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Research Article

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Production Of L-Glutamic Acid As A Function Of Urease Activity By *Corynebacterium glutamicum* X680 Using Agro-Based Raw Materials

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Abstract: Urease catalyzes hydrolysis of urea. The present investigation was undertaken to investigate the urease activity and its optimization during L-glutamic acid fermentation by a biotin auxotroph *Corynebacterium glutamicum* X680 using urea as the principal nitrogen source. Another interesting part of this study was to investigate the efficiency of this microorganism for utilization of indigenous raw materials (such as hydrolysates of cassava starch, rice bran and wheat bran) as a cheap carbon sources instead of glucose. Among different raw materials, hydrolysate of cassava starch appeared to be the most suitable. However, the production efficiency is significantly less ($p<0.01$) with cassava starch compared to glucose. When the medium is supplemented with equivalent amount of (10g%) cassava starch hydrolysate, the L-glutamic acid accumulation was reported to be 18.2mg/ml with maximum urease activity (0.18U/mg protein) with pH7 at 30^0C.Thus, among the different agro-based wastes were examined, cassava starch hydrolysate appeared to be the best alternative of glucose to minimize L-glutamic acid production cost.

Key words: *Corynebacterium glutamicum* X680, urease, L-glutamic acid, hydrolysates of cassava starch

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1. INTRODUCTION

Urease or urea amidohydrolase (EC. 3.5.1.5) is a nickel containing enzyme catalyzes the hydrolysis of urea to produce ammonia[1]. Bacterial urease is a gene product of ureaA, ureaB and ureaC, encoding ¥, β and α subunits of urease enzyme resulting a trimeric protein[2]. It was the first enzyme to be crystallized in 1926 and also first reported nickel containing enzyme[3-6]. Hydrolysis of urea by urease results one molecule ammonia and one molecule of carbamate. Later, in aqueous solution carbamate is converted to another molecule of ammonia and carbonic acid. Ammonia then protonated and increases the PH of the medium[3]. Ureolytic activity is found among different microorganisms including several species of bacteria [7-11]. The present study was undertaken to examine the urease activity in *Corynebacterium glutamicum* X680 (a biotin dependent auxotrophic mutant developed in our laboratory in my previous study by induced mutation from *Corynebacterium glutamicum* X60) during L-glutamic acid fermentation and also to investigate the efficiency of this microorganism to utilize indegeneous raw materials like hydrolysates of cassava starch, rice bran and wheat bran as cheap carbon sources to minimize the production cost by replacing glucose.

2. MATERIALS AND METHODS

2.1 Microorganism

Corynebacterium glutamicum X680, a biotin auxotroph was developed by induced mutation in my previous study[12]. It was used throughout the present investigation.

2.2 *Composition of growth medium*

The bacterial growth medium was composed of : glucose, 2%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.025%, biotin, 3µg/ml; water, 1L and agar, 4%.

2.3 *Composition of production medium and culture conditions*

Submerged fermentation was carried out with pH, 7.0; period of incubation, 72h; volume of medium, 30ml; size of inoculum, 4%($8X10^6$ cells); age of inoculum, 48h; temperature, 30^0C; 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.

2.4 *Analytical method for L-glutamic acid estimation*

L-glutamic acid was qualitatively estimated by descending paper chromatography. The spots were visualized with ninhydrin spray. Colorimetric estimation was done for quantitative assay[12]. The compound was confirmed with FTIR.

2.5 *Estimation of dry cell weight*

After centrifugation, bacterial pellet was dissolved by 2ml 1(N) HCl and then neutralized by calcium carbonate. The remaining cells were washed twice and dried at 100^0C until a constant dry cell weight was attained[13].

2.6 *Preparation of hydrolysates of cassava starch, rice bran and wheat bran*

Agricultural residues like cassava starch, rice bran and wheat bran were grinded and sieved to a particulate size less than 600µm. About 100g of each powder was poured into 1L Erlenmeyer conical flask containing 500ml water and kept it for overnight. Then concentrated H_2SO_4 was added to reduce the pH of the medium around 1 and autoclaved it at 121^0C for 15 minutes at 15lb pressure. The medium was cooled at room temperature and neutralized by adding $CaCO_3$. Each treated residue was added to the fermentation medium replacing equivalent amount of glucose[14].

2.7 *Estimation of Ph*

PH of the broth was estimated using pH meter (model: MXCL20X1).

2.8 *Urease assay*

Both the crude and purified (partial) urease was assayed. After certain periods of incubation, the broth was centrifuged at 10,0000rpm for 10 minutes. The supernatant was collected and partially saturated with ammonium sulphate followed by dialysis using 2M phosphate buffer at 0^0C for 24h. Urease activity was assayed routinely by measuring ammonia using Conway method[16].

3. STATISTICAL ANALYSIS

All data were expressed as mean±SEM, where n=6. The data were analyzed by using 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.

4. RESULTS

4.1 Effects of hydrolysates of cassava starch, wheat bran extract and rice bran

Fig 1 shows the growth pattern and urea consumption by *Corynebacterium glutamicum* X680 over a period of 72h. Hydrolysates of cassava starch, wheat bran, and rice bran potentiated growth was proved to be the best alternative source of carbon with highest urea consumption. About 72.6% urea was utilized within 72h.

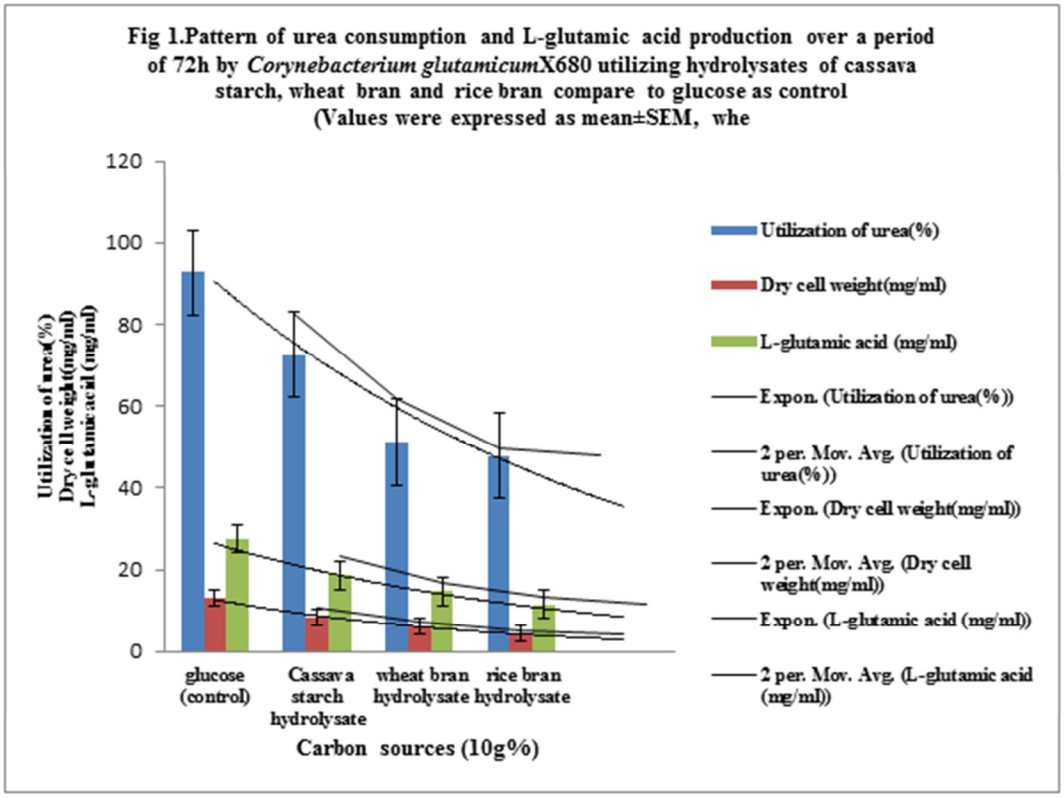


Fig 1. Pattern of urea consumption and L- glutamic acid production over a period of 72h by *corynebacterium glutamicum* x680 utilizing hydrolysates of cassava starch, wheat bran and rice bran compared to glucose as control (values were expressed as mean ± SEM, Whe

4.2 *Effect of different urea concentration on L-glutamic acid production using cassava starch hydrolysate as cheap carbon source*

L-glutamic acid production is characterized by assimilation of nitrogen. Initial 1% nitrogen concentration showed suitable for production using cassava starch hydrolysate as a cheap source of carbon (Fig 2). Higher concentrations inhibited bacterial cell growth as well as L-glutamic acid production.

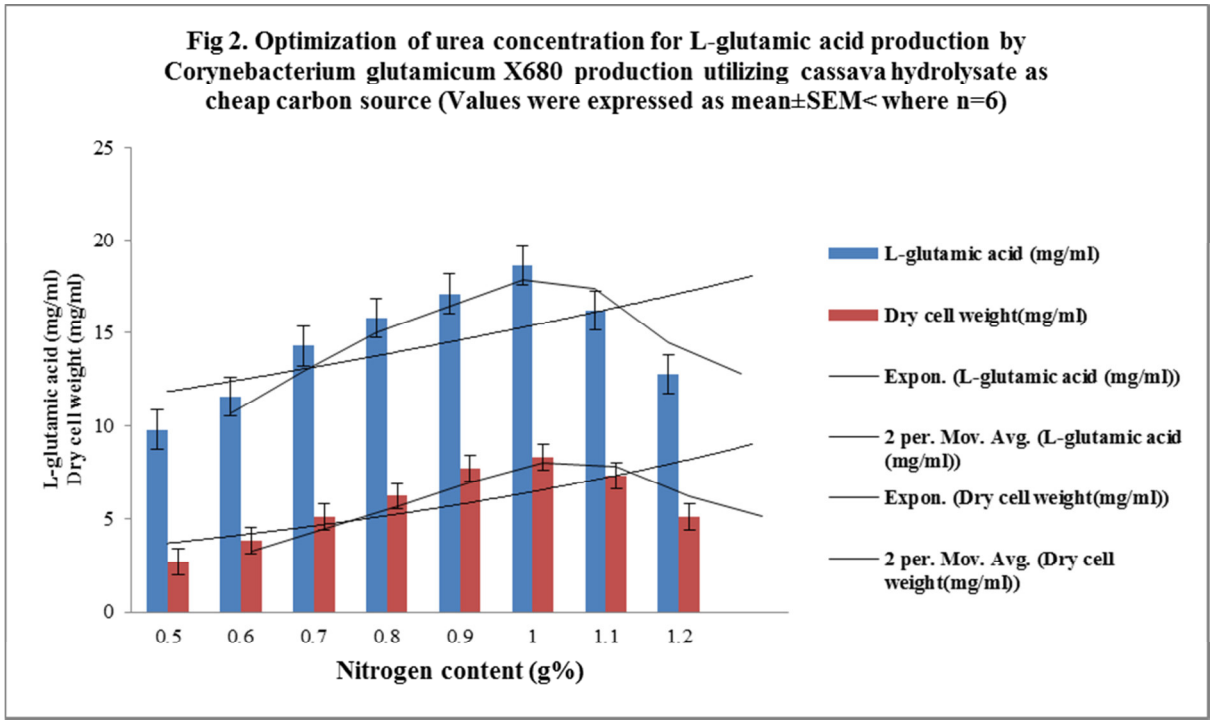


Fig 2. Optimization of urea concentration for L –glutamic acid production by corynebacterium glutamicum X680 production utilizing cassava hydrolysate as cheap carbon source (values were expressed as mean±SEM< where n=6)

4.3 Estimation of crude urease activity during L-glutamic acid production using hydrolysates of different as cheap carbon sources

Urease activity was confirmed with the color change of Christensen's agar medium (containing urea as the principal nitrogen source) from yellow to red-pink due to change in pH by liberation of ammonia. Specific enzyme activities (urease) was estimated when the cells were subjected to submerged fermentation using different indigenous raw materials as cheap carbon sources (Fig 3). Among indigenous raw materials studied, maximum urease activity was obtained with cassava starch hydrolysate (10g %) after 72h of incubation with urea (1g%nitrogen content).

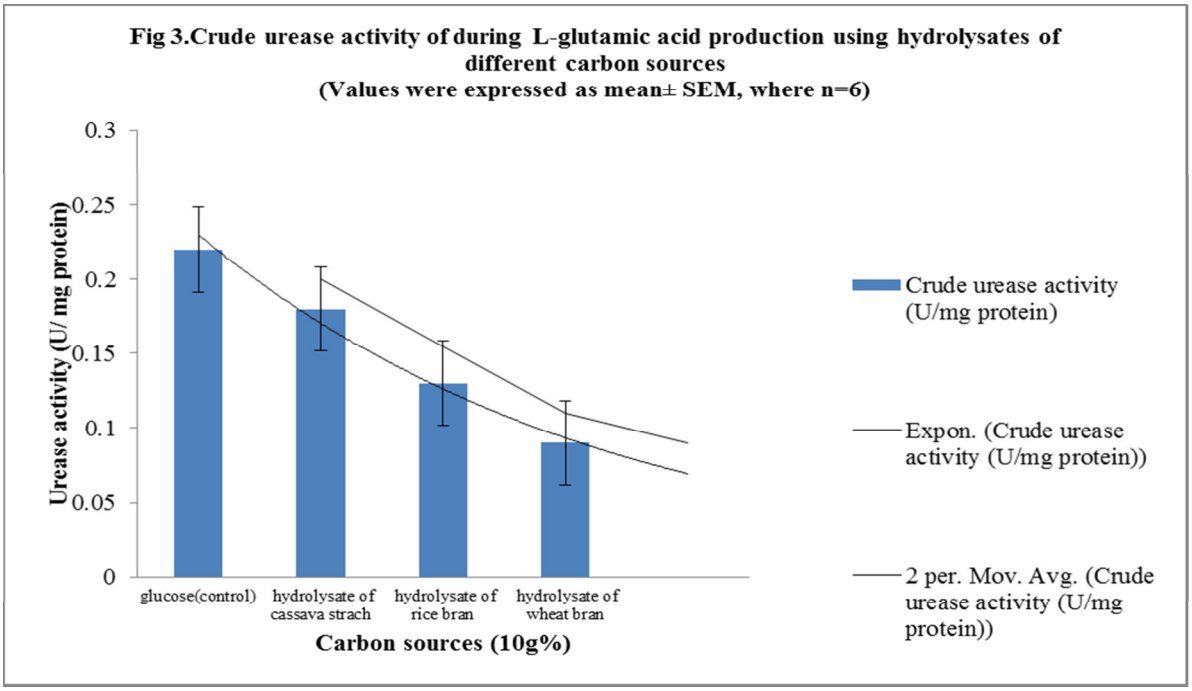


Fig 3. Crude urease activity of during L –glutamic acid production using hydrolysates of differenct carbon sources (values were expressed as mean±SEM, where n=6)

4.4 Purified urease activity as a function of ammonical and amino nitrogen; residual sugar, pH using cassava starch as a cheap carbon source

Table1 shows purified (partial) urease activity at different time intervals with changes of ammonical and amino nitrogen; residual sugar, pH using cassava starch as a cheap carbon source. Urease activity increases up to 72h of incubation with concomitant increase in pH of the medium.

Table 1. Changes of ammonical and amino nitrogen; residual sugar, pH as a function of purified(partial) urease activity (U/mg protein)

Incubation time(h)	Ammoniacal nitrogen (g%)	Amino nitrogen (g%)	Urease activity (U/mg protein)	Residual urea (g%)	Residual sugar (g%)	pH
24	0.06±0.001	0.01±0.000	0.06±0.000	0.33±0.001	4.3±0.027	7.0±0.008
48	0.15±0.001	0.06±0.000	0.11±0.001	0.48±0.003	3.8±0.016	7.1±0.016
72	0.21±0.004	0.17±0.001	0.18±0.003	0.73±0.001	1.2±0.003	7.3±0.006
96	0.18±0.002	0.11±0.003	0.13±0.003	0.61±0.001	0.8±0.038	7.4±0.0712
120	0.11±0.001	0.09±0.001	0.08±0.000	0.41±0.002	0.4±0.001	7.7±0.016

Values were expressed as mean±SEM, where n=6

4.5 Effect of pH and temperature on urease activity

Effects offive different pH levels (6, 6.5, 7, 7.5, 8) and seven different temperature (26, 27, 28, 29, 30, 31, 32^{0}C) were investigated on urease activity during L-glutamic acid production by the mutant *Corynebacterium glutamicum* X680 using 10g% cassava starch hydrolysate and 1g% urea as carbon and nitrogen sources respectively. Maximum activities were obtained with pH7 at 30^{0}C (Fig 4 and 5).

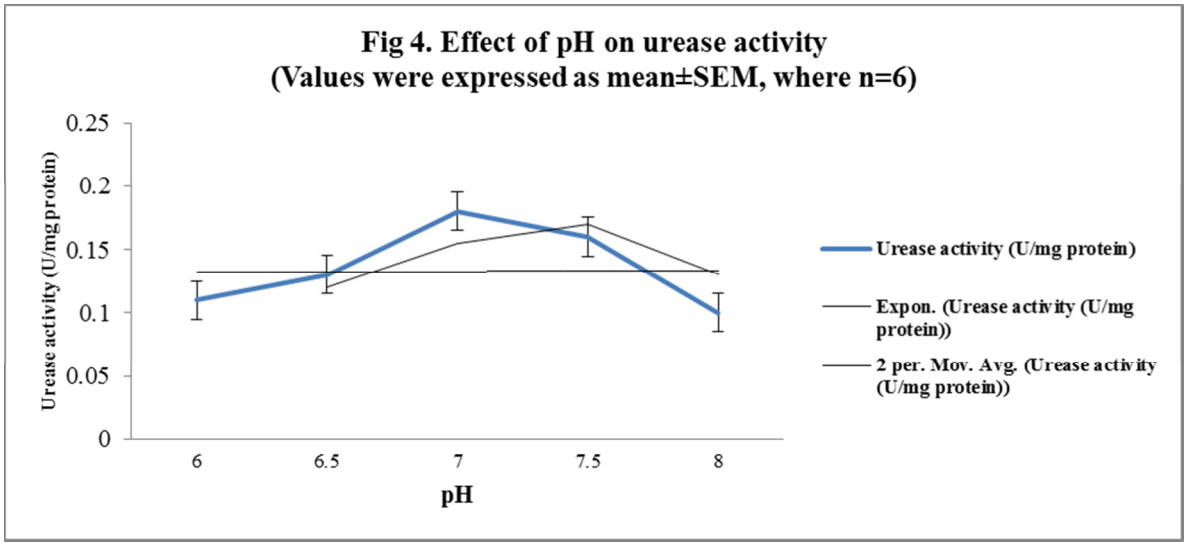


Fig 4. Effect of Ph on urease activity (values were expressed as mean±SEM, *Where n=6)*

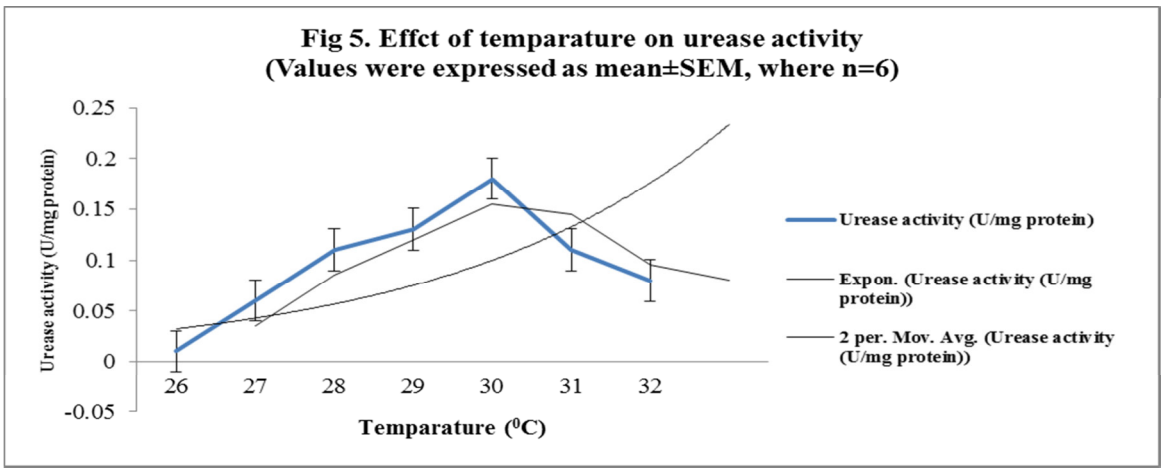
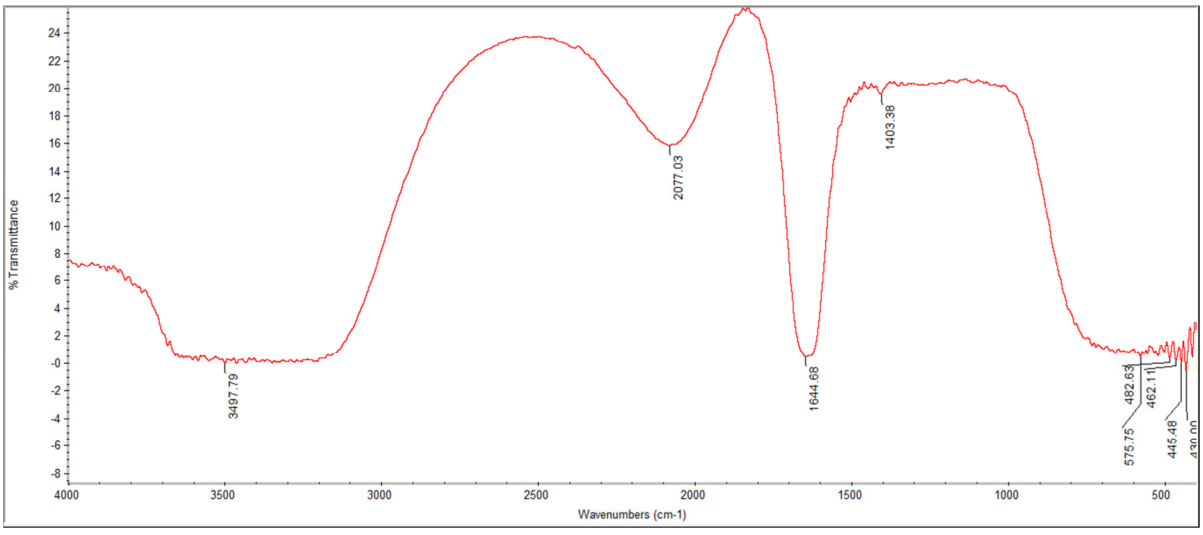


Fig 5. Effect of temperature on urease activity (values were expressed as mean±SEM, where n=6)



The product L-glutamic acid was initially identified by descending paper chromatography and finally confirmed by FTIR (Fig 6).

Fig 6. FTIR of the product confirming it as L-glutamic acid

5. DISCUSSION

Bacterial urease is a multimeric nickel containing enzyme which requires some accessory proteins to operate[17]. Those accessory proteins are required to transport and incorporate nickel into the active centre of the apoprotein[18]. A helix turn helix motif (called 'flap') is essential for urease activity[19]. It is highly mobile and assumes two conformations: in open conformation it allows urea to move into the active site and in close conformation it restricts the entry of urea into the

enzyme [20,21]. Bacterial ureases are highly conservative showing sequence similarities from different sources[22]. Burbank et al (2012) isolated soil bacteria from different sources which could exhibit ureolytic urease activity[23]. Urease activity was extensively studied by Liu (2017)[24]. He reported initial concentration of urea and pH affected urease activity and maximum activity was recorded after 96h fermentation. Very recently, Zhou et al (2019) extensively studied the urease activity in *Staphylococcus aureus*[25].

6. CONCLUSION

This experiment reveals that though glucose appeared to be the most suitable carbon source for L-glutamic acid overproduction by the mutant *Corynebacterium glutamicum* X680, however, cassava starch hydrolysate can alternatively be used as a cheap carbon source. But the production was decreased significantly ($p<0.01$) from 27.6mg/ml to 18.6mg/ml when glucose was replaced with cassava starch hydrolysate.

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8. AUTHORS CONTRIBUTION STATEMENT

The whole experimental work, interpretation of results and drafting of manuscript has been done by Dr. Suhadeep Ganguly.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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