Studies On Substrate Inhibition In The Microbial Production Of L-Glutamic Acid

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Abstract

Batch fermentation of L-glutamic acid was conducted with Corynebacterium glutamicum MTCC 2745 in the presence of glucose as a substrate. Its effects on the growth of the microbial cells and also on product formation were examined. The growth is limited as well as inhibited by the substrate depending upon its initial concentration. Limitation of growth was observed up to 50 kgm^{-3} of glucose used. Above 50 kgm^{-3} and so on, it was found to be inhibited by the substrate. At an initial glucose concentration of 300 kgm^{-3}, complete inhibition was noticed and no product was formed. The observed maximum specific growth rate was also affected by the substrate in the same manner.

Keywords: L-glutamic acid; *Corynebacterium glutamicum;* Batch fermentation; Substrate inhibition; Specific growth rate

1. Introduction

L-glutamic acid (LGA) is commercially one of the important amino acids. Its sodium salt, i.e. monosodium L-glutamate (MSG) is widely used as a flavour enhancer throughout the world. LGA and its derivatives are also used as raw materials for synthesis of many other value-added products[1]. As reported by Kumagai[2], the estimated worldwide production of LGA was one million tons in 1996. Hermann [3] reported that the annual production level of MSG is around 1.5 million tons and the market is growing by 6 % per year. A recent survey shows that the annual production in 2009 was more than 2 million tons [4].

Industrially, it is manufactured by batch/fed-batch fermentation processes[5] using various strains of *Corynebacterium* and *Brevibacterium* collectively known as *Corynebacterium glutamicum*. These strains are gram positive, nonsporulating, nonmotile, coccal or rod-like, aerobic and nonpathogenic.

Controlled supply of air and limitation of biotin, and / or addition of penicillin or treatment with surfactants are important for efficient production of L-glutamic acid [6]. Glucose has commonly been used as a carbon source in laboratory scale investigations.

The studies on the inhibition effects of glucose on growth and product formation are significant from the stand point of development of the model for the bioprocess. It is also important for further development and design of the fermentation process for commercial application.

The inhibition of growth by the substrate (glucose) as well as by the product (LGA) occurs in fermentation[7]. It was reported[6] that the graphical relationship between specific growth rate (μ) and substrate concentration (S) does not follow a simple Monod kinetics.

In the present work attempts have been made to identify the range of glucose concentrations which are responsible for the limitation and inhibition effects on growth of L-glutamic acid-producing bacteria,

Corynebacterium glutamicum MTCC 2745. It also shows the effect of substrate concentration on specific growth rate and product formation.

2. Materials and Methods

2.1 Microorganisms and Inoculum

The wild strain of *Corynebacterium glutamicum* MTCC 2745 supplied by the Microbial Type Culture Collection (MTCC) IMTECH Chandigarh, India was used in the present study. Seed culture was prepared by transferring cells from agar slants into 500 ml *Erlenmeyer* shake flask containing 100 ml of the culture medium.

2.2 Agar Slant and Seed Culture Medium

The constitution of the medium for preparing agar slants and the incubation conditions were given by the supplier. The composition of agar nutrient medium was (kgm^{-3}): beef extract, 1; yeast extract, 2; peptone, 5; sodium chloride, 5; and agar, 15. pH was adjusted to 7.0 and the culture on the slants was incubated for two days at 30 ^{0}C. The slants were preserved at 4 ^{0}C, and subcultured twice a month.

The composition of the medium for preparing seed culture was (kgm^{-3}): glucose, 50; urea, 5; corn steep liquor, 5×10^{-3} (v/v); K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4$ $7H_2O$, 0.4; $FeSO_4$ $7H_2O$, 0.01; $MnSO_4$ H_2O, 0.01; biotin, 5×10^{-6}; and thiamin-HCl, 80×10^{-6}. Biotin, thiamin-HCl and urea were sterilized by membrane filter (0.2 μm, Schleicher & Schull, Germany) whereas glucose and minerals were sterilized separately by autoclaving at 15 psi (121 ^{0}C) for 15 min. All components were mixed together aseptically before incubation. The initial pH was adjusted to 7.0 with aqueous solution of potassium hydroxide and hydrochloric acid. The cultures were incubated at 30 ^{0}C with a shaking speed of 120 rpm for 18 h in an orbital shaking incubator (CIS-24, Remi, India) before transferring them to the production medium.

2.3 Fermentation (Production) Medium

The composition of the production medium was the same as the seed culture medium except that there was no corn steep liquor used and the concentrations of biotin and urea were, 1×10^{-6} kgm^{-3} and 8 kgm^{-3}, respectively**.** A series of batch experiments were carried out in 500 ml Erlenmeyer shake flasks containing 200 ml of the fermentation medium with different initial glucose concentrations (S_0) ranging from 10 kgm^{-3} to 300 kgm^{-3}. The size of inoculums was 4%. The samples from the fermentation broth were collected and analyzed for biomass (X), product (P) and substrate (S) at every 2 h of time intervals for the initial 16 h of fermentation.

Agitation speed, temperature, pH and sterilization conditions were kept the same as those for the seed culture.

2.4 Separation of Biomass from the Broth

Free cells from the fermentation broth were separated by centrifugation using a centrifuge (R-24, Remi, New Delhi) at 10,000 rpm for 5 min. The clear supernatant was decanted from the centrifuge tubes and preserved at 4 ^{0}C for further analysis of the substrate (glucose) and the product (L-glutamic acid).

2.5 Determination of Free Cell Concentration

Bacterial growth was estimated by measuring the optical density (absorbance) at 610 nm with the help of a spectrophotometer (Lambda 35, Perkin Elmer, USA) between the absorbance 0.2 – 0.9 taking care of the Lambert-Beer's law. Whenever required the samples were diluted with double distilled water for attainment of desired range of absorbance. For estimation of cell dry weight (CDW), known volume of the sample with known absorbance was filtered by filtration membrane (0.45 μm, Millipore, USA). The retained biomass was washed twice with double-distilled water, and thereafter, dried in an oven at 110 ^{0}C

for 8 h as described by Posten and Cooney[8]. The differential weight of the membrane gives the dry weight of the cells. A standard graph was plotted for further estimation of CDW from optical density.

2.6 Estimation of Glucose and L-glutamic Acid

The DNS method [9] was used for the estimation of glucose whereas L-glutamic acid was estimated by the colorimetric method also known as copper complex method[10].

3. Results and Discussions

3.1 Substrate Inhibition

The time course of fermentation at selected initial glucose concentrations (S_0 = 10, 40, 50, 60 and 80 kgm^{-3}) and compounding concentrations of X and P are depicted in Figure 1. With S_0 = 10 kgm^{-3}, the fermentation is almost complete at 16 h and the product concentration (P) flattens. However, with the increase in S_0, X and P values also increase, and the fermentation is incomplete after 16 h. It is found that up to S_0 = 50 kgm^{-3}, X and P show increasing trend. As S_0 increases from 50 kgm^{-3}to 60 kgm^{-3} and so on, the concentrations of the biomass and the product show a decreasing trend. For example, at the end of 16 h, X = 2.38 kgm^{-3} and P = 7.10 kgm^{-3} for S_0 = 50 kgm^{-3}. For S_0 = 60 kgm^{-3}, X and P are 2.16 kgm^{-3} and 6.20 kgm^{-3}, respectively.

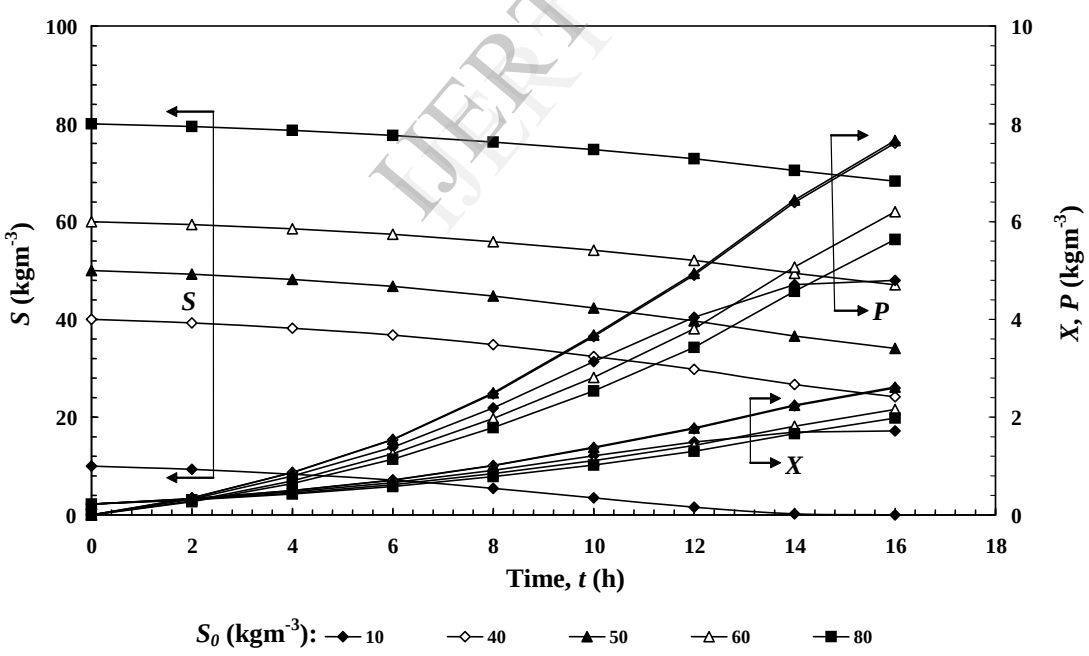

Figure 1 Time course of fermentation with different initial substrate concentrations.

In order to ascertain the substrate concentration at which the fermentation ceases, a number of batch fermentation experiments were carried out with increasing S_0 up to 300 kgm^{-3}. It is found that at S_0 = 300 kgm^{-3}, the fermentation completely ceases. This situation can be clearly observed from Figures 2 – 6.

Figure 2 shows the plots of X against fermentation time for different S_0 ($10 \le S_0 \le 300$ kgm^{-3}), whereas Figure 3 demonstrates the plots of P against time of fermentation with S_0 as a parameter. Figure 2

shows that for S_0 = 300 kgm^{-3}, no change in X is observed during the course of fermentation. From Figure 3, it is seen that the product concentration (P) is zero at all times for S_0 = 300 kgm^{-3}.

Figures 4 and 5 depict, respectively, the variation of X and P with S_0. It is observed that X and P increase with increasing S_0 up to 50 kgm^{-3}, at which they peak and then start decreasing with increase in S_0

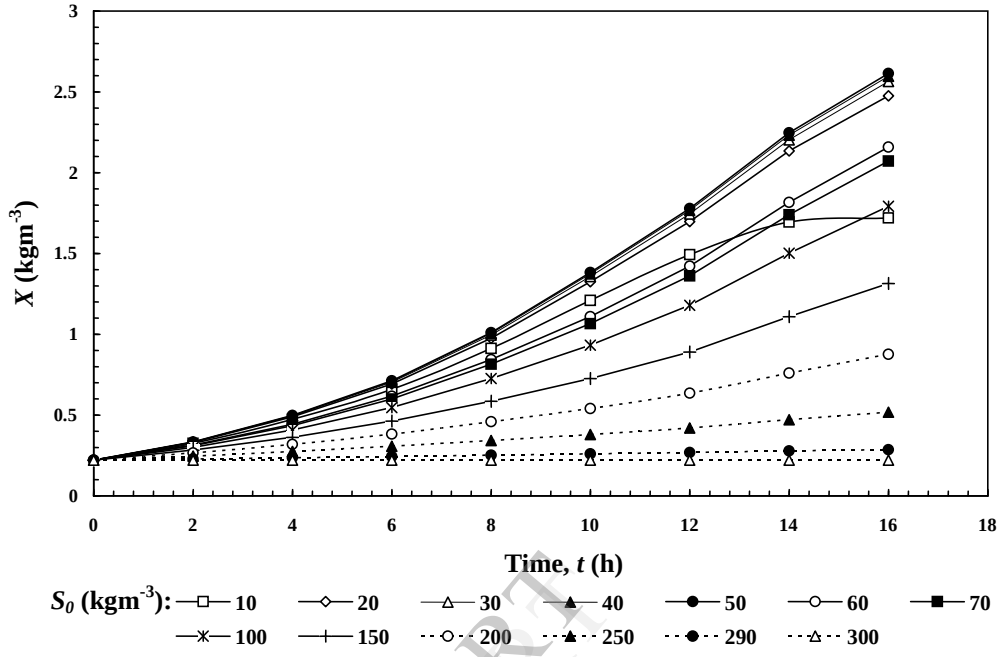

Figure 2 Time course of fermentation for growth of *C. glutamicum* at different initial substrate concentrations.

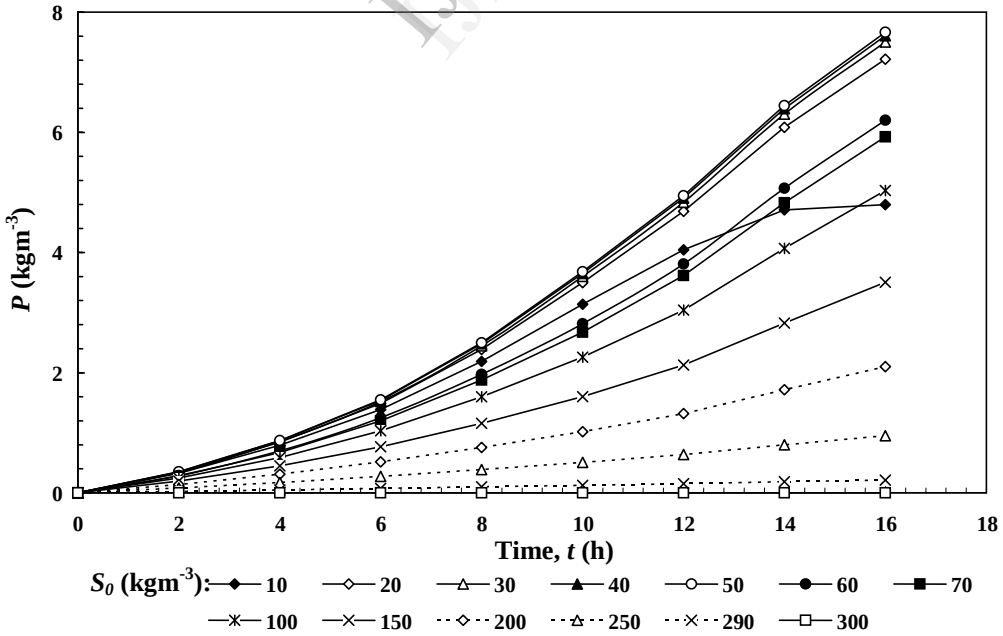

Figure 3 Time course of fermentation for product formation at different initial substrate concentrations.

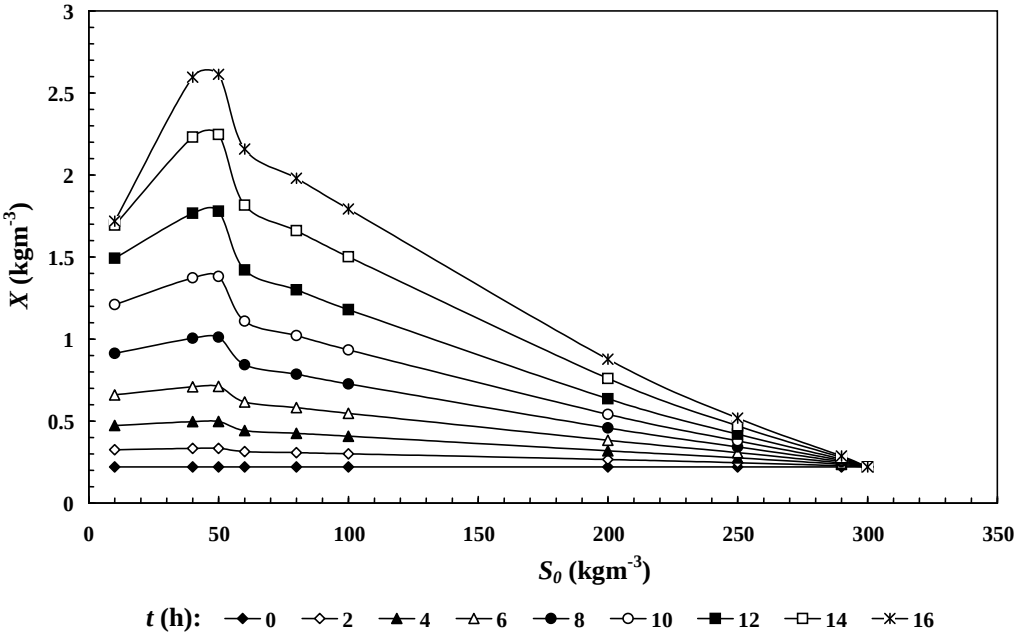

Figure 4 Variation of the growth with initial substrate concentration at various time intervals.

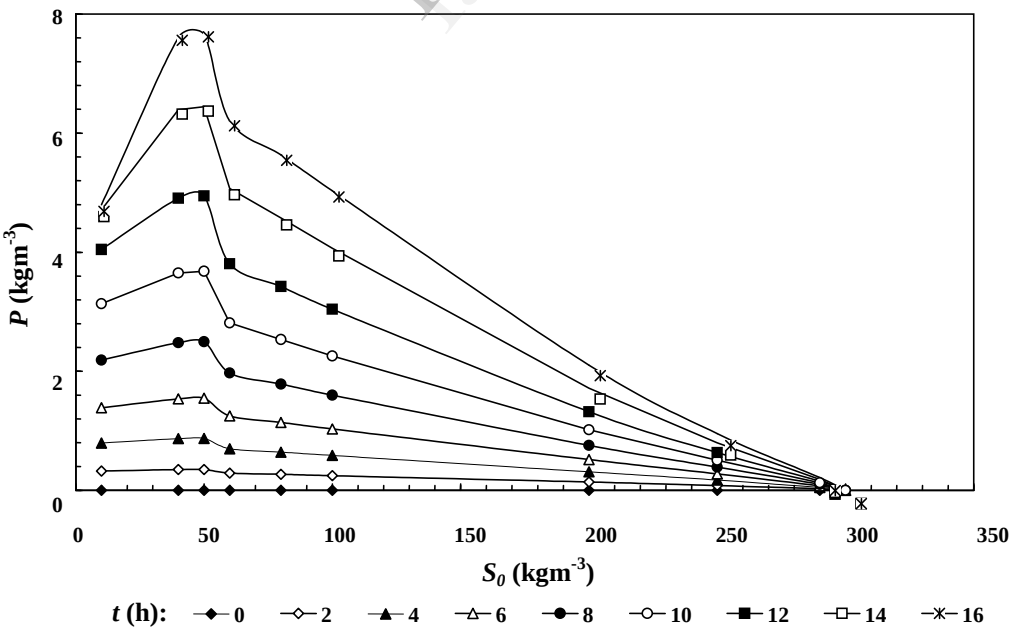

Figure 5 Variation of the product formation with initial substrate concentration at various time intervals.

and converge asymptotically at S_0 = 300 kgm^{-3}. At this S_0, X remains X_0 and P is also zero. These figures demonstrate that the optimum S_0 for LGA fermentation with *Corynebacterium glutamicum* MTCC 2745 is 50 kgm^{-3} and that the growth and product formation are limited by the substrate. For S_0 > 50 kgm^{-3} and so on, substrate inhibition retards the growth of the cells and product formation too.

In order to demonstrate the impact of initial substrate concentration on specific growth rate of the cells, the observed maximum specific growth rates for all fermentations with initial concentrations ($10 \le S_0 \le 300$ kgm^{-3}) were calculated using the ln X vs. t plot and plotted against the corresponding initial substrate concentration (S_0) as shown in Figure 6. It is found that the observed maximum specific growth rate increases with the increasing substrate concentration up to S_0 = 50 kgm^{-3}. For S_0 above 50 kgm^{-3}, the observed maximum specific growth rate also decreases continuously and becomes zero at S_0 = 300 kgm^{-3}. This is also a clear indication of substrate limitation and inhibition in L-glutamic acid fermentation. Figure 6 reinforces the conclusions drawn from Figures 1–5.

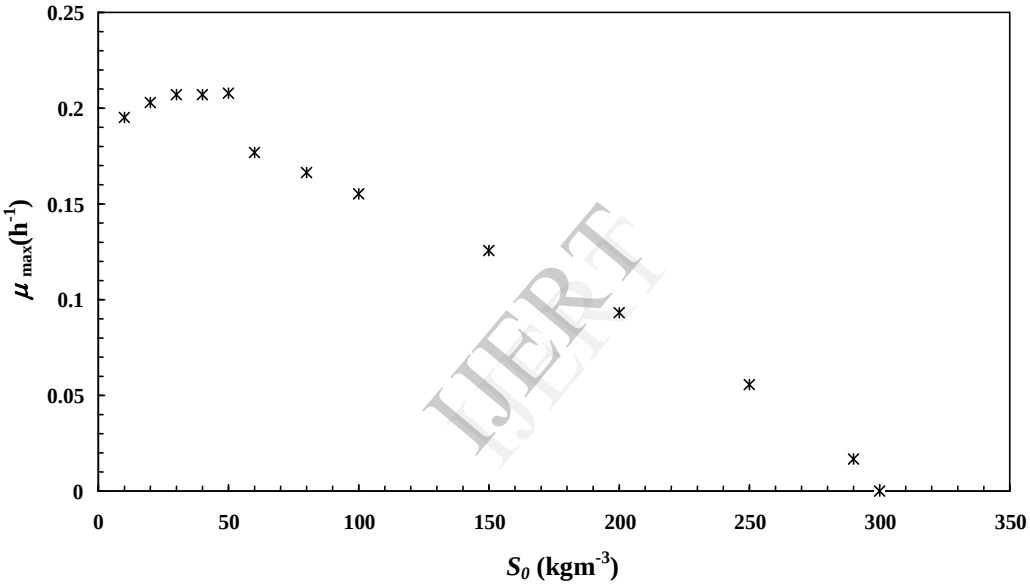

Figure 6 Effect of initial substrate concentration on the observed maximum specific growth rate (μ_{max}) of *Corynebacterium glutamicum* MTCC 2745.

4. Conclusion

The substrate inhibition and limitation effects occur in L-glutamic acid fermentation. The growth of *Corynebacterium glutamicum* MTCC 2745 is limited by the substrate (glucose) up to the initial glucose concentration of 50 kgm^{-3}. Above 50 kgm^{-3} and so on, the growth is inhibited. At the initial glucose concentration of 300 kgm^{-3}, it is completely inhibited and there is no product formation too. The initial substrate concentration also affects the observed specific growth rate in the same manner.

Nomenclature

S	Substrate concentration,	kgm^{-3};	X	Biomass (cell) concentration,	kgm^{-3}
S_0	Initial substrate comncentration,	kgm^{-3}	μ	Specific growth rate of cells,	h^{-1}
P	Product concentration,	kgm^{-3}	μ_{max}	Maximum Specific growth rate,	h^{-1}

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