Optimization of cell mass production of the probiotic strain *Lactococcus lactis* in batch and fed-bach culture in pilot scale levels

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Lactococcus lactis is highly efficient probiotics microorganism with wide range of benefits on human health. This study was conducted to design and establish industrial platform for high cell density cultivation of this novel probiotic strain, *L. lactis* (WICC-B25). During bioreactor cultivation of lactic acid bacteria in industrial scale, there are two main problems during process namely: low cell growth rate and high lactate production. These both together resulted in low cell mass production. Therefore, this study was focused on cultivation parameters optimization for high cell mass production with minimal lactate formation in shake flask and bioreactor levels. Cell growth was studied in 16-L stirred tank bioreactor under controlled and uncontrolled pH conditions. The maximal cell mass of about 5.6 g L^{-1} was obtained after 14 h cultivations under uncontrolled pH condition. This value was almost 35% higher than those cell mass obtained in pH controlled culture. Based on this data, fed-batch cultivation strategy was developed using mixed substrate feeding solution (containing all medium component) and mono-feeding (using glucose alone). For both cultures, fed was adjusted to constant rate of 3 g L^{-1} h^{-1} and, DO was cascaded to agitation speed to prevent the drop of DO to value less than 30% saturation as oxygen is limited factor for high cell mass production. Culture of mono-glucose feeding yielded the maximal cell mass of about 20 g L^{-1} after 30 h. On the other hand, the cell mass obtained in mixed substrate feeding culture was only 8.4 g L^{-1}. Thus, we can conclude that fed-batch cultivation under uncontrolled pH, mono-glucose feeding and DO stat of 30% is suitable cultivation strategy for high cell mass production of *L. lactis* in bioprocess industry.

Keywords. Probiotics, *Lactobacillus lactis*, fed-batch culture, cell mass production

1. Introduction

During the last yeast, increasing interest have been observed on the role of natural products to promote human wellness and protection against the increased number of diseases. Among these natural substances, use of living microorganisms (probiotics) for human nutrition as effective nutraceuticals are widely used. Probiotic foods are a group of health promoting food or (natural foods) of large and increased world wide market [1-3]. Probiotics have been defined as a preparation of or a product containing viable, defined micro-organisms in sufficient number, which alter the microbiota by implantation or colonization in a compartment of the host and by that exert beneficial health effects in this host [4,5]. However, the principle of using harmless living microorganisms to prevent disease, promote longecity and well being has long history as it was first proposed by Metchnikoff in the early 20th century [6]. Nowadays, microorganisms of different groups are widely used in many probiotic application as single strains or in combination. However, bacteria belong to *Lactobacilli* and *Bifidobacteria* groups are typical found in many probiotic products for human and animal use. Non-bacterial probiotics such as *Saccharomyces boulardii* are also other microorganisms of interest and used based on their probiotic and biotherapeutic activities against many diseases [7,8]. However, most of commercially used probiotic products include different strains of *Lactobacilli* such as *L. acidophilus, L. casei*, *L. plantarum* and *Lactococcus lactis*. It have been shown that *Lactobacillus* strains in general shows probiotic activity and health benefit when used in certain number in the range of 10^7-10^9 cfu day^{-1} [9,10]. This based on the fact that large number of applied microorganisms will be deactivated during the passage through the gastrointestinal tract (GIT) due to high acidity and bile salt lethal effect. Thus, to develop effective probiotic product, large number of cells are required for each dose. Therefore, studies on the growth kinetics and cultivations of probiotic strains in high cell mass is of growing interest for many fermentation industries who produce living probiotic cells and starter culture. Of different lactic acid bacteria, *L. lactis* is of high interest and main ingredient for many probiotic formulas. This strain is widely used for the production of buttermilk and different types of cheese as well. This bacteria belong to Gram-negative and non-spore former group. and grow usually in pair or short chain. Physiologically, they belong to homo-fermentative producing mainly lactic acid in culture. Other interesting feature of this microorganism is also the ability for nisin production in reasonable concentrations [11,12]. The present work was focused on bioprocess optimization for high cell mass production of *L. lactis* for probiotic application in semi-industrial scale in batch and fed-batch cultures using different feeding strategies.

2. Materials and Methods

2.1. Microorganism

Lactococcus lactis (WICC B-25) was obtained from Wellness Industries Culture Collection (WICC), Chemical Engineering Pilot Plant, UTM, Malaysia. This strain was delivered in frozen glycerol solution. Cells were first propagated on MRS agar medium, incubated at 37°C for 24 h. The arisen colonies were harvest by glycerol solution (50%) and put in series of 2 ml. Cryogen tubes. These tubes were frozen immediately at -20°C for 24 hours followed by further storage as working cell bank at -80°C for further use.

2.2. Inoculum preparation

For each experiment, 1 crygenic vial from working cell bank (containing 1 ml) was taken and used to inoculate 250 ml Erlenmeyer flask containing 50 ml MRS broth (Difco Laboratoreis, MI, USA). The inoculated flasks were incubated on rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 37°C for 24 h. The grown cells were used to inoculate either shake flask or bioreactor containing cell production medium.

2.3. Medium for cell mass production

The medium used in this study for biomass production was optimized previously in our laboratories and composed of [g L^{-1}]: yeast extract, 6.0; peptone, 6.0; KH_2PO_4, 1.5; ammonium citrate, 1.0; tween 80, 1.0; sodium acetate, 1.0; citric acid, 0.5; $MgSO_4.7H_2O$, 0.4 and $MnSO_4.7H_2O$, 0.05. The pH was adjusted to 6.8 before sterilization. Glucose in concentration of (30 g L^{-1}) was sterilized separately and added to medium before inoculation.

2.4. Cultivation conditions

For shake flask experiments, cultivations were carried out using 250 ml Erlenmeyer flasks containing 50 ml liquid medium. Inoculum was in form of 24 h old vegetative culture as described previously and used to inoculate the production medium to give final cell concentration of 0.1 OD_{600}. The inoculated flasks were incubated at 37°C on rotary shaker at 200 rpm for 24 hours. In case of bioreactor experiments, cultivations were conducted in 16-L pilot scale *in situ* sterilizable bioreactor with working volume of 8-L. The stirrer of the bioreactor was equipped with two-4 bladded rushton turbines. Unless otherwise mentioned, temperature and agitation speed were adjusted at 37°C and 200 rpm, respectively, throughout the experiment. Aeration was performed by sterile air and adjusted to rate of 1.0 (v v^{-1} min^{-1}) using integrated thermal mass flow controller. pH was measure on-line using *in situ* sterilizable pH electrode (Mittler Toledo, Switzerland) and controlled at constant value, in case of pH controlled culture, by addition of 4 M NaOH and 2 M HCl solutions, connected to acid/base controller equipped with peristaltic pumps. Dissolved oxygen concentration was first adjusted to 100% saturation before inoculation and measured using polarigraphic DO electrode (Mittler Toledo, Switzerland). In case of fed-batch cultures, the DO was cascaded to the agitation speed and aeration and set at 30% saturation. Antifoam reagent (Silicon antifoam, Sigma, USA) was added to suppress foaming during the experiment.

2.5. Sample preparation and cell dry weight determination

Samples in form of 3 flasks of 50 ml broth for each in case of shake flask or 30 ml in case of bioreactor cultivations were taken at different interval times during cultivations. Immediately after sampling, optical density was measured at 600 nm using spectrophotometer (DR/2500, Hach Co., Loveland, CO, USA). Cell dry weight was also determined after drying the cells in oven 1t 100°C for constant weight. One OD_{600} unit was equivalent to 0.29 g L^{-1}. The rest of sample was centrifuged immediately at 5000 rpm. The supernatant was taken and filtered using microbiological filter (0.22 μm, Ministar, Sartorius, Göttingen, Germany) and kept in deep freeze at -20 °C for further analysis.

2.6. Glucose and lactic acid determination

Glucose and lactic acid were determined using High Performance Liquid Chromatography (Waters, Milford, MA, USA). Lactic acid was determination using UV detector (996 PDA, Water) at 210 nm and organic acid column (250 mm × 4.6 mm ID spherisorb Octyl type column). The adsorbed substances were eluted with 0.5 M H_3PO_4 at flow rate of 0.5 ml min^{-1} at 40°C. For glucose determination, the same equipment was also used equipped with a carbohydrate separation column (250 mm × 4.6 mm ID μ Bondapak) and using IR detector (2410 Water). The mobile phase in case of glucose was acetonitrile:water (80:20) at a flow rate of 1.0 ml min^{-1} at 85°C. This equipment was connected with computer and software (Empower Software) for calculation of concentrations.

3. Results and Discussion

3.1 Kinetics of cell growth and lactate production in shake flask culture

Cultivation of *L. lactis* was carried out using the optimized cultivation medium and conditions in shake flask level to investigate the kinetics of cell growth, glucose consumption, lactate production and change of pH in batch culture. As shown in figure 1, cells grew exponentially with growth rate of 0.188 g L^{-1} h^{-1} and reached its maximal value of 2.1 g L^{-1} after 12 hours. As cells entered the stationary phase, cell mass decreased gradually with time in very low rate and reached 1.8 g L^{-1} at the end of cultivation time. This strong exponential growth without significant lag phase was based on the high inoculum quality, rich nutrients inside the production medium and the optimal cultivation conditions. This was also attributed to the cultivation of cells at 37°C rather than lower temperature as many other authors do. It have been also observed that for many *Lactococcus* and *Lactobacillus* species of probiotic activities, cells grew exponentially without lag-phase when cultivated at 37°C [13,14]

During the cell growth phase, glucose concentration decreased gradually in culture with rate of 2.44 g L^{-1} h^{-1}. However, during cell growth phase, lactate accumulated in culture concomitantly with cell growth without any lag phase. As shown, lactate accumulated in culture with rate of 0.3 g L^{-1} h^{-1} reaching its maximal concentration of 4.3 g L^{-1} after 14 hours. After that time, lactate concentration kept almost the same for the rest of cultivation time. Based on the production of acid in culture it has been observed that the culture pH was dropped accordingly. The initial pH of culture was adjusted to 6.5 and decreased gradually during with the cultivation time. The decrease in pH value was significantly observed during the exponential growth phase and lactic acid production phase. This result directly indicated that once glucose was limited in culture, growth phase (log phase) was terminated and cells entered the stationary phase. This gives direct indication that glucose is the limiting substrate in shake flask culture for *L. lactis* cultivation. However, it have been reported by other authors that *L. lactis* grow optimally at pH 6.5 and growth arrested only at around pH 4 [15].
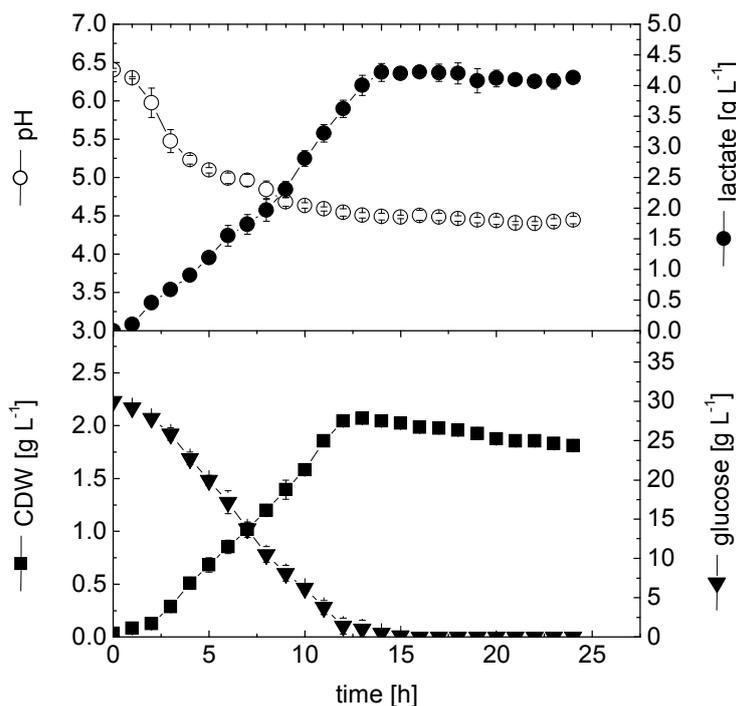

Fig. 1. Cell growth, glucose consumption, lactate production and change in pH during *L. lactis* cultivation in shake flask culture.

3.2 Kinetics of cell growth and lactate production in bioreactor cultures under controlled and uncontrolled pH conditions

The aim of this experiment was to evaluate the effect of pH control on the kinetics of cell growth and cellular metabolism in regards to glucose consumption and lactate production. Cultivations were conducted in 16-L *in situ* sterilizable stirred tank bioreactor with working volume of 8-L. This type of bioreactor provides same aeration and agitation mode of those applied in large scale. Cultivations were carried out using the same medium composition and inoculum size used in shake flask. Figures 2 and 3 represent the data of cell cultivations under uncontrolled and controlled pH conditions, respectively. As shown, cells grew exponentially in both cultures with different rates without

any significant lag phase. However, uncontrolled pH condition was more favourable for cell mass production. The cell specific growth rates were 0.22 h^{-1} and 0.15 h^{-1} for uncontrolled and controlled pH cultures, respectively. In culture of uncontrolled pH, the maximal cell mass obtained was 5.64 g L^{-1} after 14 h. This value was almost 35% higher than those obtained in controlled pH culture. These differences in growth rate and cell yields is linked to the rate of glucose consumption, which also reflects the cell physiological status and activity. In case of uncontrolled pH culture, glucose concentration decreased gradually with consumption rate of 2.55 g L^{-1} h^{-1} this value was almost 66% higher than those obtained in controlled pH culture. On the other hand, both cultures produced more lactate compared to shake flask culture. The rates of lactate production were 0.33 g L^{-1} h^{-1} and 0.21 g L^{-1} h^{-1} in uncontrolled and controlled pH cultures, respectively.

In case of uncontrolled pH culture (figure 2), the pH was slightly increased during the first 2 hours of cultivation reaching 6.9 and dropped significantly during the exponential growth phase and reached about pH 4.0 as cell entered its stationary phase and kept more or less constant for the rest of cultivation time. These data all together show that controlling the pH of culture decreased not only cell mass but also other metabolic activities of glucose consumption and lactate production. It have been reported that although *L. lactis* is able to survive at low pH, glycolysis is strongly affected at pH values below 5, showing reduced rate of glucose consumption [13]. However, this negative effect was not obvious as glucose was fully consumed when cells entered the exponential phase after only 14 h.

In technical point of view, cultivation of cells under uncontrolled pH conditions not only ease in monitoring and control but also the obtained cells will be more adapted to acidity (as pH dropped to about 4 during the growth phase). This will and ease their future application as probiotic bacteria should possess some acid resistance. It have been reported by other authors that cells grow under acidic are usually adapted acid tolerance through accumulation of some acid stress proteins inside the cells [16].
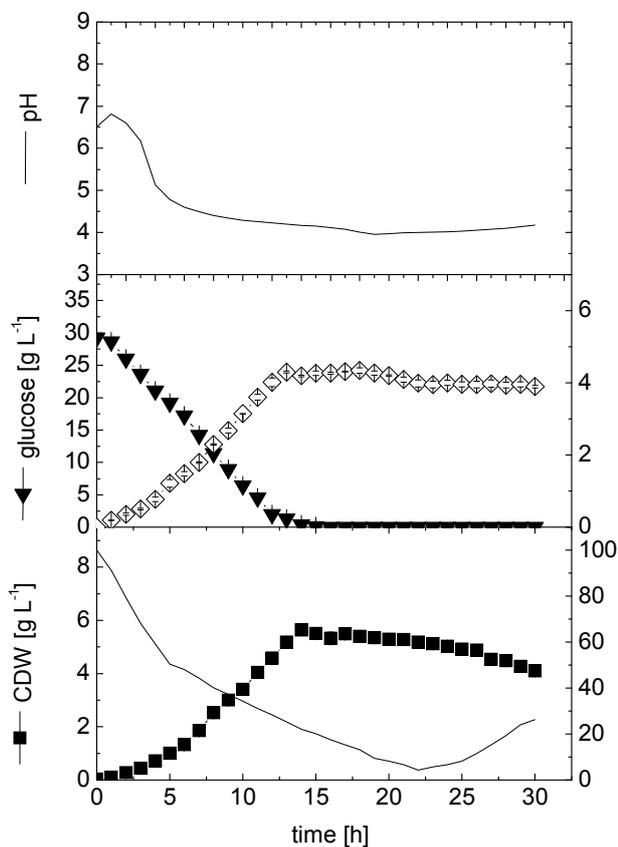

Fig. 2 Cell growth and change of different medium components during cell cultivation in bioreactor at uncontrolled pH
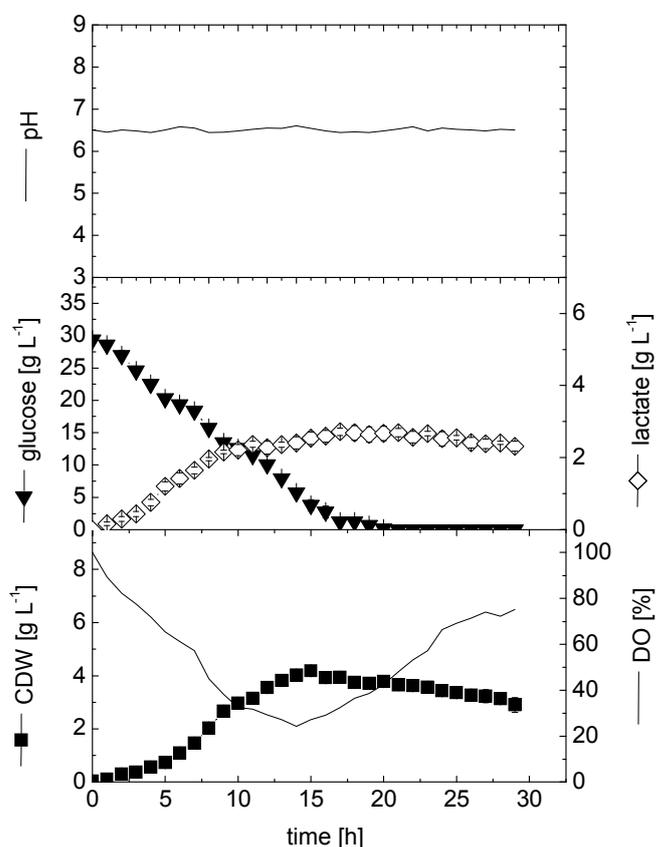
Fig. 3 Cell growth and change of different medium components during cell cultivation in bioreactor under controlled pH

3.3 Fed-batch cultivations of L. lactis using different feeding solutions.

Based on data obtained from the previous experiments, fed-batch cultivations were conducted without pH control of culture. Fed-batch cultivations were designed to improve cell mass through elimination glucose limitation during the cultivation process by two different substrates feeding strategies. In the first strategy, feeding was carried out after 10 hours using ten times (×10) concentrated solution of medium components and added to the bioreactor using peristaltic

pump with rate of 3 g L^{-1} h^{-1} (based on glucose concentration). This feeding rate was based on the data of glucose consumption rate which obtained in the previous experiment. During fed-batch phase, both of agitation speed and air-flow were cascaded with DO value and increased gradually during cultivation to keep DO value higher than 30% saturation. This strategy was performed to prevents oxygen limitation which occur as function of high cell mass production. The agitation upper and lower values of the cascade were, 200 and 800 rpm, respectively. Whereas, aeration range was between 1.0 and 2.5 v v^{-1} min^{-1}. Cultivation of *L. lactis* in fed-batch culture was studied before by many authors. The main target for fed-batch cultivation in other studies was to improve the production process for primary or secondary metabolites such as lactic acid [17] and nisin [18,19]. The aim of the present experiment was to set fed-batch cultivation strategy suitable for high cell mass production using previously optimized medium which promotes *Lactobacillus* cell growth with minimal lactic acid production.
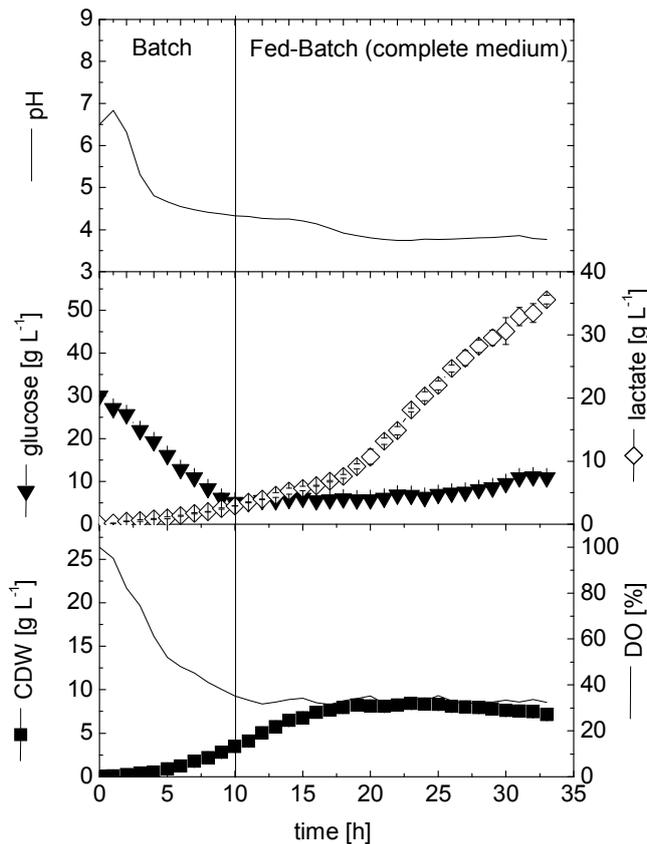

Fig. 4 Kinetics of cell growth, glucose consumption, lactate production and change in cultivation parameters during cultivation in fed-batch culture (fed by complete medium components)
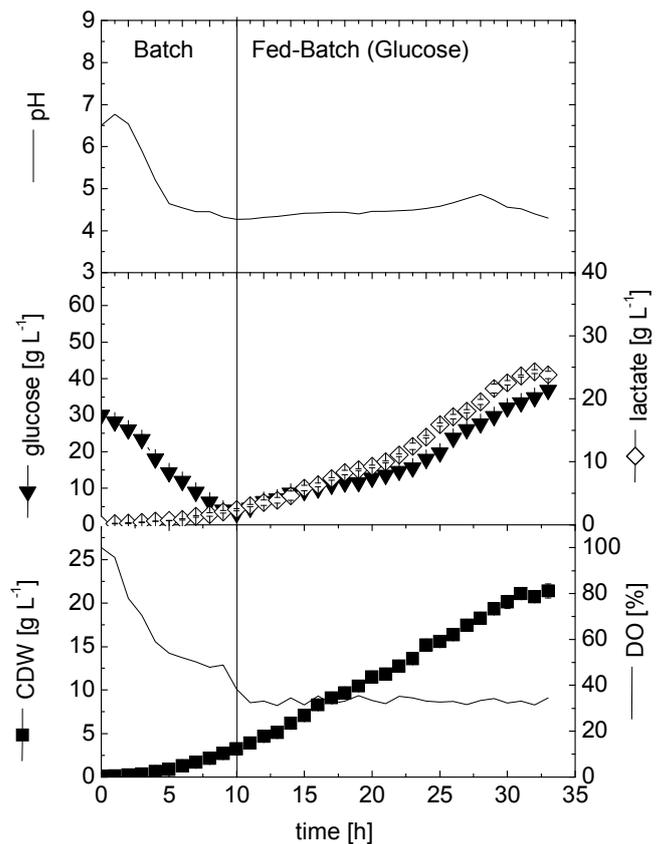
Fig. 5 Kinetics of cell growth, glucose consumption, lactate production and change in cultivation parameters during cultivation in fed-batch culture (mono-feeding by glucose)

On comparing the kinetics of cell growth in culture fed either by complete medium or by glucose only (figures 4,5 and Table 1). It is clear that cell growth in culture of mono-glucose feeding was 2.5 folds higher than other culture fed by complete medium components. This was irrespective to that the growth rate in both cultures was the same during the early phase of feeding. Cells grew exponentially in both cultures with the same specific growth rate of 0.1 h^{-1} during the first 6 hours feeding. After that time, cells cease to grow in culture fed with complete medium and kept more or less the same for the rest of cultivation time. Whereas, cells grew exponentially in the other culture upto 30 hours.

On the other hand, lactate production was higher in culture fed with complete medium. As shown in figures 4 and 5, the rate of lactate production in complete medium fed culture was 1.49 g $L^{-1}h^{-1}$ compared to only 0.98 g L^{-1} h^{-1} in case of glucose-fed culture. Since lactate production was continuously increased during the fed-batch phase in both cultures, the final concentration of lactate reached about 35.6 g L^{-1} in complete medium fed culture. This value was almost 48 % higher than those obtained in glucose fed culture. The obtained cell mass in this study was higher than those obtained by Bai *et al.* [17] who used glucose as feeding substrate. In their study the maximal cell mass was only 2.5 g L^{-1} and lactic acid production more than 200 g L^{-1}. The difference between our results with those obtained by Bai *et al*. is mainly due to the different medium formulation used in our study which enhances cell growth on the cost of lactate production. This is also in agreement with the other authors who reported that medium composition is the main factor which control the process direction toward lactic acid production or vigorous biomass growth [20].

Conclusion

In summary, kinetic data for all experiments conducted in this study are summarized in table (1). On comparing these results together we can conclude that on scaling up the process from shake flask to bioreactor level, the biomass production was improved by 169% accompanied by the increase of lactic acid production by only 78%. Further increase in biomass production was achieved using fed-batch cultivation strategy of mono-glucose feeding. This yielded almost 10 folds increase in cell mass compared to those obtained in shake flask batch culture.

Table 1 Kinetic parameters of cell growth, glucose consumption and lactate production during different modes of submerged cultivations of *L. lactis* in shake flask and in bioreactor.

Parameter	Shake flask	Bioreactor			
		Batch cultivation		Fed-batch cultivation	
		Uncontrolled pH	Controlled pH	Full medium feeding	Glucose feeding
X_{max} [g L^{-1}]	2.1	5.64	4.20	8.41	21.34
dx/dt [g L^{-1} h^{-1}]	0.187	0.58	0.30	0.25	0.85
μ [h^{-1}]	0.20	0.22	0.15	0.10	0.10
$-Q_{gluc}$ [g L^{-1} h^{-1}]	2.44	2.55	1.54	2.3 (batch)	2.3 (batch)
$Lact_{max}$ [g L^{-1}]	2.44	4.34	2.70	35.60	24.1
Q_{lact} [g L^{-1} h^{-1}]	0.31	0.33	0.21	1.49	0.98

X_{max}: maximal cell dry weight; dx/dt: growth rate; μ: specific growth rate; $-Q_{gluc}$: glucose consumption rate; $Lact_{max}$: maximal lactate production; Q_{lact}: lactate production rate.

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