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BATCH AND CONTINUOUS PRODUCTION OF LACTIC ACID USING *LACTOBACILLUS BULGARICUS* (ATCC 8001)

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ABSTRACT

In this work lactic acid was produced in batch and continuous fermentation processes. The objective of this work was to investigate the effects of initial substrate concentration (30-120 g/l) and hydraulic retention time (HRT: 10-40 h) on cell growth, lactose utilization and lactic acid production. Whey lactose was used in batch and continuous systems; obtained results were compared. Substrate and product inhibitions were investigated at high initial substrate concentration. The results of continuous system indicate that production of lactic acid has increased with an increase in HRT along with increasing substrate concentration. The optimum initial lactose concentration for maximal lactic acid production in batch and continuous fermentation were found to be 90 and 120 g/l, respectively. The maximum concentrations of lactic acid in batch and continuous cultures were 32.1 and 42.9 g/l, respectively. The results showed that optimal lactic acid production was obtained at HRT of 30 hours. Mathematical projection model and kinetic studies for lactic acid production in batch cultures were performed. It was found that initial lactose concentration of 60g/l resulted in maximum specific growth rate of $0.215h^{-1}$.

Keywords: Lactose; Lactic acid; Fermentation; *Lactobacillus bulgaricus;* Whey

INTRODUCTION

Generally, dairy industries generate large quantities effluents from milk processing for cheese production plants (Najafpour *et al.*, 2008). Disposal of whey is one of the major pollutants may cause oxygen depletion and destroy aquatic life. Each liter of whey contains about 50 g of lactose and 10 g of proteins with a high nutritional value for microbial growth which is a suitable raw material for lactic acid production (Panesar *et al*,. 2007). In order to avoid the environmental pollution caused by synthetic polymers, biodegradable plastic such as polylactate can replace the synthetic polymers. Therefore, lactic acid production should be economical and environmental friendly through utilization of dairy wastes (Wee *et al*,. 2006).

On the other hand lactic acid is widely used in industry such as an acidulant, flavor and preservative in food, pharmaceutical, leather and textile industries (Tango and Ghaly 2002). Lactic acid can be produced via chemical synthesis and microbial fermentation. In recent years, fermen-

tation process has become more appropriate for production of lactic acid while demand for naturally producing lactic acid has increased (Abdel-Rahman *et al,*. 2013, Kadam *et al.,* 2006). Moreover, the processing costs for fermentation processes may be lower than those for synthetic processes (Vodnar *et al,*. 2010).

Substrate for the lactic acid fermentation process can be chosen from a variety of carbon sources, such as glucose, xylose, sucrose or lactose (Guo *et al,*. 2010, Pagana *et al,*. 2013, Saito *et al,*. 2012, Tay and Yang 2002). The choice of substrate depends upon its availability, cost and pretreatment required for fermentation (Yen and Kang 2010).

There are several species of bacteria that are capable to produce lactic acid from lactose such as *L. bulgaricus* (Fakhravar *et al.,* 2012), *L. plantarum* (Liu *et al.,* 2010), and *L. casei* (Khiralla *et al.,* 2009, Korbekandi *et al.,* 2007). Fermentation processes can be carried out in batch, fed batch and continuous modes (Roukas and Kotzekidou 1996, Son and Kwon 2013, Zhang *et al.,* 2011). However, each of these processes has some advantages and disadvantages (Chotisubha-anandha *et al.,* 2011). Selection of fermentation processes may vary with respect to type and nature of the substrate, microbial growth, and fermentative media (Abdel-Rahman *et al.,* 2013).

In batch fermentation, the kinetic model provides information to predict the rate of cell mass or product formation (Shafiq *et al.,* 2004). A process might be developed on a trial and error basis but that is an extremely costly approach both in terms of time and equipment. A more profitable approach is to use mathematical modeling for the process (Choi *et al.,* 2013). Then one can examine the consequences of changing parameters without any expenses of running costly experiments (Mercier *et al,*. 1992).

The purpose of present work is to investigate the effects of initial substrate concentration and hydraulic retention time on cell growth, lactose utilization and lactic acid production from whey lactose using continuous system and batch cultures. Since high initial substrate may create inhibition, the extreme points were identified and data for batch and continuous processes were compared. Moreover, the kinetic parameters in batch culture were evaluated for microbial growth, substrate utilization and lactic acid production.

MATERIALS AND METHODS

Microorganism and Medium: The selected microorganism in this study was *Lactobacillus bulgaricus* (ATCC 8001). This strain was obtained from Iranian Research Organization Science and Technology (IROST). Man-Rogosa -Sharpe (MRS) medium was used for cultivation of lactobacilli bacteria. The composition of MRS medium is as follows: yeast extract, 5 g; meat extract, 5 g; peptone, 10 g; K_2HPO_4, 2g; diammonium citrate, 5 g; glucose,

20g; sodium acetate, 2 g; $MgSO_4 \cdot 7H_2O$, 0.58 g; $MnSO_4 \cdot 4H_2O$, 0.2 g, in 1 liter medium. The media were sterilized at 121 ^oC for 15 minutes before inoculation.

Substrate preparation: Cheese whey was obtained from a dairy plant (Kalle, Mazandaran, Iran). The whey was first filtrated in order to separate the coagulated proteins. Then lactose in presence of dilute acid was hydrolyzed to galactose and glucose (1 ml HCl in 100 ml whey). After 24 h, the whey was neutralized with 1 M NaOH solution. The pH of pretreated whey was adjusted to 6.5. A 0.3% (w/v) yeast extract was added to whey. Deproteinated and hydrolyzed whey was prepared as suitable media for fermentation.

Experimental set-up: The bioreactor was fabricated from a cylindrical stainless steel column with an internal diameter of 114 mm and total height of 250 mm. A temperature sensor is embedded inside the reactor. This sensor is connected to a PID controller and the reactor temperature was adjusted via a heating jacket which is wrapped external surface area of the reactor. The fermentation experiments were conducted without pH control. The bioreactor sterilized with sodium hypochlorite solution (NaOCl: 200 ppm) for 60 min and washed with hot distilled water. The experimental set up used for the continuous production of lactic acid is illustrated in Fig. 1. The temperature of bioreactor was fixed at 42°C. The agitation rate for uniform mixing was 180 rpm and initial pH was 6.5.

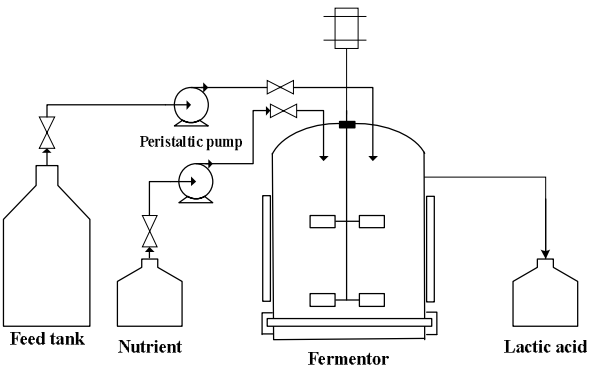

Figure-1: Schematic diagram of the laboratory-scale bioreactor for lactic acid production

In continuous culture, after 12 hours of incubation, when the cell concentration has reached to stable condition, the fresh medium was fed into the bioreactor with defined flow rate. The system was allowed to come to a steady state condition. Samples of the culture were taken in interval 6 hours till to confirm whether the steady state has achieved.

When the cell, lactose and lactic acid concentrations remained constant, steady state condition was performed. As the steady state condition was identified, a new flow rate was assigned. The HRTs of 5 to 40 h were based on working volume of the bioreactor, which were calculated by the fresh feed flow rates from 40-315 ml/h.

Analytical methods: Lactic acid and lactose concentrations were measured by HPLC (Shimadzu-Japan) equipped with a Shim-pack CLC-ODS column. The column, maintained at 75°C, was eluted with 4 mM H_2SO_4 at a flow rate of 0.4 ml/min for 20 min. The retention time of lactic acid under these conditions was 18 min. The samples were centrifuged at 5000 rpm for 5 minutes and then filtered through 0.2µm paper filter (Whatman). To obtain desired peak height, sample size of 10µl of the clear solution were injected to HPLC.

Growth rates were monitored by measuring the absorbance at 620 nm using a Unico 2100 spectrophotometer. The dry cell weight was measured through the calibration curve of dry cell weight concentration versus optical density.

RESULTS AND DISCUSSION

Batch fermentation: The effects of initial lactose concentration on microbial cell population and lactic acid production were investigated. Figure-2 shows biomass of *L. bulgaricus* in batch fermentation process using lactose as carbon source. The range of lactose concentration was 30-120 g/l. The data indicate that the cell dry weight concentration is related to substrate concentration. High substrate concentrations and product formed may cause growth inhibition; as the microorganisms may be intoxicated in an undesired condition. With initial lactose concentration 90g/l, maximum dry cell weight of 5.0 g/l was obtained. For the substrate concentration increased to 120g/l the concentration of biomass decreased to 3.2 g/l; this proved that substrate inhibition existed.

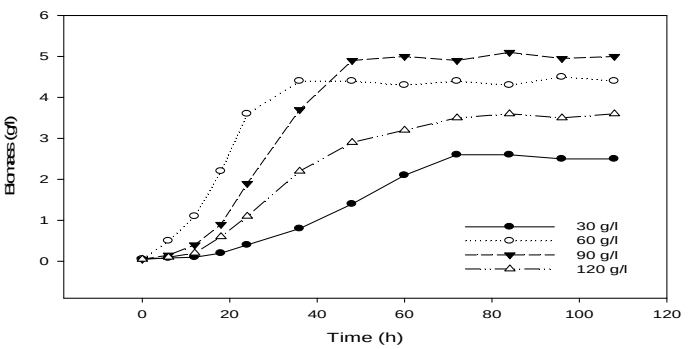

Figure-2: Effect of initial substrate concentration on biomass concentration

The effect of initial lactose concentration on product formation is shown in Fig. 3. The highest concentration of lactic acid obtained at 90 g/l lactose concentration was 32.1g/l. The yield of product formation was 44.1%. When the concentration of lactose increased, the lactic acid production and yield decreased, that was due to inhibitions by high substrate and product concentrations. At high substrate concentration of 120 g/l, the concentration of lactic acid dropped to 14.9 g/l that was due to existing inhibitions in batch process.

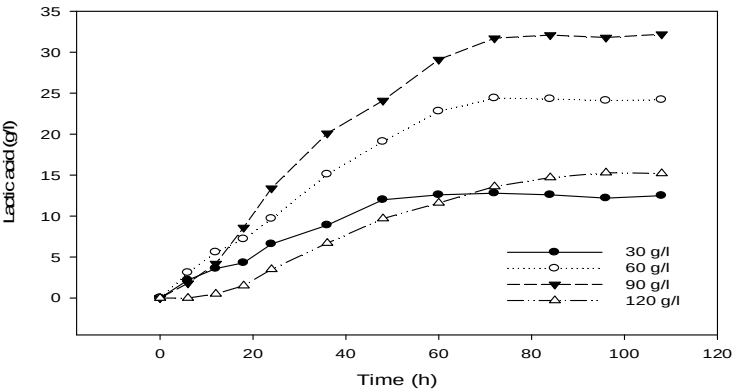

Figure-3: Effect of initial substrate concentration on lactic acid production

In comparison to similar work conducted by other investigators, the reported concentration of lactic acid at controlled pH was slightly higher than data of present work (Büyükkileci and Harsa 2004, Chiarini *et al*,. 1992). When pH was uncontrolled, the pH dropped to value lower than 3. This condition is unfavorable for the growth activity of the fermentative lactic bacteria and the microorganism was unable to fully utilize the substrate.

Fig.-4 demonstrates utilization of various concentration of lactose as substrate. It appears that the consumption of lactose has decreased when the initial lactose concentration was increased. In the course of fermentation, with initial lactose concentration of 30 g/l, 88.2% of the substrate was consumed. At initial lactose concentration of 120 g/l, consumption of lactose dropped to 50.9%.

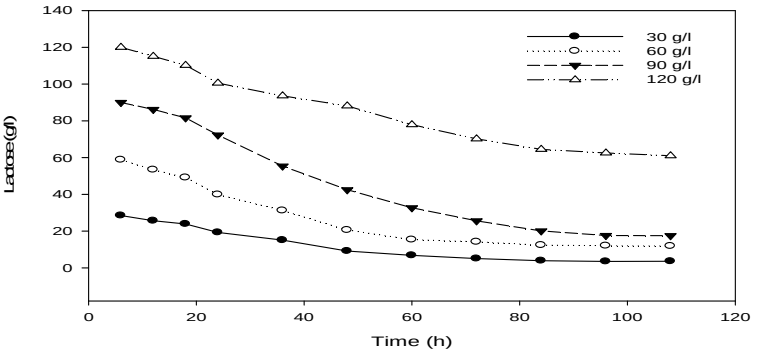

Figure-4: Effect of initial substrate concentration on lactose utilization

The yield of lactic acid production in batch fermentation was summarized in Table 1. The results indicate that maximum yield of lactic acid production is 50.9% with initial lactose concentration of 60 g/l. The obtained yield was 72.3% of the theoretical yield of lactic acid production.

Table-1: Yield of lactic acid at various initial lactose concentrations in batch fermentation

Initial lactose concentration (g/l)	Yield (%)
30	47.1
60	50.9
90	43.2
120	26.4

Logistic model described the *lactobacillus bulgaricus* growth kinetic. The logistic equation leads to define lag phase, an exponential and stationary phases. The specific growth rate predicted by the model is explained by the following equation:

$$\frac{dx}{dt} = \mu_{max} x\left(1 - \frac{x}{x_{max}}\right) \quad (1)$$

Where μ_{max} maximum specific growth is rate (h^{-1}) and x_{max} is the maximum cell dry weight concentration (g/l). The above expression is known as the Riccati equation, which can be easily integrated to give the logistic equation as follows:

$$x = \frac{x_0 \exp(\mu_{max} t)}{1 - \left(\frac{x_0}{x_{max}}\right)(1 - \exp(\mu_{max} t))} \quad (2)$$

Matlab software was applied to predict the logistic growth kinetic parameters of the culture. The kinetic parameters are summarized in Table 2. The high coefficient of determination (R^2) shows the high accuracy and capability of the model to interpret the experimental data. According to Table 2, initial lactose concentration of 60g/l has the highest specific growth rate of 0.215 h^{-1}.

Table-2: Kinetic parameters of logistic model for *L. bulgaricus* growth at different initial lactose concentration

Initial lactose concentration, g/l	$x_{max}\ (g/l)$	$\mu_{max}\ (1/h)$	R^2
30	2.68	0.072	0.986
60	4.4	0.215	0.997
90	5.02	0.139	0.998
120	3.5	0.110	0.974

Continuous fermentation: The batch fermentation results show the existence of inhibitory effects on growth due to high initial lactose concentration. To reduce substrate inhibition, continuous fermentation was used to maintain high substrate consumption while keeping the level of inhibition low by feeding fresh nutrients to the fermentation broth. In continuous fermentation hydraulic retention time (HRT) is an important factor, which must be taken into consideration for achieving maximum yields of lactic acid production. This parameter was investigated in our research via evaluating lactic acid production at several HRT (5-40 h). The bioreactor was cultured with *L. bulgaricus.* It was continuously operated for duration of 23 days without any interruption. The effect of hydraulic retention time (HRT) on the cell concentration, lactic acid production and lactose consumption at several initial lactose concentrations was studied. Figure-5 showed the effect of HRT on the cell concentration at various initial lactose concentrations. It is clear that the maximum concentration of cell dry weight increased with an increase HRT for all initial lactose concentrations. The maximum concentration of dry cell weight obtained at initial lactose concentration 90 g/l and HRT 30 h was 5.0 g/l. In continuous fermentation, at low initial lactose concentration no inhibitory effect was observed. However, at high initial lactose concentration (150g/l) strong inhibition effect was identified (see Fig. 5). Even in continuous process the inhibition effect shifted substrate

concentration to higher than concentration reported in the batch bioreactor (lactose concentration shifted from 90 to 120 g/l).

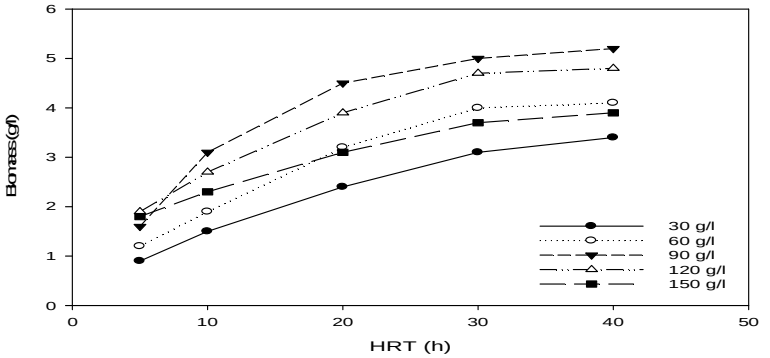

Figure-5: The effect of HRT on the cell concentration

The effect of the HRTs on lactic acid production at various initial lactose concentrations is shown in Fig. 6. It is indicated that the lactic acid concentration increased when the HRT increased. The maximum lactic acid concentration with initial lactose concentration of 120 g/l at HRT 30 h was 42.9 g/l. There was no significant increase in lactic acid production for the HRT of greater than 30 h. The relatively low concentration of lactic acid was due to the fact that the pH of the medium decreased from 6.5 to 2.8. Therefore, the activity of the microorganism at acidic condition drastically dropped; that caused substrate utilization reduction. If the pH of the system to be controlled then rate of lactic acid production definitely increase. Unfortunately, the deficiency of the fabricated bioreactor was uncontrolled pH of the media.

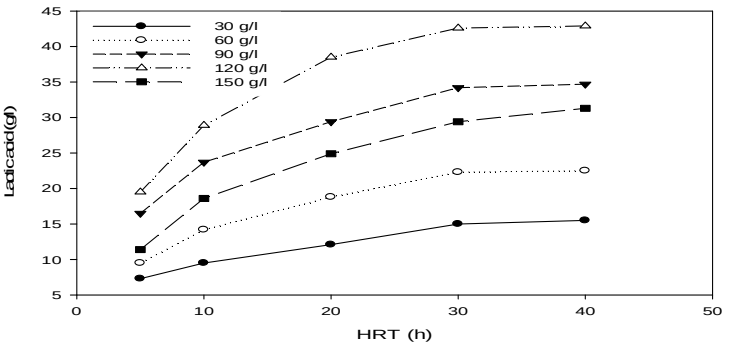

Figure-6: The effect of HRT on the lactic acid production

Substrate consumption with respect of HRT is shown in Fig. 7. It appears that the consumption of lactose at all initial lactose concentrations decreased with an increase in HRT. However the sugar was not completely utilized even after 40 hours. At HRT greater than 30 the concentration of substrate in fermentation media has reached to constant level. This means that no more substrate was utilize for HRT of greater than 30 h.

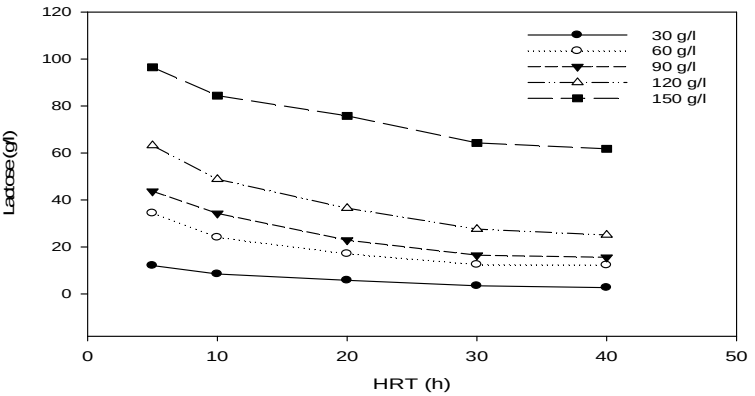

Figure-7: The effect of HRT on the lactose utilization

Lactic acid yield at several HRTs and initial lactose concentrations are summarized in Table 3. Lactic acid yield has increased with an increase of HRT for all initial lactose concentrations. This indicates that the conversion rate of lactose to lactic acid was more efficient than at a high residence time of lactic acid formation. The maximum lactic acid yield of 56.8% was obtained with initial lactose concentration of 30 g/l. The yield of HRT at greater than 30 h was initially constant; then the value dropped.

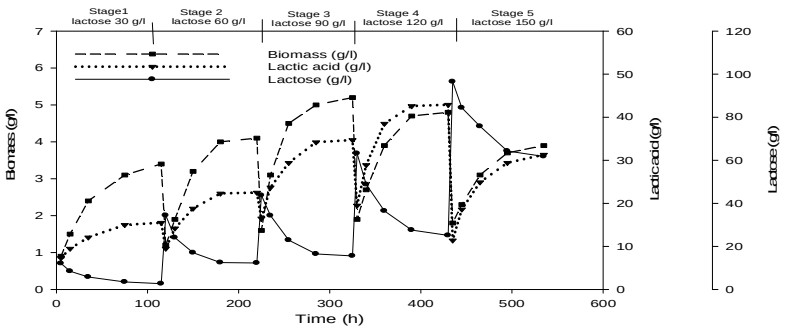

Figure-8: The performance of continuous fermentation at various initial lactose concentrations

Table- 3: The lactic acid yield at different HRTs and initial lactose concentrations

Initial lactose concentration (g/l)	HRT (h)	Yield (%)
30	5	40.7
	10	44.2
	20	49.7
	30	56.6
	40	56.7
60	5	37.1
	10	39.5
	20	43.7
	30	46.8
	40	47.1
90	5	35.6
	10	42.5
	20	43.8
	30	46.5
	40	46.6
120	5	34.3
	10	40.6
	20	46.1
	30	46.2
	40	45.7
150	5	21.3
	10	28.3
	20	33.5
	30	34.8
	40	35.4

The performances of CSTR in continuous mode of operation for duration 23 days are shown in Fig. 8. The sudden changes are due to shifting operation variables or replacing fresh media. The bioreactor in overall demonstrated quite stable and successful for production of lactic acid.

CONCLUSIONS

The effect of HRT and initial lactose concentration on the performance of lactic acid fermentation from lactose with uncontrolled pH was investigated. The lactic acid concentration has increased as the HRT and initial lactose concentration increased. The batch fermentation results showed the existence of an inhibitory effect on growth due to high initial lactose concentration. For instance, an increase in lactose concentration from 90 to 120g/l led to decrease in concentration of biomass and lactic acid. To reduce substrate inhibition, continuous fermentation was applied to maintain the substrate concentration at a low level by feeding fresh nutrients to the fermentation broth. The data for batch and continuous fermentation were compared. The results of continuous fermentation showed that lactic acid production has increased with an

increase in HRT. The slightly low concentration of lactic acid obtained in present work was due to the fact that the pH of the medium drastically decreased. In order to avoid decrease in lactic acid production, the process required to control pH of fermentation media.

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