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Evidence for auxotrophic nature of *Corynebacterium glutamicum* X680

***Subhadeep Ganguly**

Department of Physiology, Vidyasagar College,
39, Sankar Ghosh Lane, Kolkata-700006 (India)
*Address for correspondence: res_biol@rediffmail.com

Abstract

The present investigation was undertaken to determine the specific nutritional deficiency of the newly developed mutant *Corynebacterium glutamicum* X680 maintenance of its L-glutamic acid over production. In this connection, I added cyanocobalamin, folic acid, thiamine-HCl, riboflavin, nicotinic acid, pyridoxine-HCl, inositol, calcium pantothanate, paraamino benzoic acid (PABA) and biotin one by one in the minimal salt medium (2µg/ml each) to evaluate their specific requirement for this mutant. The mutant showed nutritional requirement for biotin only.Various concentrations of biotin were tested. The mutant required biotin auxotroph with 3 µg/ml. Different kinetic parameters like rate of product yield (qp), specific product yield (Yp/x) and rate of glucose consumption (Yp/s) were studied. Production of L-glutamic acid decreased significantly ($p<0.01$) above and below this concentration. The growth pattern and productivity varied with different concentrations of biotin. ***Conclusion:*** The mutant strain proved to be a biotin auxotroph.

L-glutamic acid is a non-essential proteinogenic amino acid globally popular for its flavor enhancing property and unique taste 'umami', the fifth modality of taste which remained unexplored till the first half of the twenty first century[5]. India's market demand for L-glutamic acid is expected to be USD 471 million by 2020. However, the total demand is meeting up by import only. China appears to be the largest Asian country for L-glutamic acid production.

Fermentation of L-glutamic acid was started via discovery of L-glutamic acid over producing strain *Corynebacterium glutamicum* in late 50s[24]. Since then several trials have been made to improve its production. Biotin (vitamin H or B7) is a water soluble vitamin. Its deficiency is rarely reported in human. However, in biotin- auxotrophic microorganisms, it is required to uptake from the medium[12]. Biotin synthesis occurs through the synthesis of pimelic acid followed by biotin ring assembly[3]. Biotin ring assembly is catalyzed by 8-amino-7-oxononanoate synthase, 7,8 diaminonanoate synthase, dethiobiotin synthase and biotin synthase encode by *bio*A, *bio*D and *bio*B genes[4,14,25,28]. In *Corynebacterium*

glutamicum pimeloyl COA synthesis system is absent, but enzymes for biotin ring assembly exist.

The present study was undertaken to detect the specific auxotrophy of the newly developed mutant *Corynebacterium glutamicum* X680. In this connection, cyanocobalamin, folic acid, thiamine-HCl, riboflavin, nicotinic acid, pyridoxine-HCl, inositol, calcium pantothanate, paraamino benzoic acid (PABA) and biotin were examine one by one to detect specific auxotrophy of this strain.

Microorganism: Corynebacterium glutamicum X680 developed in my previous investigation from a wild strain *Corynebacterium glutamicum* X60 was used throughout the study[7].

Composition of growth medium: The bacterial growth medium was composed of: glucose, 2%; peptone, 0.5%; yeast extract, 0.1%; beef extract, 0.3%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.H_2O$, 0.025% ; agar, 4% and H_2O, 1L[7].

Composition minimal salt medium for L-glutamic acid production: L-glutamic acid production was carried out using basal salt medium containing: glucose, 10%; urea, 0.8%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.H_2O$, 0.025% ;yeast extract, 0.2% and H_2O, 1L. The pH was adjusted at 7.0. The submerged fermentation was carried out at 30°C for 72h[7].

Analysis of amino acid: Descending paper chromatography was used for the detection of L-glutamic acid. Solvent system used was composed of n-butanol:acetic acid:water (2:1:1). The spots were visualized by spraying 0.2% ninhydrin in acetone. The quantitative estimation was done by colorimetric estimation method[32].

Addition of vitamins: Sterilized solutions of cyanocobalamin, folic acid, thiamine-HCl, riboflavin, nicotinic acid, pyridoxine-HCl, inositol, calcium pantothanate, paraamino benzoic acid (2µg/ml each) were aseptically added to the minimal salt medium one by one.In the second phase, only biotin in different concentrations (1-10 µg/ml) was added to the medium and incubated it for 72h at 30°C.

Estimation of dry cell weight: After centrifugation, 2 ml 1(N) HCl was poured into the precipitate of the bacterial cells to dissolve it. Calcium carbonate was added to neutralize it. The remaining cells were washed twice and dried at 100°C for 16h till the cell weight remained constant[8].

Estimation of residual sugar: The residual sugar of the fermentation broth was estimated by dinitrosalicylic acid (DNS) method. Fermentation broth (1 ml) was added to 3 ml water containing 2 ml DNS in a test tube and boiled it for 10 minutes. The test tube was cooled under running tap water and optical density (OD) was measured colorimetrically using 530nm filter in a colorimeter. The residual glucose concentration was estimated using a standard curve of glucose[10].

Kinetic study: Different kinetic parameters such as maximum volumetric rate of L-glutamic acid formation (Qp), specific L-glutamic acid yield (Yp/x), product yield in

terms of glucose utilization (Yp/s) and rate of L-glutamic acid formation (qp) were determined using the following equations:

$dP/dt = Yp/x.dX/dt$; where $dX/dt = \mu X$……(a)

$Yp/x = dP/dX$……...……(b)

$Yp/s = -dP/dS$……...……(c)

$qp = 1/X.dP/dt$……(d)[21].

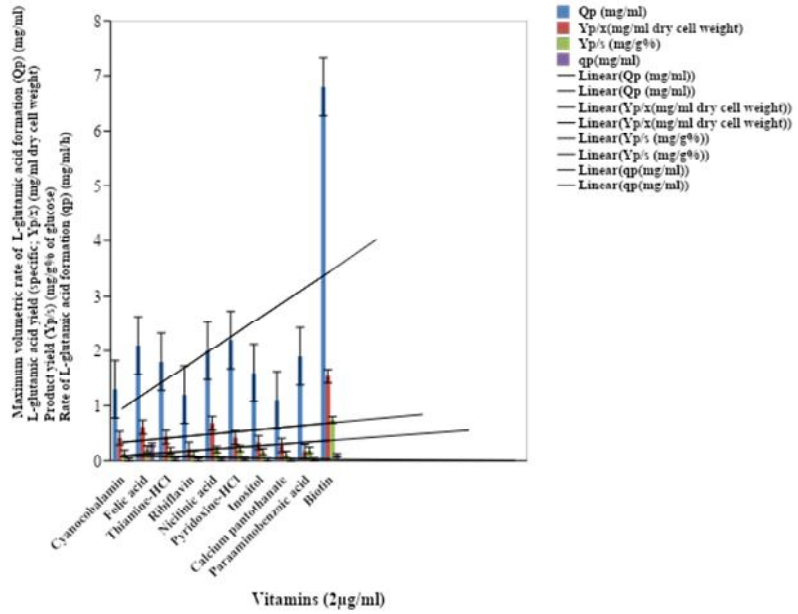
Statistical analysis: All data were expressed as mean±SEM, where n=6. The data were analyzed by one way ANOVA followed by Dunett's post hoc multiple comparison test using prism 4.0 (Graph pad Inc., USA). A '*p*' value less than 0.05 was said to be significant and less than 0.01 as highly significant.

Clean pyrex glass ware was used to prevent contamination. Borosil glass goods and analytical grade reagents and Borosil glass goods were used throughout the study. The medium was sterilized in an autoclave at 15lb pressure for 15 minutes.

Evidence for the auxotrophic requirement for *Corynebacterium glutamicum* X680 as a producer of L-glutamic acid:

To determine the specific auxotrophic requirement for the mutant *Corynebacterium glutamicum* X680, we added different vitamins (cyanocobalamin, folic acid, thiamine-HCl, riboflavin, nicotinic acid, pyridoxine-HCl, inositol, calcium pantothanate, paraamino benzoic acid (PABA) and biotin), 2µg/ml each in the fermentation broth separately. We have found that biotin showed maximum rate of acceleration on L-glutamic acid production by the mutant (Fig 1).

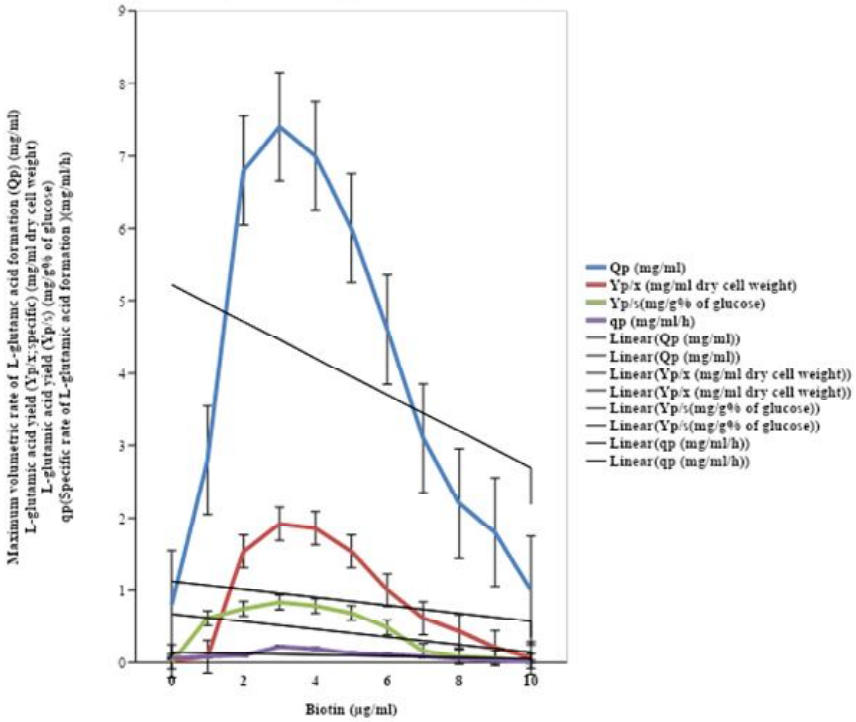
Fig 1. Kinetics of L-glutamic acid formation by *Corynebacterium glutamicum* X680 an evidence of auxotrophic nature for Vitamin B complex (Values were expressed as mean±SEM, where n=6)



Optimization of biotin for L-glutamic acid production by an auxotrophic mutant *Corynebacterium glutamicum* X680 :

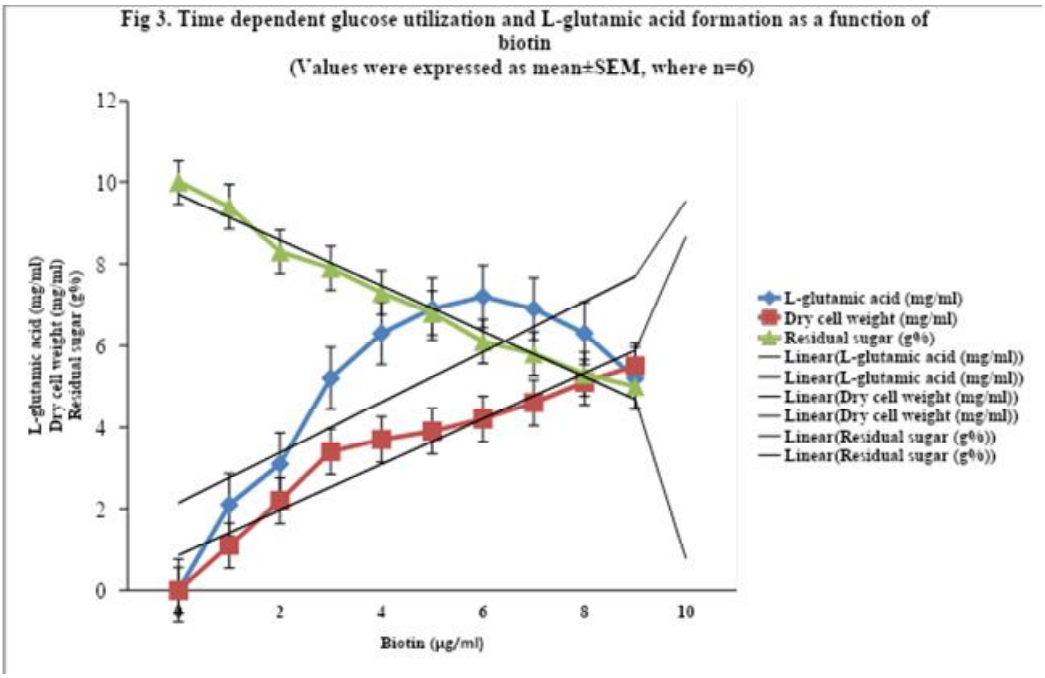
Among different concentrations (1-10µg/ml) of biotin examined, maximum rate of L-glutamic acid formation was obtained with 3 µg/ml which led to significant ($p<0.01$) acceleration of product formation as well as specific product yield in terms of dry cell weight and also the rate of product formation as a function of maximum rate of substrate utilization (Fig. 2). It was also evident that excessive biotin in the broth also inhibited bacterial growth and product formation.

Fig 2. Optimization of biotin for L-glutamic acid formation by *Corynebacterium glutamicum* X680
(values were expressed as mean±SEM, where n=6)



Time dependent glucose utilization and specific product formation (L-glutamic acid) as a function of biotin :

To examine the glucose utilization pattern in presence of 3µg/ml biotin for L-glutamic acid fermentation, residual sugar, dry cell weight and L-glutamic acid were estimated at different time intervals (12, 24, 36, 48, 60, 72, 96 and 108h) during the course of fermentation. Maximum product formation occurred at 72h of incubation (Fig. 3). However, with the advancement of incubation period, cell mass and substrate utilization increased gradually.



Corynebacterium glutamicum is a Gram +ve, non-motile, non-sporulating, rod shaped bacterium which could able to over produce L-glutamic acid in presence of biotin[1]. Biotin auxotrophy in this strain is due to lack of BioF homolog[13]. Biotin addition in the medium promotes its growth[31]. Biotin auxotrophy of this strain leads to over production of L-glutamic acid[26]. Biotin limitation changes the synthesis of fatty acid and mycolic acid which leads to over production of L-glutamic acid[26]. It ultimately inhibits acetyl CO-A carboxylases[6]. L-glutamic acid over production is due to inhibition of TCA cycle enzyme oxoglutarate dehydrogenase[2]. However, extracellular relese of L-glutamic acid depends on cell membrane permeability[30]. Lower concentrations of biotin lead to alterations in the external layer of the cell membrane. In electron microscopic view such membrane appeared to be a disordered one. It is correlated to extracellular release of L-glutamic acid[15]. Different studies reported different levels of biotin, however, recommended level is between 0.001-5.0µg/ml[11,16,17,19,20,22,27]. Under high biotin concentrations, cells produce large quantity of oleic acid which increases phospholipid content, leading to reduction in extracellular release of L-glutamic acid[9]. On the otherhand in presence of excess biotin, excessive cellular growth leads to over production of lactic acid. However, Niaz *et al.*[21] reported maximum L-glutamic acid production by *Corynebacterium glutamicum* NIAB SS-27 in presence of 10 µg/ml biotin in the medium[23]. Very recently, Wen and Bao[29] reported L-glutamic acid over production by *Corynebacterium glutamicum* S9114 in presence biotin rich corn stover hydrolysate[29]. But in my study we obtained maximum

production of L-glutamic acid with addition of 3µg/ml biotin in the basal salt medium.

This experiment clearly shows that, *Corynebacterium glutamicum* X680 is a biotin auxotroph which could produce L-glutamic acid in presence of biotin. Furthermore, maximum L-glutamic acid production (up to 7.2mg/ml) was obtained in presence of 3µg/ml biotin in the production medium. Too low as well as excess biotin content in the medium inhibit its production.

The author declares no conflict of interest.

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