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High production of L-glutamic acid from date juice extracts by *Corynebacterium glutamicum* using fed-batch cultures: pulsed and continuous feeding modes

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Abstract: *In the present work, L-glutamic acid production by Corynebacterium glutamicum fermentation on date juice extracts applying two fed-batch feeding modes, pulsed and continuous, were investigated. According to the obtained results, the continuous feeding fed-batch mode was found to be the most efficient process. Moreover the continuous feeding rate mode with a feeding medium containing date juice sugars enriched with ammonium sulfate was found even more favorable as it enhances the L-glutamic acid production by approximately 2.35 fold more than the batch culture and by about 1.17 fold more than the pulsed feeding. In this respect, comparing the traditional batch culture to the continuously fed culture with a medium containing date juice sugars with ammonium sulfate showed increases of 135.47% in L-glutamic acid production, 104% in productivity, 39.09% in biomass, and 47.69% in the yield respectively allowing us to reach a final L-glutamic acid concentration of about 138 g/L, the highest ever published.*

Keywords: *Corynebacterium glutamicum*, L-glutamic acid, date juice, fed-batch fermentation, feeding mode

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Introduction

L-glutamic acid is an important industrial amino acid for producing the flavoring agent monosodium glutamate. It is widely used in the food industry, pharmaceuticals, medical sciences, cosmetics, and polymer precursors. The annual production of glutamic acid reaches now around 2.2 million tons [1]. Several strains of *Corynebacterium* and *Brevibacterium*, now collectively known as *C. glutamicum* are used for this industrial production [2]. The induction process of glutamate excretion by *C. glutamicum* requires either biotin limitation [3], treatments either with penicillin [4], fatty acid ester surfactant [5], or a temperature up-shift of the culture broth [6]. As for the substrate, glucose is one of the major carbon sources for the production of L-glutamic acid. It is also produced using different kinds of raw materials including submerged fermentation of palm waste hydrolysate [7], cassava starch [8], sugar cane bagasse [8], and date waste [10].

As mentioned earlier [11], Algeria produces annually over 720,000 tons of different varieties of dates. However, approximately 30% of the production be are lost due to over-ripening, improper handling, processing or marketing. This part is not suitable for human consumption and is generally used for feedstock. The waste recycling process of date fruit is considered the most important biological process within the environmental system that aims to keep the environmental balance. Biological reactions are one of the safest and most successful methods, in which microorganisms such us *C. glutamicum* play an important biological role and restore the balance within the environmental system, in addition to their success in transforming date palm wastes to products of an economic return. To better use this poor quality dates and date by-products, investigations for food product manufacture, such as in bakery products, ice-cream, caramel products, ethanol, vinegar [12], oxytetracycline [13], single-cell protein [14], and lactic acid [15, 16, 17] have been performed. Date flesh contains 73 to 83% of sugars which are glucose, fructose and sucrose. They also contain proteins, lipids, minerals and some vitamins [18], which makes date extracts suitable fermentation substrates.

During fermentation the inhibition of L-glutamic acid biosynthesis, due to the high concentration of carbon substrate, is considered as one of the main problems generated by the batch process [19, 20]. As a result, running the fermentation in a fed-batch mode in which the carbon source concentration is maintained at a reasonably low level is essential for obtaining both high yield and productivity. An efficient method for feeding the nutrients and an appropriate feed timing into the bioreactor are essential parameters to achieve high productivity in fed-batch fermentations. Several feeding strategies, such as constant feeding, intermittent feeding and exponential feeding, can be used in such cultivations [21]. For L-glutamic acid production, only a few papers reported fed-batch fermentation leading to a high L-glutamic acid concentration. Kishimoto et al. [22] suggested a fermentation process in which L-glutamic acid can be produced with ethanol at a concentration of 25 g/L during fed-batch culture. Das et al. [7] described the production process of L-glutamic

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acid from palm waste hydrolysate by using the strain *Brevibacterium lactofermentum* ATCC 13869, which utilizes glucose as a carbon source. A concentration of 88 g/L of L-glutamic acid has been then obtained. An other study carried out by Nampoothiri and Pandey [23], used the strain *Brevibacterium* sp DSM 20411 on cassava starch hydrolysate allowed them to reach 21 g/L of L-glutamic acid. In fed-batch fermentation using 5% w/v sugar concentration, L-glutamic acid produced was 25 g/L Delaunay et al. [24] reported the production of 80 g/L of L-glutamic acid on a glucose medium under fed-batch fermentation using *C. glutamicum* 2262, with temperature shifts-up from 33°C to 37°C, 38°C, 39°C, 40°C or 41°C.

In the present work, the production of L-glutamic acid by *C. glutamicum* 2262 from date juice sugar extracts using batch and fed-batch (pulsed and continuous feeding) cultures is investigated. The present experiments allowed us to reach a L-glutamic concentration of about 138 g/L which is one of the highest published at the moment.

Materials and methods

Microorganism and preparation of inoculum

The strain used throughout this study was the thermo-inductible *C. glutamicum* strain number 2262 [6]. This strain obtained from the Laboratory Réactions et Génie des Procédés (LRGP-CNRS, Nancy, France) is provided by Amylum-Orsan (France). The inoculum was grown in shake-flask culture at 33°C, in MCGC medium [25], which has the following composition: glucose, 34 g/L; urea (Panreac), 4 g/L; thiamine (Sigma), 20 mg/L; biotin (Sigma), 2 mg/L; $CaCl_2$ (Fluka), 84 mg/L; $MgSO_4.7H_2O$ (Panreac), 0.4 mg/L; $FeSO_4.7H_2O$ (Panreac), 40 mg/L; $FeCl_3$ (Panreac), 4 mg/L; $ZnSO_4.7H_2O$ (Merck), 1 mg/L; $CuCl_2.2H_2O$ (Riedel-De Haen), 0.4 mg/L; $MnSO_4.H_2O$ (Prolabo), 4 mg/L; Na_2HPO_4 (Fluka), 15 g/L; KH_2PO_4 (Prolabo), 3 g/L; NaCl (Prolabo), 1 g/L; $(NH_4)_2SO_4$ (Sigma), 8 g/L.

Preparation of date juice substrate

The substrate used was obtained from date rejects. The method used for sugar extraction from these dates was adapted from Nancib et al. [26]. The dates were thoroughly cleaned manually to remove dust and foreign materials. The seeds were separated by manual splitting. Tap water was added at a ratio of two parts to one part of the dates (by weight). The mixture was heated at 80°C for 2 h with continuous stirring, and then centrifuged at 10,000 rpm for 10 min to separate the cellulosic debris, while the supernatant was used essentially as a carbon source in the fermentation medium. Immediately before each experiment, an appropriate quantity of date juice was diluted to a defined concentration of total sugars. The total sugar content corresponding to the addition of the glucose, fructose, and sucrose contents, of the collected supernatant was therefore determined.

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Fermentation medium

The fermentation medium for L-glutamic production consisted of date juice with a 88 g/L concentration of the total sugars. To reduce precipitates formation salts and date juice sugars were sterilized separately at 121°C for 20 min whereas vitamins were added after sterilization by filtration (0.22 µm). After cooling, the date juice sugars were supplemented with ammonium sulfate, 8 g/L; biotin, 272 µg/L; thiamine, 0.01 g/L; glycine betaïne, 12 g/L; and minerals ($CaCl_2$, 0.01 g/L; KH_2PO_4, 2.5 g/L; K_2HPO_4, 2.5 g/L; $MgSO_4$, 0.6 g/L; and $MnSO_4$, 2.5 mg/L).

General fermentation conditions

The experiments were carried out in a 2 L jar bioreactor (Biolafitte, France), equipped with lipseal stirrer assembly, pH, dissolved O_2 and temperature automatic controllers, foam controller and multi-channel peristaltic pump for feeding. The culture was grown at 33°C with an air rate of 102 L/h. The culture pH was maintained at 7.78 by automatic addition of (4N) NH_4OH, using a peristaltic pump. The increase in culture temperature (from 33 to 38.4°C) to induce L-glutamic acid biosynthesis was performed when the exponential phase was attained (5 h). The agitation speed was set at 662 rpm to ensure complete mixing of the fermentation broth. The inoculum was incubated at 33°C for 14 h on a shaking incubator at 330 rpm, before inoculation into the bioreactor with 11.5% inoculum volume.

Batch fermentation

Firstly the bioreactor containing 1 L of date juice medium at the concentration of a total sugar of 88 g/L is sterilized at 120°C for 20 min. An 11.5% inoculum grown in the MCGC medium was then added aseptically into the bioreactor. At 5 h of culture, corresponding to the beginning of the exponential phase, the culture temperature was increased to 38.4°C to induce L-glutamic acid biosynthesis. In the course of the entire fermentation, 4N NH_4OH is added to maintain pH at 7.78. The samples were withdrawn at desired intervals and analyzed for cell biomass, residual total sugars of date juice, and L-glutamic acid.

Fed-batch fermentation

Following the batch fermentation step, the fed-batch fermentation period was performed using either pulsed or continuous feeding. When using a pulsed feeding mode, 160 mL containing 210 g/L of total sugars of date juice (glucose, fructose, and sucrose at proportions of 29/23.5/47.5%) was fed into the bioreactor at pulses feeding times of 10, 20, and 30 h of culture, with the residual total sugar concentration of date juice in the range of 0-5 g/L in the fermentation medium. When using a continuous feeding mode, the date juice solution (210 g/L of total sugars) was fed continuously at the rates of 10, 20, and 30 mL/h fixed at 10, 20, and 30 h of culture respectively.

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Analytical methods

During the fermentations, samples were collected to determine biomass, residual total sugars of date juice, and L-glutamic acid concentrations. The cell biomass concentration was estimated by optical density measurement at 570 nm and calibrated to the cell dry weight. After centrifugation of the sample (10,000 rpm, 10 min), the amount of L-glutamic acid and total sugars of date juice (glucose, fructose, and sucrose) date juice were enzymatically determined. For that glucose and fructose were determined with the enzymatic Biopharm Roche kits using respectively glucose-6-phosphate dehydrogenase and hexokinase plus phospho-glucose isomerase. Sucrose was evaluated using glucose determination after invertase hydrolysis. L-glutamic acid was determined with the enzymatic Biopharm kit using glutamate dehydrogenase and diaphorase.

Results and discussion

Batch culture

The profiles of the evolution of the growth of *C. glutamicum* 2262 (biomass, residual total sugars of date juice, and L-glutamic acid production) on the fermentation medium, are shown in Figure 1. Compared with pulsed and continuous feeding fed-batch, the batch culture had the lowest glutamic acid concentration, biomass and productivity (see below). As shown in Table 1, at 30 h of culture, the maximum concentration of L-glutamic acid observed was 58.57 g/L and the maximum biomass, yield and productivity of L-glutamic acid were 18.52 g/L, 0.65 g/g and 2.25 g/L.h, respectively. This might result from the substrate inhibition and limitation effects that occur in L-glutamic acid fermentation as reported by Khan et al. [27]. These authors found that above 50 g/L of glucose, the L-glutamic acid biosynthesis is inhibited.

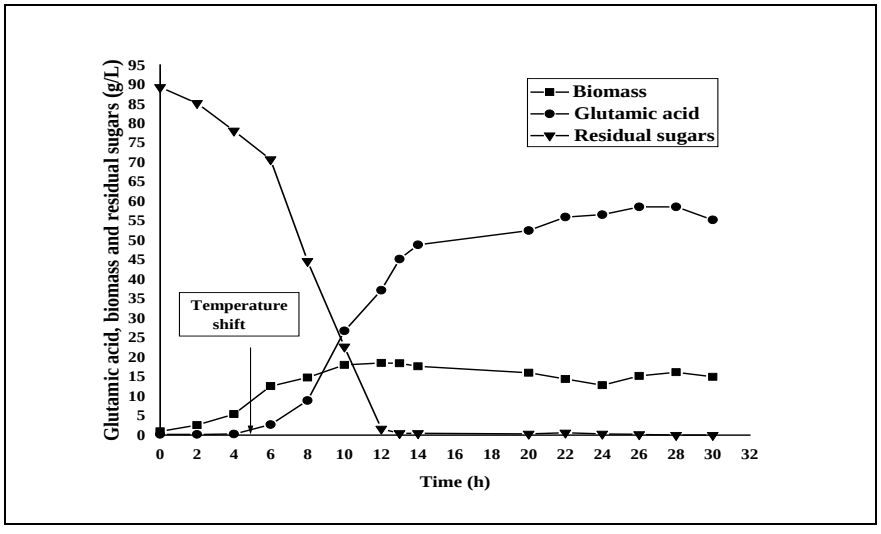

Figure 1. Kinetics of batch fermentation of *C. glutamicum* 2262 on fermentation medium. The arrow indicates the time when the temperature was changed from 33°C to 38.4°C

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Table 1. Comparison of glutamic acid production by batch, and fed-batch cultures of *C. glutamicum* 2262 by date juice

Parameters	Batch culture	Pulse Fed-batch	Continuous feed fed-batch		
			[a]	[b]	[c]
L-glutamic acid (g/L)	58.57	117.7	120	137.92	109.7
Productivity (g/L.h)	2.25	3.92	4	4.59	3.65
Yield (g/g)	0.65	0.78	0.80	0.96	0.72
Used substrate (%)	100	100	100	98.58	99.17
Biomass (g/L)	18.52	29.6	33.3	25.76	26.56

[a]: Fed-batch using continuous feed by feeding medium containing only date juice solution at the concentration of a total sugar of 210 g/L.

[b]: The same feeding medium but enriched with ammonium sulfate at a concentration of 16 g/L.

[c]: The same feeding medium with ammonium sulfate at the same concentration and minerals ($CaCl_2$, 0.01 g/L; KH_2PO_4, 2.5 g/L; K_2HPO_4, 2.5 g/L; $MgSO_4$, 0.6 g/L; and $MnSO_4$, 2.5 mg/L).

Fed-batch cultures

As indicated above several fed-batch fermentations were conducted to see if L-glutamic acid can then reach higher concentrations. The feeding modes were either pulsed or continuous feeding.

Pulse fed-batch cultures

In these experiments, the same amount (160 mL) of date juice at the concentration of a total sugar of 210 g/L was added into the bioreactor at 10, 20 and 30 h of culture, while the residual total date juice sugars concentration was within the range of 0-5 g/L. As shown in Figure 2, at 30 h of culture, biomass and L-glutamic acid production increased to 29.6 g/L and 117.7 g/L, respectively, compared to 18.52 g/L and 58.57 g/L obtained in the batch culture. Under these conditions, the yield and productivity of L-glutamic acid were improved significantly. The obtained values were 0.78 g/g and 3.92 g/L.h, respectively (Table 1). Compared with the batch culture, feeding mode by pulse showed around 100% increase in L-glutamic acid final title, 60% in biomass production, 20% in yield, and 75% in productivity, respectively.

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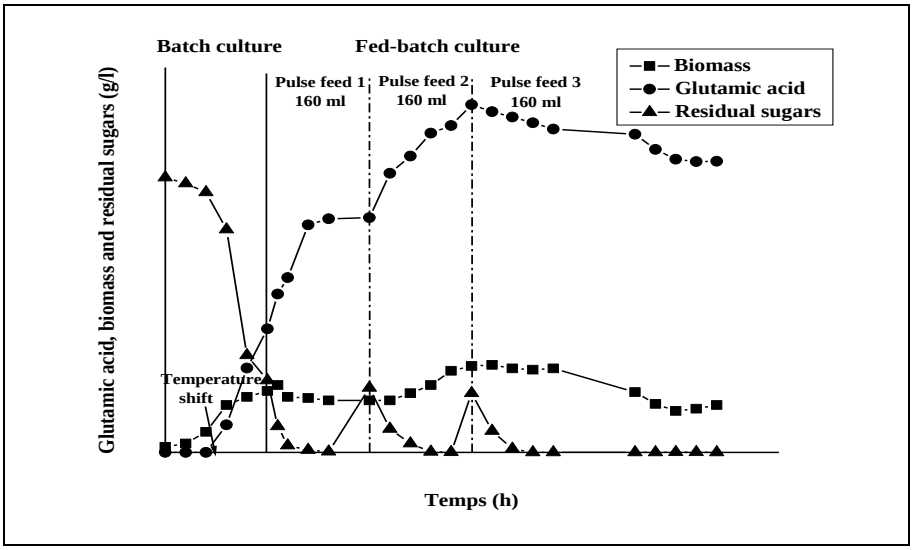

Figure 2. Kinetics of fed-batch fermentation of *C. glutamicum* 2262 using pulsed feeding fermentation (3 pulse feeds of 160 ml containing 210 g/L of total date juice sugars at 10, 20 and 30 h). The arrow indicates the time when the temperature was changed from 33°C to 38.4°C

Continuous fed-batch cultures

The influence of the feeding medium supplementation during three continuous feeding rate fed-batch assays was investigated: (A): feeding medium containing only date juice extract at the concentration of a total sugar of 210 g/l; (B): the same medium after enrichment with ammonium sulfate at a concentration of 16 g/L as the nitrogen source, and (C): the same medium enriched with ammonium sulfate (16 g/L) and with salts ($CaCl_2$, 0.01 g/L; KH_2PO_4, 2.5 g/L; K_2HPO_4, 2.5 g.l^{-1}; $MgSO_4$, 0.6 g/L; $MnSO_4$, 2.5 mg/L). The concentration of 16 g/L in ammonium sulfate and those of the different used salts have been chosen because they were the optimum concentrations found during preliminary assays (data not shown). Note also that because the results were higher with the continuous fed-batch cultures than with the pulse fed-batch cultures, the assays with ammonium sulfate enrichment were carried out only with the assay performed with a continuous feeding.

Figure 3A shows the corresponding results when using a feeding medium containing only date juice sugars at a concentration of 210 g/L, indicating at 30 h of culture increases in biomass, L-glutamic acid productions, as well as for the yield and productivity as compared to those obtained during batch and pulsed fed-batch culture. In these conditions, the values obtained at 30 h of culture are 33.3 g/L, 120 g/L, 0.80 g/g, and 4 g/L.h respectively (Table 1).

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Date juice extracts alone may not be sufficient to provide the necessary nitrogen need. In fact as reported by Abou-Zeid et al. [18], they have a low level of nitrogen. Hence, the addition of nitrogen source was thought to be useful and perhaps even necessary. In the present work an ammonium salt (sulfate) was chosen because it is the prefered nitrogen source of *C. glutamicum* [28]. So far a new fermentation using the previous feeding medium enriched with ammonium sulfate at a concentration of 16 g/L has been used. Similary to the previous fermentation, after the batch period, the feed rates were fixed at 10, 20, and 30 mL/h at 10, 20, and 30 h of culture respectively. The results indicated in Figure 3B, show that at 30 h of culture a L-glutamic acid concentration of about 138 g/L is obtained corresponding to increases of 135% and 17% compared to the batch and pulsed fed-batch cultures respectively. Under these conditions, yield and productivity were also increased to 0.96 g/g and 4.59 g/L.h, respectively in comparison with 0.65 g/g and 2.25 g/L.h during the batch fermentation and with 0.78 g/g and 3.92 g/L.h during the pulsed fed-batch fermentations. Furthermore, in comparison with the continuous feeding rate cultures containing only date juice sugars, the continuous feeding rate date juice sugars enriched with ammonium sulfate allowed us to reach increases of 14.93% improvement in L-glutamic acid concentration, 20% in yield, and 14.75% in productivity. These results are coherent with previous studies and particularly this of Li et al. [28] who used also ammonium sulfate where with an ammonium sulfate of 2% they observed maximum volumetric productivity of 2 g/L.h. Besides this it should be noted that ammonia remains the preferred source of nitrogen because it not only serves for regulating the pH of the fermentation medium but in many microorganisms also serves for regulating the synthesis of glutamate dehydrogenase (GDH) [24]. Therefore, in L-glutamic fermentation in the presence of a high concentration of NH^+ ions, GDH activity responsible for glutamate synthesis could be a predominant or important activity. By the way, Tesch et al. [29] using studies on carbon flux by NMR in *C. glutamicum* ATCC-13032 have shown that 72% of glutamate is synthesized using GDH and 28% with glutamine synthetase (GS)/glutamine amide α-ketoglutarate amino-transferase (GOGAT).

From these results, it can be concluded that the continuous feeding (here at 20 mL/h) could be more efficient for L-glutamic acid production than a pulsed feeding in fed-batch cultures. Indeed the L-glutamic acid concentration is then increased by approximately 1.17 fold than in pulsed feeding and about 2.35 fold than what it is in batch cultures at 30 h. Subsequently, it seems that this culture mode could be very suitable for this amino acid production. These findings can be compared to some obtained by fed-batch fermentation using *Bacillus* spp [30]. For example with the strains *Bacillus* sp. R22EG1 and R20EG2 the results using a continuous feeding were respectively 1.53 and 1.42 fold higher than those obtained with pulsed feeding, and 1.64 and 1.59 fold higher than those obtained during batch fermentation.

In the present work, the L-glutamic acid concentration of 138 g/L was much higher than those obtained by other authors. The results published by Kishimoto et al. [2] (on ethanol), Das et al. [7] (on palm

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waste hydrolysate), Nampoothiri and Pandey [23] (on cassava starch hydrolysate) and Delaunay et al. [24] (on glucose), which were 25, 88, 25 and 80 g/L, respectively. However it must be noted that a similar or equal final L-glutamic acid concentration obtained also on palm date extract but this time with a strain treated with penicillin and after optimisation has been obtained by Tavakkoli et al. [31]. It was then 142,25 g/L. So apparently 138 g/L is the highest published concentrations obtained for L-glutamic fermentation with *C. glutamicum*, and this with date extracts as substrate, which seems to be noticeable and could be deepened in the future.

One further fermentation assay has been performed in the same conditions as those for the just previous continuous fed-batch assay, but the feeding solution containing date juice sugars and ammonium sulfate was then enriched in salts ($CaCl_2$, 0.01 g/L; KH_2PO_4, 2.5 g/L; K_2HPO_4, 2.5 g/L; $MgSO_4$, 0.6 g/L; and $MnSO_4$, 2.5 mg/L). As shown in Figure 3C, the salts' enrichment did not improve the performances. At 30 h of culture, L-glutamic acid concentration (109.74 g/L) was lower than the previous one (138 g/L). This figure shows also in these conditions decreases in the glutamate title and the biomass concentration. Table 1 show a productivity of 3.65 g/L.h and a yield of 0.72 g/g lower than those obtained in the continuously fed-batch fermentations, using a feeding medium containing only date juice sugars and ammonium sulfate. These observed decreases could probably be due to a higher osmotic pressure created by the presence of high concentrations of salts in the fermentation medium or to salt actions. In fact, during fed-batch fermentation, the feeding medium of date juice supplemented by salts inevitably contributes to an increase of the osmotic pressure which may lead to a decrease of L-glutamic acid biosynthesis. Such a negative impact has been shown in the past [32, 33, 34]. The corresponding authors studied the influence of high medium osmolality on L-glutamic acid production. Guillouet [32] reported that the addition of a high concentration of NaCl (5M) to increase the medium osmolality resulted in lower specfic growth rates and reduced glucose to biomass conversion yields. The osmotic pressure above 2 osmol/kg is unfavourable to L-glutamic acid production with *C. glutamicum* ATCC 17965. Debay [33] studied also the effect of salt (NaCl) added to the feeding medium during fed-batch culture. The results obtained showed that high concentrations of NaCl disrupted the metabolism of *C. glutamicum*, deflecting the carbon stream towards the synthesis of high lactate concentrations. Gourdon et al. [34] reported that in medium in which the osmolarity was modified by the addition of NaCl, the specific rates of growth of *C. glutamicum* and sugar consuption decreased as a fuction of the osmolarity at values higher than 750 mosmol/kg. We could therefore suppose that the increased osmotic pressure could be involved in the presently observed decline of L-glutamic acid production during the fed-batch culture of *C. glutamicum* 2262. This could be also deepened in the future.

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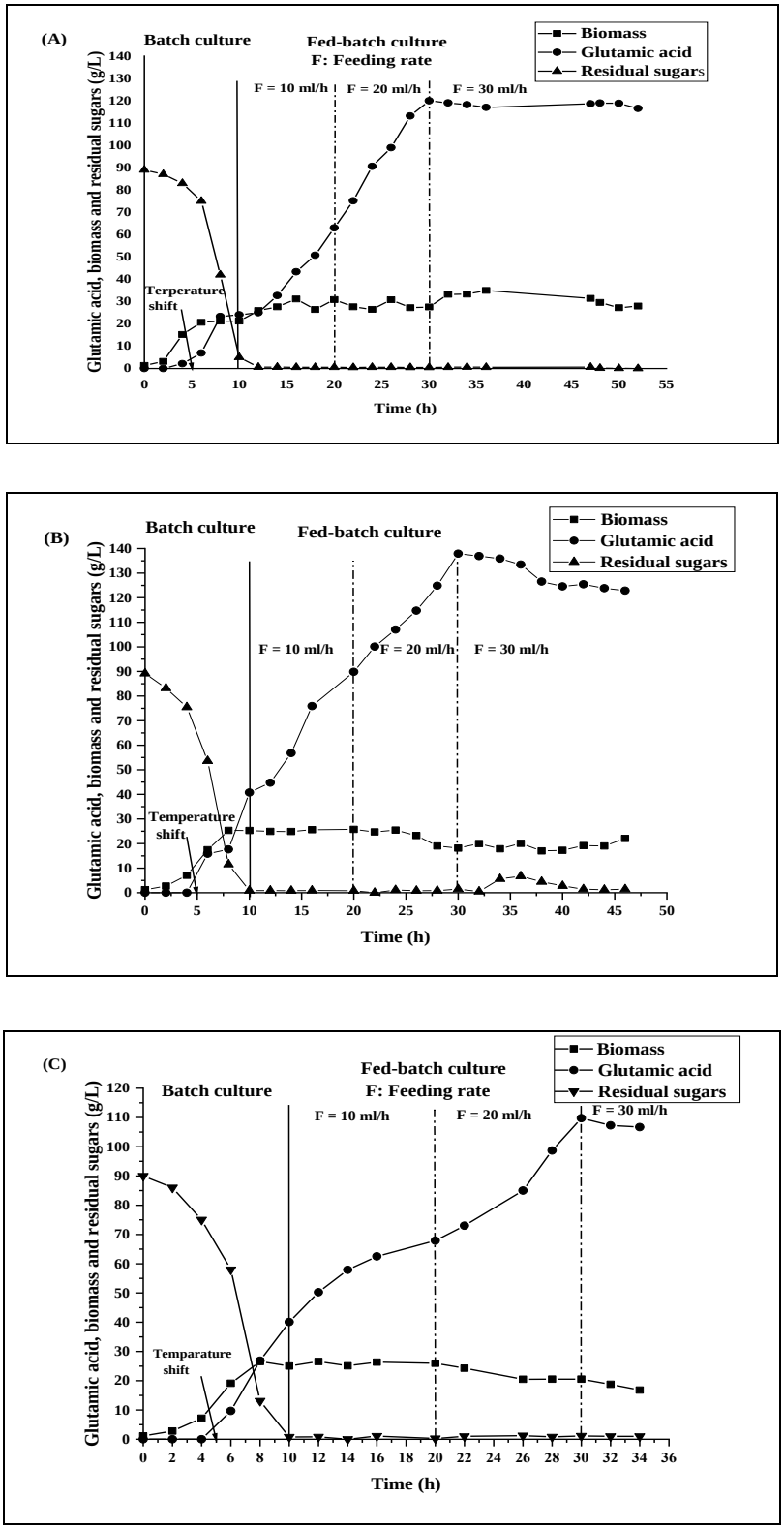

Figure 3. Kinetics of fed-batch cultivation of *C. glutamicum* 2262 using continuous feeding rate at rates (F) 10, 20 and 30 mL/h

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The arrow indicates the time when the temperature was changed from 33°C to 38.4°C.

(A): Fed-batch using continuous feed by feeding medium containing only date juice solution at the concentration of a total sugar of 210 g/L. (B): The same feeding medium but enriched with ammonium sulfate at a concentration of 16 g/L. (C): The same feeding medium with ammonium sulfate at the same concentration and minerals ($CaCl_2$, 0.01 g/L; KH_2PO_4, 2.5 g/L; K_2HPO_4, 2.5 g/L; $MgSO_4$, 0.6 g/L; and $MnSO_4$, 2.5 mg/L).

Conclusion

In the present work, batch and fed-batch fermentation for the production of L-glutamic acid using *C. glutamicum* 2262 on date juice with two different feeding modes, pulsed and continuous, were performed. The continuous feeding rate using a solution with date juice sugars and ammonium sulfate provided better results than pulsed feeding in fed-batch culture. This mode increased the L-glutamic acid production by approximately 1.17 fold compared to pulse feeding, and about 2.35 fold compared to batch culture. So the enrichment with ammonium sulfate allowed us to reach one final concentration of around 138 g/L which seems to be one of the highest L-glutamic acids obtained concentrations. This seems to be due to a particular property of the substrate date juice extracts allowing us to think that this last fact should be deepened in the future. Another point that could also deepen is the negative effects of added salts in the fermentation medium which are probably due to an osmotic pressure negative action.

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