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Roles of Penicillin G and surfactants on extracellular secretion of L-glutamic acid as a function of membrane permeability

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Abstract

The present investigation was undertaken to examine the effect of penicillinG and surfactants on L-glutamic acid production by a biotin auxotroph *Corynebacterium glutamicum* X680. Addition of penicillin G, 6U/ml, 8h after incubation led to maximum extracellular secretion of L-glutamic acid. Among different fatty acid derivatives (surfactants) studied, Tween60 (polyoxyethylene sorbitan monostearate), 0.5mg/ml, appeared to be the most suitable fatty acid derivative in extracellular secretion of L-glutamic acid from the bacterial cells in presence of biotin when added to the medium after 6h of incubation. Surprisingly, the stimulatory effect was dependent on addition time which was also reflected in the bacterial growth curve of *Corynebacterium glutamicum* X680.

L-glutamic acid was identified as 'umami' substance in 1908 by Professor K. Ikeda[5]. But 'umami' was unrecognized as fifth basic taste modality till twenty first century. Its large scale production was started in 1957 through the discovery of *Micrococcus glutamicus* (later renamed as *Corynebacterium glutamicum*) from Japanese soil by Kinoshita and his coworkers[6]. Since then its global production is increasing every year[7]. *Corynebacterium glutamicum* is able to secrete L-glutamic acid, hence it is widely used for industrial production of stereo-specific amino acids[5,6,17].

In several studies, it has been clearly shown that L-glutamic acid production under biotin limitation in biotin-auxotroph *Corynebacterium glutamicum* is accompanied with membrane alterations[4]. Penicillin G inhibits cell wall formation cell wall formation by inhibiting transpeptidase which catalyses cross linking between peptidoglycan chains and thereby increases L-glutamic acid production[8]. Several surfactants (including fatty acid derivatives) are also used to elicit L-glutamic acid release from different bacterial cells[1,7,11].

In the present study, we were intended to examine the stimulatory effect of penicillin G and surfactants on L-glutamic acid production by a biotin auxotrophic mutant *Corynebacterium glutamicum* X680.

Microorganism:

A biotin auxotrophic mutant

Corynebacterium glutamicum X680 was used throughout the study[2].

Composition of the growth medium:

The bacterial growth medium was composed of (g%): glucose, 2%; peptone, 0.5%; yeast extract, 0.1%; beef extract, 0.3%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.0025%; agar, 4% in double distilled deionized 1L water. The pH was neutrally adjusted.

Culture conditions:

The following synthetic medium was recommended for improved and steady rate of L-glutamic acid production (27.6 mg/ml) with the free cells of *Corynebacterium glutamicum* X680 in submerged fermentation: pH, 7.0; period of incubation, 72h; volume of medium, 30ml; size of inoculum, 4%($8X10^6$ cells); age of inoculum, 48h; temperature, 30°C; shaker's speed (agitation), 150rpm; glucose, 12g%; urea, 1g%;calcium carbonate, 4g%; biotin, 3µg/ml; potassium dihydrogen phosphate, 0.3g%; dipotassium hydrogen phosphate, 0.3g%; magnesium sulphate, heptahydrate, 2mg%; zinc sulphate, heptahydrate, 10µg/ml ; ferrous sulphate, heptahydrate, 10 µg/ml and biotin, 3µg/ml.

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[18].

Estimation of L-glutamic 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[12].

Determination of pH of the medium:

pH of the medium was estimated by pH meter (XPXL1802).

Estimation of residual sugar:

Residual sugar was estimated by DNS method[12].

Addition of penicillin G and surfactants:

Potassium salt of penicillin G, Tween 20, Tween 40, Tween 60 and Tween 80 with varying concentrations were added to the medium.

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 considered significant and less than 0.01 as highly significant.

All the chemicals used were in AR grade. Borosil glass goods were used throughout the study.

Penicillin G targets peptidoglycan cross-linkages in bacterial cell walls, where as non-ionizing surfactants especially Tweens

impairs surface integrity[7,12]. Here, we characterize how glutamate production by *Corynebacterium glutamicum* X680 is triggered by addition of penicillin G and surfactants in the medium.

Effects of penicillin G:

L-amino acid formation results as a function of biomass formation. Initially the effect of penicillin G on colony formation of *Corynebacterium glutamicum* was investigated. Colony formation is almost totally inhibited even at a very low concentration of penicillin G (Fig 1). Effect of different concentrations of penicillin G (added to the broth 2h after the onset of fermentation) on bacterial growth, L-glutamic acid production, final pH of the medium and residual sugar content was investigated (Fig. 2). Antibiotic was added at different time intervals (0, 2, 4, 6, 8 and 10h) of incubation. Maximum production (up to 33.2mg/ml) was obtained with 6U/ml penicillin G addition after 6h from the onset of incubation (Fig 3). However, pH and residual sugar remained unaltered. On addition of antibiotic penicillin G, 6U/ml, 6h after incubation, the production was increased significantly ($p<0.01$) up to 35.6mg/ml compare to control (which could accumulate L-glutamic acid up to 29.2mg/ml).

Effects of surfactants :

Tweens (80, 60, 40 and 20) with 0.2mg/ml concentration of each were added to the fermentation broth at two different time intervals (0 and 5h) from the onset of the fermentation. Tween 20 did not show any apparent effect on L-glutamic acid production. However, other three Tweens at a concentration of 0.2 mg/ml showed stimulatory effect on L-glutamic acid production. Tween 60 showed maximum effect in this context. When Tween 60 was added to the broth before and during fermentation medium containing biotin, 3µg/ml; the growth of *Corynebacterium glutamicum* X680 was reduced with the concomitant increase of L-glutamic acid release. Fig 4 shows the pattern of changes in the growth in presence of Tween 60, 0.2mg/ml at different time intervals during the course of fermentation. It was clearly depicted that addition of Tween 60, 6h after the incubation resulted significant reduction in bacterial growth, however addition during incubation brought too much inhibition of bacterial growth and drastic reduction of L-glutamic acid production. However, addition of Tween 60 after 6h of incubation had almost no effect on bacterial growth and L-glutamic acid production (Fig 4). It indicated that in such cases, bacterial growth was independent of addition time of Tween 60.

As described in Fig 5, maximum effect of Tween 60 exhibited with the amount of 0.5mg/ml, less than of this optimum amount, almost no effect and the production was not influenced by further increase of the concentration (Fig 5).

Takinami *et al.*,[12] studied the effect of free fatty acids (C8 and C18) and esters on L-glutamic acid production by *Brevibacterium lactifermentum* No.2256 and concluded that the effects show dissimilarities between polyethylene glycol derivative and ether[3]. Takinami *et al.*,[13] examined the effects of different fatty acids and their derivatives on L-glutamic acid production by *Brevibacterium lactifermentum* No. 2256 and reported

Fig 1. Colony formation of Corynebacterium glutamicum X680 in presence of different concentrations of penicillin G (Values were expressed as mean±SEM, where n=6)

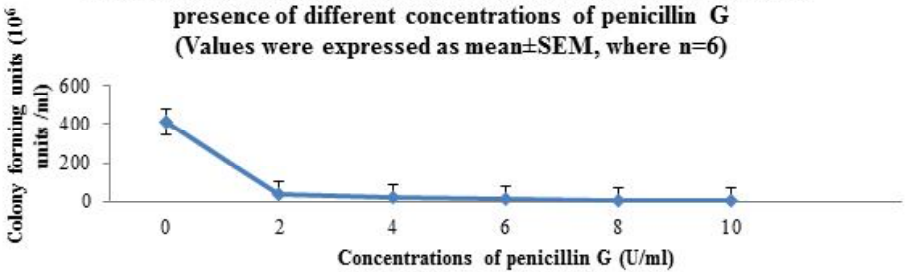


Fig 2. Effect of different concentrations of penicillin G on L-glutamic acid production, bacterial growth and residual sugar content (Values were expressed as mean±SEM, where n=6)

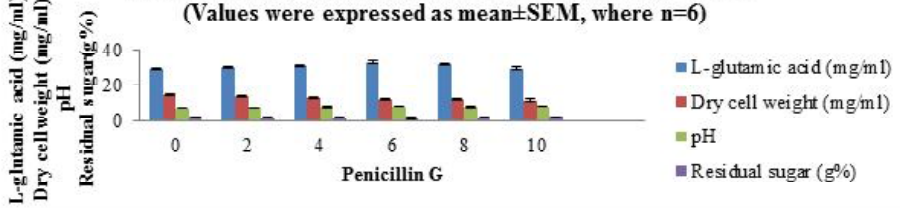


Fig 3. Effect of penicillin G (6U/ml) on L-glutamic acid production, bacterial cell growth, pH and residual sugar content (Values were expressed as mean±SEM, where n=6)

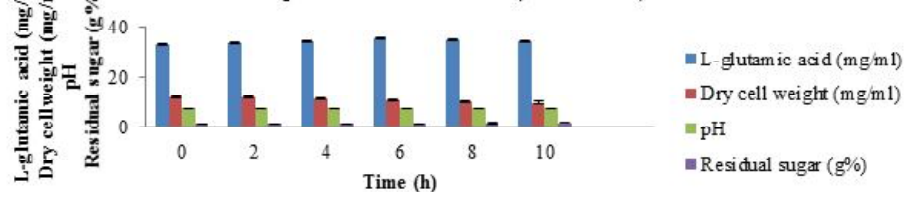
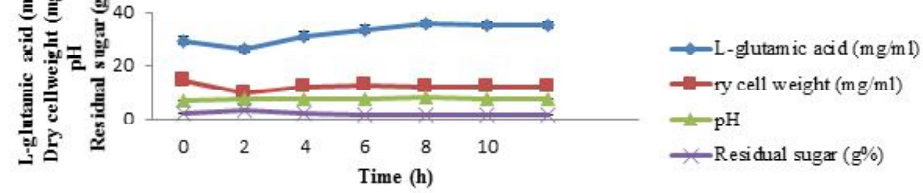
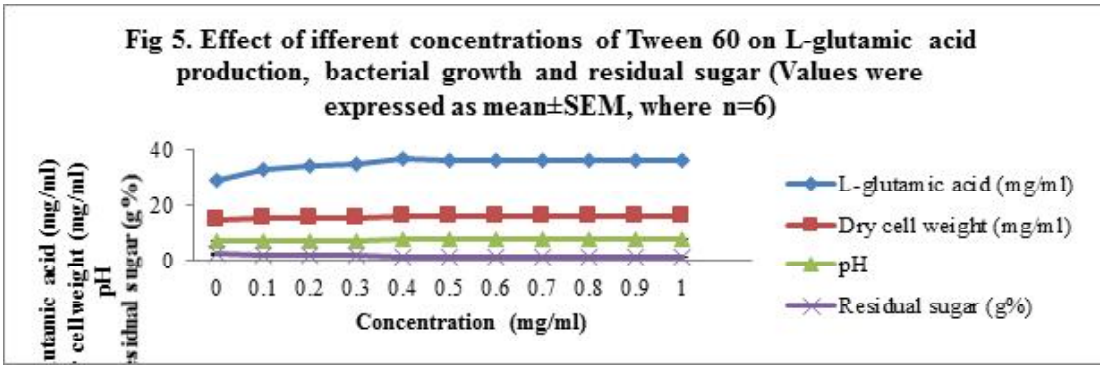


Fig 4. Effect of Tween 60 in relation to time of addition on L-glutamic acid production, bacterial growth and residual sugar content (Values were expressed as mean±SEM, where n=6)





remarkable effect of Tween 60 on L-glutamic acid production[13]. Takinami *et al.*,[14] reported Tween 60 had partial inhibitory effect on bacterial growth and stimulatory effect on L-glutamic acid production[14]. Oleic acid exhibited dose-dependent stimulatory as well as inhibitory (dualistic) effect on growth of *Brevibacterium lactifermentum* No.2256 as well as L-glutamic acid production[15]. Very recently, it has been demonstrated that Tween 80 inhibits pressure induced loss of metabolic activity, protein release and membrane preambilization, however membrane damage is not a prerequisite for loss viability or metabolic activity[9].

It was evident from the present study that in *Corynebacterium glutamicum* X68 (biotin dependent auxotrophic mutant) penicillin and Tween60 significantly ($p<0.01$) improve extracellular secretion of L-glutamic acid. Maximum effects were observed with penicillin G, 5U/ml and Tween60, 0.5mg/ml. On addition of both Penicillin G (5U/ml) and Tween 60, 0.5 mg/ml, 6h after incubation, lead to significant increase of L-glutamic acid production ($p<0.01$) from 27.6 mg/ml to 36.2 mg/ml.

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Conflict of interest :

The author declares no conflict of interest.

References :

1. Duperray, F., D. jezequel, A., Ghazi, L. Letellier and E. Shechter (1992). *Biochemistry Biophysics Acta, 1103:* 250-258.
2. Ganguly, S. (2019). *Journal of The Indian Chemical Society, 95:* 705-710.
3. Ghooi, R.B. and S.M. Thatte (1995). *Med hypotheses, 44:* 27-131.
4. Hoischen, C. and R. Kramer (1990) *Journal of Bacteriology, 172:* 3409-3416.
5. Ikeda, M. (2003) *Advances in Biochemical Engineering and Biotechnology, 79:* 1-35.
6. Kinoshita S, S. Udaka and M. Shimono (1957). *The Journal of General and Applied Microbiology, 3:* 193-205.
7. Lubitz, D. and V.F. Wendisch (2016). *BMC Microbiology, 16:* 235-246.

8. Nara, T., S. Kinoshita and H. Samejima (1964). *Agricultural and Biological Chemistry, 28:* 120-124.
9. Reitermayer, D., T.A. Kafka, C.A. Lenz, R.F. Vogel (2018). BMC Microbiology *18:* 72-84.
10. Sano, C. (2009). *American Journal of Clinical Nutrition, 90:* 728S-732S.
11. Takinami, K., T.Tsunoda and H. Okada (1963). *Agricultural and Biological Chemistry, 27:* 858-863.
12. Takinami, K., H. Okada and T. Tsunoda (1964). *Agricultural and Biological Chemistry, 28:* 14-19.
13. Takinami, K., H. Yoshi, H. Tsuri and H. Okada (1965). *Agricultural and Biological Chemistry, 29:* 351-359.
14. Takinami, K., Y.Yamada, H. Okada (1966). *Agricultural and Biological Chemistry, 30:* 674-682.
15. Takinami, K., Y. Yamada, H. Okada (1967). *Agricultural and Biological Chemistry, 31:* 223-228.
16. Takimani, K., H. Yoshi, Y. Yamada, H. Okada and K. Kinoshita (1968). *Amino acid Nucleic acid 183 :* 120-160.
17. Uaaka S. (1960). *Journal of Bacteriology, 79:* 745-755.
18. Zamir, A., and N.A. Lichtenstein (1955) *Analytica Chemica Acta,12:* 577-579.