the increase of dry cell weight and the production of L-methionine along with the accumulation of α-ketoglutarate and pyruvate in the fermentation broth. The pH of the medium decreased sharply with the advancement of fermentation period (Fig.1).

The amino nitrogen level increased up to 72h of incubation as an indication of the product formation. Cell nitrogen and ammonical nitrogen increased gradually throughout the fermentation period. But pH remained unchanged upto 72h then declined gradually (Fig.2).

**Discussion:** Su and Yamada (1960) examined the biochemical changes in the fermentation broth during L-glutamic acid fermentation by *Brevibacterium divaricatum* nov. sp. They also reported alteration of pH and accumulation of α-ketoglutarate, pyruvate and succinate during the fermentation period.17 Ganguly and Banik (2011) examined the biochemical changes occurred in the fermentation medium during the production of L-glutamic acid by a biotin auxotrophic mutant *Micrococcus glutamicus* AB1018, and reported that the production was increased up to 72h of incubation along with the sharp fall of pH and gradual increase of dry cell weight. Amino nitrogen, cell nitrogen and ammonical nitrogen increased continuously with the sharp fall of residual nitrogen. Ghosh et al. (2012) examined the biochemical changes in the fermentation broth during citric acid fermentation by the mutant *Aspergillus niger* AB180119. The production was increased up to 8th day of incubation, and then declined. Dry cell weight increased continuously. Ammonial nitrogen increased steadily but amino nitrogen and urea nitrogen decreased continuously.

**Conclusion:** In this present study, dry cell weight increased gradually and the production of L-methionine along with the accumulation of α-ketoglutarate, pyruvate and succinate in the fermentation broth with sharp decrease in residual sugar content. The pH of the medium decreased sharply with the advancement of fermentation period after 72h. The amino nitrogen level increased gradually up to 72h of incubation as an indication of the product formation. Cell nitrogen and ammonial nitrogen also increased throughout the fermentation period. The study attributed to a reflection about the metabolic diversity in this mutant which could be considered for other metabolite production by this mutant in further study.

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