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Research Article

Production of Alginate by *Azotobacter vinelandii* in semi-industrial scale using batch and fed-batch cultivation systems

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ABSTRACT

The alginate production by *Azotobacter vinelandii* in batch and fed-batch culture was studied in semi-industrial scale level using 16-L and 150-L stirred tank bioreactor (STR). During batch cultivation in 16-L STR, the maximal volumetric alginate production of 0.98 g/L was obtained in uncontrolled pH culture, whereas, the maximal specific alginate production of 0.15 g alginate/g cells was obtained in controlled pH culture. As glucose was found to be the limiting substrate in this process, fed-batch cultivation was developed using C-source constant feeding strategy in combination with other key medium components to improve volumetric alginate production. The maximal volumetric and specific alginate productions were 7 g/L and 0.45 g/g, respectively, in fed-batch culture. Based on these data, the production process was scaled up using constant power input (P/V) criteria to 150-L stirred tank bioreactor. Bioprocess scaling up using this strategy improved further the volumetric alginate production up to 20 g/L concomitant with increased specific alginate production reaching 0.65 g/g after 48 h cultivation.

Keywords: Alginate production, *A. vinelandii*, batch process, Fed-batch process, semi-industrial production

1. INTRODUCTION

Alginic acid (CAS No. 9005-32-7) is a linear polysaccharides with common molecular formula $(C_6H_8O_6)_n$ with a molar mass varied between 10,000 and 600,000. This polysaccharide is composed of linear chain of copolymers of 1-4 linked β-D-mannuronic acid and its C-5 epimer, α-L-guluronic acid, in varying proportions [1]. Alginate is negatively charged biopolymer and presence in viscous solution form as solution (in absence of divalent cations) or in gel like structure (in the presence of divalent or trivalent cations). The importance of alginates is based on their wide industrial applications as thickening agents, stabilizers, gelling agents and emulsifiers in food, textile, paper making industries [2, 3]. In addition, alginates are widely used as microencapsulating agent for probiotics to increase their viability during process and application as food additives [4, 5]. As alginates are known as biocompatible, biodegradable and safe biopolymers, thus they are widely used in many pharmaceutical and medical applications as anti-inflamatory agent, radioactive suppressive agent and wound healing [6-9]. Moreover, based on chemical structure of alginate of abundance of free hydroxyl and carboxyl groups distributed along the polymer chain backbone, different modifications of these two active groups were conducted to improve their chemical and physical properties for more applications [10]. On the wide applications of alginates, the worldwide annual industrial production is estimated to be 30,000 metric tons in 2009 [11]. This biopolymer was first discovered as one of the main components of brown algae cell wall such as in *Macrocystis pyrifera* and *Laminaria digitata* and *Liminaria byperborea*. Nowadays, beside algae, many bacterial strains mainly belong to *Azotobacter sp.* and *Pseudomonas sp.* are widely used for the production alginic acid in industrial scale. These types of bacteria are able to produce alginates as extracellular polysaccharides during the late exponential growth phase. However, *A. vinelandii* is the most preferred alginate producer strain for large scale production based on two main factors. First, this strain is reported as safe strain according to its GRAS (Generally Regarded As Safe) status according to FDA (Food and Drug Administration) and second, only alginates derived from this type of microorganism display contiguous sequences of L-guluronic acid residues similar to those of algal alginate [12]. Moreover, it was also reported that bacterial alginate is more cost effective compared to algae [13]. Therefore, alginate production by *A. vinelandii* was interesting research topic by many researchers. As other microbial metabolites, cultivation conditions and medium composition play crucial role in the kinetics of cell growth and biopolymer yield. Among different cultivation conditions affecting alginates production, aeration and oxygen supply are key factors affecting the alginate

production and molecular weight of the final product [14-16]. This study was undertaken first to investigate the effect of pH control on the kinetics of cell growth and alginate production using industrially optimized medium using 16-L stirred tank bioreactor. Then, fed-batch cultivation process was designed by constant feeding of concentrated complex medium of balanced nutrient composition. Based on these results, cultivation was conducted in 150-L stirred tank bioreactor to investigate the scalability for further industrialization of this process.

2. MATERIAL AND METHODS

2.1. Microorganism

The bacterial strain used in this study was *Azotobacter vinelandii* NRRL B-14641. This strain was kindly provided in lyophilized form by ARS culture collection (Peoria, IL, USA). The lyophilized cells were activated first in vegetative medium ATCC 14 of the following composition (g/L): sucrose, 20.0; yeast extract, 0.5; KH_2PO_4, 0.2; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.2; $CaSO_4.2H_2O$, 0.1; $FeCl_3$, 0.002; $Na_2MoO_4.2H_2O$, 0.001. The pH of this medium was adjusted to 7.0 before sterilization. The inoculated cultures were cultivated in incubator shaker for 24 h at 30°C to ensure cell viability. The obtained cells were subcultured on ATCC 14 agar medium. The grown colonies were harvested in 50 % glycerol solution (v/v) and subsequently stored in cryo-vials for cell banking at -80 °C to minimize the productivity loss by subsequent cultivations of cells. Each experiment was started by revival of one glycerol vial in vegetative culture.

2.2. Inoculum preparation

Inoculum was prepared in a 250 ml Erlenmeyer flask containing 50 ml ATCC 14 broth medium. After sterilization for 15 min at 121°C, 50 ml YPM medium was inoculated with 250 µl of glycerol culture. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 30°C for 24 h. Cells were used thereafter to inoculate either 250 ml Erlenmeyer flasks or stirred tank bioreactor with inoculum concentration of 10% (v/v).

2.3. Alginate production medium

The production medium used in this study was composed of the following in (g/L): glucose, 20.0; yeast extract, 5.0; $(NH_3)_2SO4$, 2.0; $MgSO_4.7H_2O$, 0.2; $CaSO_4.2H_2O$, 0.1; $FeSO_4.7H_2O$, 0.005; $Na_2MoO_4.2H_2O$, 0.0002, triammonium citrate, 0.052. After adjusting the pH to 7, the medium was sterilized at 121°C for 15 minutes. Glucose was sterilized separately and added to the fermentation medium before inoculation. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 30°C.

2.4. Bioreactor cultivations

Two types of bioreactors of 16-L and 150-L total volume were used in this study. Both bioreactors were bottom drive stirred tank bioreactors (BioEngineering, Wald, Switzerland) with working volume of 8-L and 80 L, respectively. For 16-L bioreactor, the stirrer was equipped with two 6-blade Rushton turbine impellers ($d_{i\ (impeller\ diameter)}$ = 85 mm; $d_{t\ (tank\ diameter)}$ = 214 mm, d_i/d_t = 0.397). For 150-L bioreactor, agitation was performed using two 6-blade Rushton turbine of the following dimensions: ($d_{i\ (impeller\ diameter)}$ = 200 mm; $d_{t\ (tank\ diameter)}$ = 410 mm, d_i/d_t = 0.488). Unless otherwise mentioned, the agitation speed was adjust to 400 rpm and kept constant throughout the cultivation and aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of 1.0 v/v/min. Foam was suppressed by the addition of silicon antifoam grade A (Sigma-Aldrich Inc., MO, USA). During the cultivation process, pH value and dissolved oxygen concentration were determined using pH and DO polarographic electrodes, respectively (Ingold, Mittler-Toledo, Switzerland). In case of pH controlled culture, the pH was adjusted to 7.0 by cascading the pH controller with acid/base feeding peristaltic pumps connected with 4 M HCl and 4 M NaOH solutions.

2.5. Sample preparation and cell dry weight determination

Samples, in form of 10 ml of broth in case of bioreactor, were withdrawn at different times during cultivation in a centrifugation falcon tube (Falcon, USA). Immediately after sampling, 0.2 ml of Na4EDTA (0.5 M) and 0.2 ml of NaCl (5 M) were added to the sample to solubilise alginate. The sample was then centrifuged at 6000 rpm and 20°C for 40 min using bench top cooling centrifuge (Hettich EBA 12, Germany). After centrifugation, the supernatant was removed and stored at -20°C for alginate and glucose analysis. The cell pellet was washed twice with distilled water and finally resuspended in 10 ml distilled water. After proper sample dilution, the optical density of cells was measured by single beam spectrophotometer (DR/2500, Hach Co., Loveland, CO., USA) at 600 nm. For all samples, the cultivated broth was diluted to give values less than (1 OD_{600}) for better accuracy. The OD of culture was converted to dry cell mass through a linear correlation standard curve. Based on standard curve of this strain, One OD_{600} was almost equivalent to 0.3 g/L.

2.6. Glucose determination

Glucose concentration was determined in culture using colorimetric chemical method. This chemical method is based on the determination of color developed after the reaction between the reduced sugar and DNS (3,5-Dinitrosalisylic acid) in citrate buffer solution [17].

2.7. Alginate determination

The supernatant of sample was used for alginate analysis according to the method of Clementi *et al* [18] with some modifications. In this method, 20 ml of ethanol (95% v/v) was added to the supernatant to precipitate alginate. The mixture was then centrifuged at 6000 rpm for 30 min. The supernatant was discarded and the precipitate was washed twice using distilled water before drying in an oven at 105°C for 24 h.

3. RESULTS AND DISCUSSION

3.1. Batch cultivation in 16-L stirred tank bioreactor under controlled and uncontrolled pH conditions

This experiment was conducted in semi-industrial scale 16-L stirred tank bioreactor to investigate the effect of pH control on the kinetics of cell growth, substrate consumption and alginate production during batch cultivation. Thus, two parallel cultivations were run simultaneously to investigate this effect. For the first culture, the initial pH was adjusted to 7.2 and reserved uncontrolled throughout the cultivation process. For the second culture, the pH value of culture was kept constant at 7.2 by continuous addition of sodium hydroxide and sulphuric acid during cultivation. Other cultivation parameters such as temperature, agitation, aeration, inoculums size, were the same in both cultures. As shown in figure 1, cells grew exponentially in both cultures with the same rate of about 0.73 g/L/h during the first 8 hours. After that time, the growth rate of cells in uncontrolled pH culture was higher than in other culture. The maximal cell growth in uncontrolled pH culture of about 15.8 g/L was obtained after 26 h, whereas, the maximal cell growth obtained in other culture was 7.5 g/L after 20 h. The kinetics of glucose consumption and alginate production were also different in both cultures. In uncontrolled pH culture, the rate of glucose consumption was 2.8 g/L/h which is about 40% higher than the rate in controlled pH culture. However, glucose was limited in both uncontrolled and controlled pH cultures after 14 h and 18 h, respectively. On the other hand, after a lag phase of about 4 hours, alginate was produced in both cultures but with different rates. In pH uncontrolled culture, alginate was accumulated in culture with rate of 0.039 g/L/h (almost 116% higher than the rate of alginate production under controlled pH condition). The maximal volumetric alginate production in both cultures was 0.98 g/L and 0.66 g/L for uncontrolled pH and controlled pH cultures, respectively. For better understanding on the cell performance for alginate production in both cultures, specific alginate production was also calculated. As shown in fig. 1, the maximal alginate production yield of 0.15 g alginate/g cells was obtained in controlled pH culture. This value is almost double of those obtained in pH uncontrolled culture after 30 h cultivation. This directly indicates that the high volumetric alginate production in uncontrolled pH culture was mainly due to high cell mass. Meanwhile, the cell performance for alginate production was almost 50% less than in this culture compared to pH controlled one. On observing the pH profile of uncontrolled culture it is clearly observed that the pH value was dropped gradually during cultivation from 7.1 (initial) to almost 3.0 after 20 hours. It is well known that *Azotobacter* can grow over a wide range of pH. The obtained results are in agreement with those obtained by Vermani *et al.* [14] who observe that low initial pH supports cell growth and volumetric alginate production. However, their study was in small scale level without pH control. Thus, we can conclude that pH control is one of the key cultivation parameters for alginate production and cultivation under controlled pH condition is required to increase polysaccharide production yield. Therefore, we can conclude that, controlling pH value at 7.2 during cell cultivation showed positive effect on cell productivity. Consequently, the research approach was focused on improvement of cell mass production in controlled pH culture to increase the volumetric alginate production.

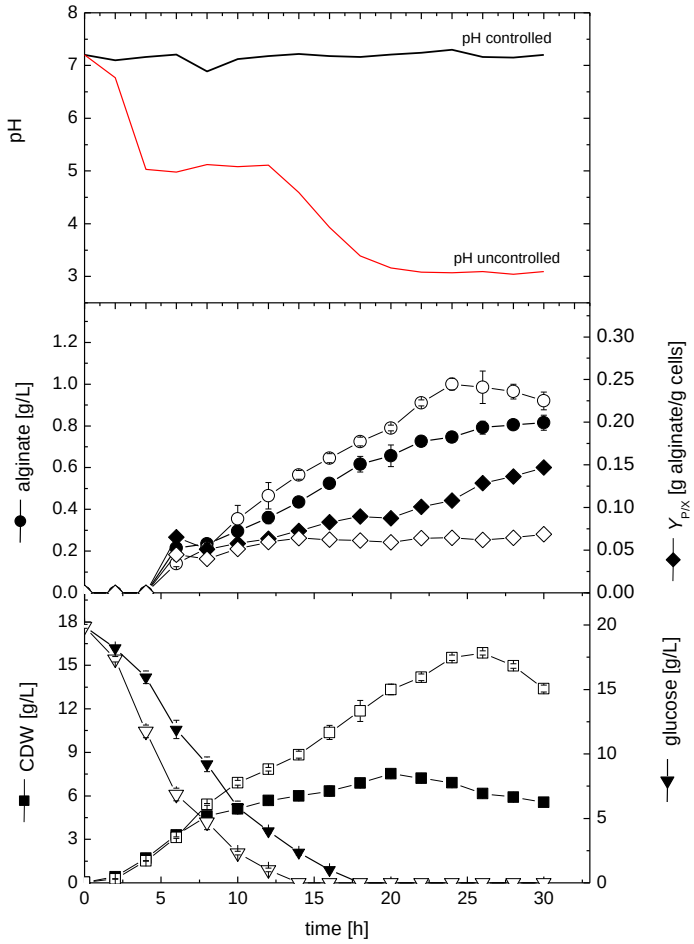

Fig. 1: Cell growth, glucose consumption and alginate production during cultivation of A. vinelandii in 16-L stirred tank bioreactor (closed and opened symbols represents controlled and uncontrolled pH cultures, respectively)

3.2. Fed-Batch cultivation in 16-L stirred tank bioreactor under controlled pH condition

Based on the results obtained in batch cultures, Fed-batch cultivation was designed to increase cell mass production for increasing polysaccharide yield in bioreactor under controlled pH condition. After a batch phase of 10 h, fed-batch cultivation was conducted using constant feeding strategy with glucose feeding rate of 2.8 g/L/h (the rate of glucose consumption as calculated in batch culture). The feeding solution was composed of (g/L): glucose, 200.0; yeast extract, 50.0; $(NH_3)_2SO_4$, 20.0; $MgSO_4.7H_2O$, 2.0; $CaSO_4.2H_2O$, 1.0; $FeSO_4.7H_2O$, 0.05; $Na_2MoO_4.2H_2O$, 0.002, tri-ammonium citrate, 0.52. As shown in fig. 2, after a short lag phase of about 2 h, cells grew exponentially and reached 6.8 g/L after 10 h (batch mode). As mixed substrate feeding solution added continuously in the cultivation, cells continue to grow and reached about 8 g/L after 14 h and kept more or less constant for the rest of cultivation time. On looking to the glucose concentration profile, glucose concentration in culture was almost zero from 12h and forward. This indicates that glucose fed to medium was consumed as a result of microbial metabolites in spite of termination of cell growth. From DO concentration profile, we can conclude that oxygen was the limited substrate for microbial cell growth as it reached about zero % saturation in spite of continuous oxygen supply. On the other hand, alginate was first produced in culture after 8 hours and accumulated in culture continuously reaching its maximal concentration of about 6.4 g/L after 32 h. The drop of oxygen to almost zero was a result of a combination of vigorous cell growth with alginate production which leads to high viscosity and low oxygen transfer in culture. Thus, we can conclude that oxygen limitation influenced cell growth but showed almost no effect on alginate production. These results are in agreement with other authors who find also that *A. vinelandii* is highly aerobic microorganism whereas alginate production reach its maximal rate under microaerophilic conditions [19, 20]. The maximal yield of alginate production of about 0.45 g alginate/ g cells was obtained after 32 h.

3.3. Fed-Batch cultivation in 150-L stirred tank bioreactor under controlled and pH condition

The aim of this experiment was to investigate the scalability of the fed-batch cultivation strategy which developed in 16-L bioreactor scale. Inoculum was prepared in two stage cultivation (in shake flask and 16-L bioreactor) and cells of mid-exponential growth phase were used to inoculate 150-L stirred tank bioreactor in with inoculums size of 5% v/v. As shown in fig. 3, immediately after inoculation, cells grew exponentially without any detected lag phase with rate of 1.35 g/L/h during the batch growth phase (the first 10 h). After that time, cells grew in lower growth rate (0.37 g/L/h) in the bioreactor and reached about 27 g/L after 48 h. Glucose was consumed in culture with high growth rate of 4.47 g/L/h

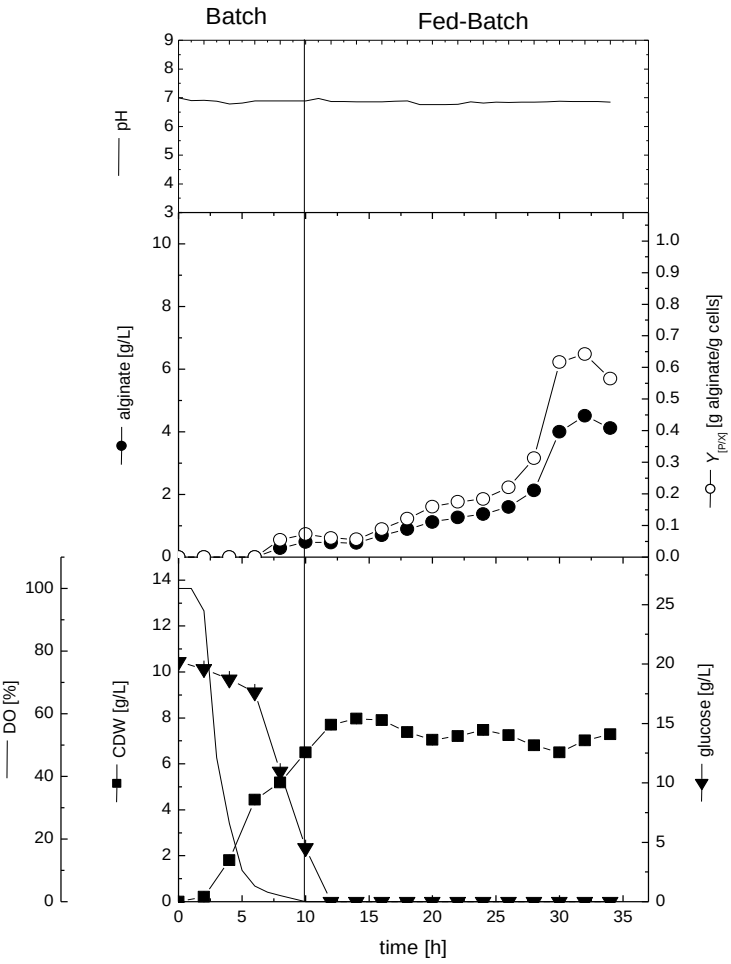

Fig. 2: Cell growth, glucose consumption and alginate production during cultivation of A. vinelandii in 16-L stirred tank bioreactor in fed-batch cultivation system.

during the early stage of cultivation and fully consumed after 8h. During fed-batch cultivation phase, no glucose was determined in medium. This indicates that the feeding rate provided was suitable in this process and no glucose was accumulated in culture. During batch cultivation phase, DO dropped rapidly in culture and reached almost zero after 8 h. Keeping oxygen in culture above zero in range between 0-2 % saturation (the optimal condition for alginate production) was challenging task in this process. Thus, DO was cascaded to both aeration and agitation to keep the DO value within this range during fed-batch cultivation. However, this was a challenging task since *A. vinelandii* is highly aerobic microorganism and the alginate produced in culture increases broth viscosity and thus resulted in a dramatic decrease in oxygen transfer in culture [21, 22]. On looking to the volumetric alginate production kinetics in this culture, after a lag phase of about 6 hours, alginate was produced in culture and accumulated in culture with rate of 0.397 g/L/h reaching its maximal value 18.6 g/L after 46 hours. However, in parallel to the increase in alginate volumetric production, specific alginate production was increased as well. This

Table 1: Kinetics of cell growth, glucose consumption and alginate production in different cultivation modes and production scales using A. vinelandii

parameters	16-L Bioreactor			150-L Bioreactor
	Batch cultivation		Fed-batch cultivation	Fed-batch cultivation
	uncontrolled pH	controlled pH	controlled pH	controlled pH
X_{max} [g/L]	15.86	7.52	8.0	27.0
P_{max} [g/L]	0.98	0.66	4.5	18.6
Q_P [g/L/h]	0.039	0.018	0.18	0.397
$Y_{P/X}$ [g/g]	0.060	0.150	0.64	0.66

X_{max}: maximal cell dry weight, dx/dt: growth rate, μ: Specific growth rate, P_{max} : maximal alginate production; Q_P: alginate production rate; Q_x: glucose consumption rate, $Y_{P/X}$: specific alginate production (g alginic acid produced per g cell dry weight). $Y_{P/S}$: specific alginate production (g alginic acid produced per g glucose consumed).

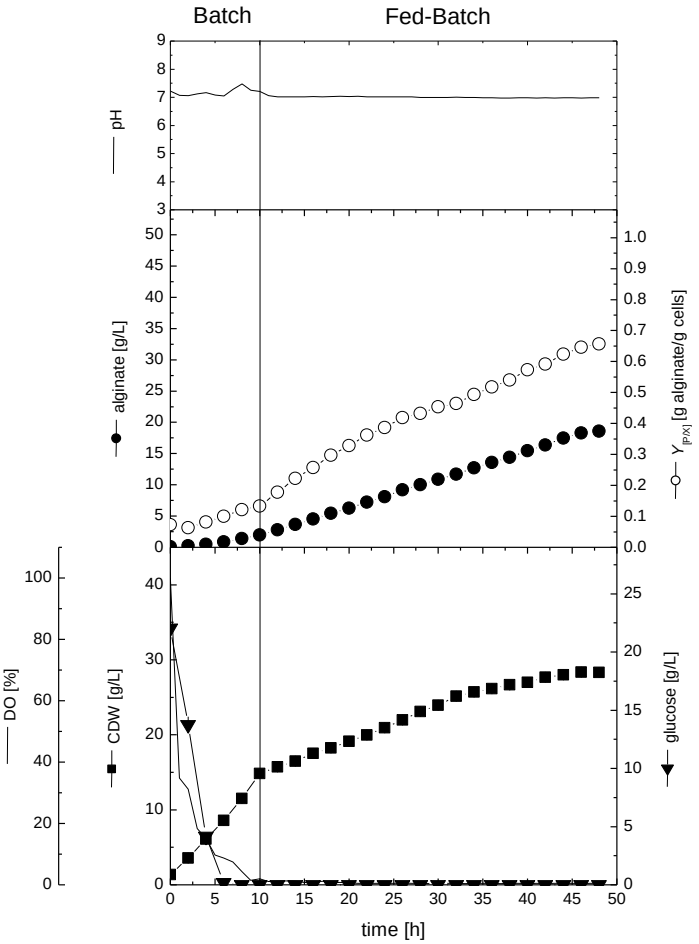

Fig. 3: Cell growth, glucose consumption and alginate production during cultivation of A. vinelandii in 150-L stirred tank bioreactor in fed-batch cultivation system.

indicates that, further increase in alginate production was due to high cell mass production in culture as the maximal specific production of alginate obtained was about 0.66 g/g. This value was almost the same of that obtained in 16-L stirred tank culture. This alginate production yield is high compared to those reported by other authors using different *A. vinelandii* strains [13, 15, 18]. Nevertheless, this was almost equal to the highest yield obtained under optimized conditions as reported by other authors [19]. On the other hand, based on the type of medium used in this study which supports high cell mass production, we achieved higher volumetric alginate production compared to other author's results.

4. CONCLUSION

The data of the kinetics of cell growth and alginate production in batch and fed-batch culture are summarized in table 1. In batch culture at 16-L bioreactor level, cultivation under controlled pH condition yielded lower cell mass but the specific alginate produced in (g/g) was more than double of the value obtained in culture with uncontrolled pH. On the other hand, when the process run in fed-batch cultivation mode in 16-L bioreactor, no significant increase in biomass was observed, whereas both of volumetric and specific alginate production increased by about 7 folds and 10 folds, respectively, compared to batch culture. Further scaling up of the cultivation in 150-L bioreactor using fed-batch cultivation strategy resulted in further increase in both volumetric and specific alginate production. The maximal alginate production achieved in this study for volumetric and specific alginate production was 18.6 g/L and 0.66 g/g, respectively. The fed-batch cultivation strategy proposed in this study showed high potential for alginate production in large scale and reached almost the maximal theoretical alginate yield.

5. ACKNOWLEDGEMENTS

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