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COMPARISON OF HYALURONIC ACID BIOSYNTHESIS BY THE RECOMBINANT *Escherichia Coli* STRAINS IN DIFFERENT MODE OF BIOREACTOR OPERATION

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ABSTRACT

Recombinant *E. coli* was used as a host to explore the biosynthesis of Hyaluronic acid (HA) in Gram-negative strain using the optimized medium. In addition, measurement of HA molecular weight produced by the recombinant strains in shake-flask and 2 L stirred tank bioreactor (batch and fed-batch cultivation) were compared too .In this study, the *hasA* and *hasE* genes expressed together in a plasmid in recombinant *E. coli* strains were successfully produced HA using glucose as a carbon source under aerobic condition. Production of 127 mg/L HA was observed in a batch fermentation process with the highest HA molecular weight (70056 Da) using strain sAE in 2 L stirred tank bioreactor with controlled DO at 30%. The fed-batch culture with constant feeding of glucose was favorable for cell growth and the highest OD_{600} achieved at 1.923. However, the HA biosynthesis and molecular weight in fed-batch culture were lower than that observed in the batch culture.

Keywords: Recombinant, Hyaluronic acid, Batch cultivation, Fed –batch cultivation

INTRODUCTION

HA can be used in various fields such as cosmetics, nutraceutical and osteoarthritis due to its biological and physiological properties **(Kogan *et al.*, 2007)**. The worldwide market value is estimated to be over US $ 1 billion **(Liu *et al.*, 2011)**. In past researches, a vast number of scientists argued that *Streptococci* spp. can be fairly difficult or expensive to mass cultivate and it was also found to be a challenge to genetically manipulate the strains. It was shown to have the potential to produce exotoxins, which is unsuitable for producing HA applied for medical applications **(Kogan *et al.*, 2007; Shih *et al.*, 2013)**. All these drawbacks may have played a part in encouraging researchers to favor the incorporation of novel biotechnological productive methods. This was to solve the pathogenic problems as well as to avoid any contaminations that may have a negative effect towards health. Thus, in the recent years, HA biosynthesis in recombinant microorganisms through metabolic engineering emerged as an attractive alternative method that could reduce safety concerns associated with the use of pathogenic microorganisms and avian products.

Appropriate process conditions are crucial for the metabolic engineered strains to reach their maximum potential, as they may allow the modified metabolic routes to behave as predicted. The first metabolic engineering study effort for recombinant biosynthesis of HA was reported by **Frohberg and Koch (2004)** who expressed the HA synthase genes of human and Chlorella virus in algal plant. There have been a few approaches for the production of HA using metabolically engineered microorganisms. Both Gram-positive and Gram negative bacteria were used as hosts in producing the HA **(Liu *et al.*, 2011).** These include *Bacillus sp.* **(Widner *et al.*, 2005; Chien and Lee, 2007a),** *Lactococcus lactis* **(Chien and Lee, 2007b; Prasad *et al.*, 2010);** for Gram-negative bacteria were *Escherichia coli* **(Yu and Stephanopoulos, 2008; Rehm 2009)**, and *Agrobacterium* sp. **(Mao and Chen, 2007).** For instance, Gram positive *Bacillus subtilis* was used as expression host, due to its ability to secrete similar cell-wall composition features with *Streptococcus* **(Widner *et al.*, 2005). Yu and Stephanopoulos (2008)** incorporated the use of recombinant *E. coli* under fed-batch fermentation using Luria broth as the fermentation medium and resulted in a HA concentration of 0.19 g/L. Another study using *E. coli* under batch fermentation using also Luria broth as the fermentation medium was able to produce a HA concentration of 0.325 g/L **(Jongsareejit *et al.*, 2007).** The HA concentration produced by the recombinant *B. subtilis* indicates that the genetic engineering approach provides new opportunities for capsular polysaccharides to be produced in a heterologous host.

Uridine diphosphate (UDP)-glucose (UDP-GlcA) and UDP-GlcNAc are two nucleotide sugar substrates required for HA synthesis and are produced as precursors of bacterial cell wall components. In addition to that, there is stiff competition for metabolites between HA synthesis and cellular growth due to glucose-6-phosphate and fructose-6-phosphate being consumed in the pentose phosphate pathway and glycolysis respectively **(Mao *et al.*, 2009).** It has been elucidated that HA is synthesized in various cells by polymerization of the monosaccharides from two nucleotide sugar (UDP)-glucuronic acid (UDP-GlcUA) and UDP-N-GlcNAc via HA synthase into a long chain **(DeAngelis *et al.*, 1993)**. As mentioned earlier, UDP-GlcNAc is commonly present in most bacteria as precursors of cell wall components. In contrast, UDP-GlcUA is not commonly used for building their cell wall **(Chien and Lee, 2007a).** Therefore, HA synthase is the only enzyme that lacking in most bacteria important for HA biosynthesis.

In this study, recombinant *E. coli* was used as a host to explore the biosynthesis of HA in Gram-negative strain using the optimized medium. In addition, measurement of HA molecular weight produced by the recombinant strains in shake-flask and 2 L stirred tank bioreactor (batch and fed-batch cultivation) were compared.

MATERIALS AND METHODS

Microorganism and Medium

Recombinant *E. coli* Rosetta (DE3) (CalbioChem, Germany) was used as an expression host of the pRSF and duet PCDF plasmids. The gene sets included two genes set *hasA* and *hasE* genes (strain sAE) in one plasmid and *hasA* and *hasE* in one plasmid with addition of *hasE* gene in separate plasmid (strain sAE-E). The cells were grown at 37°C with 200 rev/min shaking speed for 12 h either in Luria-Bertani (LB) plates or in the LB liquid medium (as an inoculum) supplemented with kanamycin (CalbioChem, Germany) (50 µg/mL) and chloramphenicol (BioWorld, USA) (35 µg/mL) for sAE strains, additional 50 µg/mL of streptomycin (CalbioChem, Germany) for sAE-E strains. The fermentation medium contained (in g/L) glucose 50, tryptone 15, yeast extract 5, KH_2PO_4 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.5. The culture medium was sterilized at 121°C for 15 min. The glucose and $MgSO_4.7H_2O$ were autoclaved separately.

The kanamycin, chloramphenicol and streptomycin were sterilized by filtration using 0.45 µm nylon syringe membrane filter (ThermoScientific, USA). Fermentations were carried out in duplicates and the mean value of each experiment was obtained.

Fermentation in Shake-flask

To investigate the effect of incubation temperature (30°C, 37°C and temperature-shift, 37°C-30°C) for HA biosynthesis by the recombinant strains (sAE and sAE-E), experiments were carried out using a 250 mL Erlenmeyer flask. The flask cultures were incubated in rotary shaker at 200 rev/min with different temperatures. To initiate the fermentation, 1 loop of colony culture was transferred to 50 mL of LB inoculum medium for 12 hours at pH 7, and 10 % (v/v) of inoculum was inoculated into the 250 mL shake flask containing 50 mL of the production medium and run for another 12 hours at initial pH 7 with different temperatures. For temperature shift, temperature was shifted to 30°C once inducer is added. Inducer of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of *hasA* genes when OD_{600} reached about 0.6.
Two different induction times (when cell growth OD_{600} = 0.6; OD_{600} = 1.0) were investigated by using 0.5 mM of IPTG. Four different IPTG concentrations (0, 0.1, 0.5, and 1.0 mM) were investigated when the cell growth reached the middle exponential phase. All experiments are run at 37°C, 200 rev/min and with initial pH 7.

Fermentation in 2 L Stirred Tank Bioreactor

Batch Fermentation

All experiments were conducted using a 2 L stirred-tank bioreactor using strains sAE and sAE-E. The dissolved oxygen of batch HA fermentation was controlled according to requirement of the experiments (20%, 30% and 50%). Dissolved oxygen was provided by sparging filtered air at a flow rate of 1 vvm and was maintained according to requirement by automatically adjusting the agitation rate from 50 to 350 rev/min with half-pitched double blade helical ribbon impeller, via sequential cascade control.

Fed-batch Fermentation

Fed-batch fermentations were carried out in a 2 L stirred-tank bioreactor equipped with half-pitched double blade helical ribbon impeller. The working volume was 1 L with initial batch culture working volume 500 mL. The fed-batch feed protocol was designed as initially the cells were cultivated in 4 h of batch phase, to stabilise the cells and control the lag phase behaviour. The cultivation was the batch phase followed by a constant glucose feed before glucose was depleted. Initial glucose (10 g/L) was added to create batch phase before the constant feed phase was started. The feed solution (10 g/L glucose and other components of medium) were pumped into the bioreactor at a feeding rate of 2 ml/min by a peristaltic pump (Watson Marlow 101 U/R, England). The fermentation was carried out for 12 h, with temperature maintained at 37°C and pH controlled at 7.0 by the automatic addition of 3 M NaOH and 3 M HCl. A polarographic oxygen electrode was used to determine the dissolved oxygen level (DOT). The dissolved oxygen never dropped below 30% air saturation. Also, 1 mM of IPTG was added to express *hasA* gene in *E. coli*.

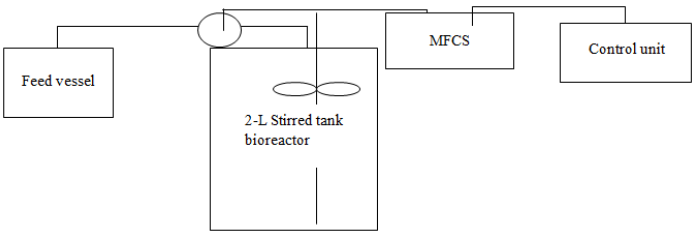

Figure 1 Schematic diagram for fed-batch fermentation set-up

The bioreactor was connected to the multifermenter control system (MFCS) for controlling the feeding rate of the substrate and also controls some of the bioreactor operating variables. Control of the peristaltic pump was facilitated using MFCS/win software, which is a fermenter supervisory control and data acquisition system (SCADA) for simultaneous control of multiple fermenters.

Analytical Methods

All samples were withdrawn at regular time intervals for analysis of cell, HA and glucose concentration. Cell growth was observed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer. Correlation between the dry cell weight (DCW) and OD was estimated from several batch experiments using the equation: DCW (g/L) = 1.489 x OD

The supernatants were used for HA and glucose determination. After the removal of the cell pellet for cell growth determination, two volumes of ethanol were added to one volume of the supernatant in a 15 ml centrifuge tube and the solution was refrigerated at 4°C for 1 h to precipitate HA. The precipitate was collected by centrifugation at 3,000 x g for 20 min and was re-dissolved with a 2 to 3-fold volume of distilled water. The HA concentration was determined using the carbazole method (**Bitter and Muir, 1962**) and the optical density was measured at 530 nm. The HA concentration was calculated using a standard curve prepared at different concentrations of HA standards (Sigma-Aldrich, Malaysia). The HA molecular weight (M_w) was analyzed by high performance liquid chromatography equipped with a cation-exchange column (Aminex HPX-87H, BioRad Labs, Hercules, CA, USA), a UV detector and the mobile phase was 4 mM H_2SO_4 at a steady flow rate 0.5 ml/min.

RESULTS AND DISCUSSION

Fermentation in Shake-flask

Effect of Temperature

The ability of 2 different types of *E. coli* strains that were cloned with *hasA* or *hasE* gene from *S. zooepidemicus* ATCC 39920 **(Samsudin *et al*., 2011)** for HA biosynthesis were investigated. The HA biosynthesis capability of the metabolically engineered *E. coli* strains were investigated in shake-flask culture with different temperature in the presence of IPTG. At three different temperatures investigated (30°C, 37°C and 37°C-30°C), a distinct stationary phase was observed following the exponential phase. As seen from **Table 1**, the cell production for all strains had very little deviance which was in the range of OD_{600} between 0.8 to 1.2. The IPTG inducer was added at 2 h of fermentation when OD_{600} reached to about 0.6 and the cell growth was inhibited once IPTG was added and decreased from 1.1 to 0.6 after 12 h fermentation (data not shown). This indicates that the host strain is susceptible to IPTG **(Chien and Lee, 2007a).** All the recombinant strains were grown better when the temperature shift from 37°C to 30°C once the IPTG was added; in contrast, cell growth was the lowest at 37°C in comparison to cell grown at 30°C. The HA biosynthesis is the highest at 37°C for all strains, indicating cell lysis at this temperature (data not shown). However, little cell lysis, based on the HA biosynthesis in medium, was observed at 30°C and temperature shift from 37°C to 30°C.
Cell growth inhibition was observed during HA accumulation. Similar phenomena were observed with all recombinant strains investigated. As can be seen from **Table 1**, the cell production was similar for sAE and sAE-E cultures. However, the HA production was consistently at least 2-fold and above higher in the strain sAE culture compared to sAE-E cultures. **Yu and Stephanopoulos, (2008)** deduced that gene coding for phosphoglucoisomerase (pgi, corresponding to *hasE* in HA synthesis operon) probably plays a critical role to obtain a higher production of HA.
An appreciable amount of HA (<110 mg/L) was observed for all strains before IPTG induction. The observed HA should result from the expression of HA synthase, due to the optimized medium employed for cell cultivation does contain 50 g/L glucose, the HA detected before IPTG induction was probably resulted from the glucose-induced expression of HA synthase. At 30°C fermentation, sAE-E produced 62.7 mg/L HA, and the highest HA produced is by sAE (86.8 mg/L). On the other hand, the maximum HA biosynthesis was observed when all fermentation run under 37°C. The sAE yielded about twice the amount of HA (109.6 mg/L) in shake-flask culture **(Table 1).** However, for strains sAE-E, the HA biosynthesis was remained low. It is possible that the two plasmids applied were not stable enough for *has* gene to express. In order to express two heterologous enzymes simultaneously, the energy burden not only slows down the cell growth but also reduces the expression of these two enzymes **(Chien and Lee, 2007b)**. The successful expression of these two heterologous enzymes demonstrates that PRSF vector constructed is effective for protein expression.

Table 1 Effect of temperature on cell growth and HA biosynthesis by different recombinant *E. coli* strains in shake-flask culture after 12 hours of fermentation

Temperature	30°C	37°C	37°C-30°C
sAE strain			
OD_{600} (nm)	0.964	0.849	1.043
HA (mg/L)	86.8	109.6	74.8
MW HA (Da)	11021	26802	9872
sAE-E strain			
OD_{600} (nm)	0.972	0.877	0.989
HA (mg/L)	62.7	28.7	30.8
MW HA (Da)	11339	27344	10145

With co-expression of a heterologous phosphoglucoisomerase (sAE), as consequence, the HA concentration was enhanced from 75.7 mg/L to 109.6 mg/L. The results were comparable to sseA and sseAB strains developed by **Yu and Stephanopoulos, (2008)** which are 27.8 and 203.5 mg/L HA, respectively. It is interesting to note that over-expressing of *hasA* alone does not seem to hinder

the cell growth and the *hasA* is not titrating away sugars that are required for cell growth, at least not to dangerously low levels. The over-expression of *hasA* caused a significant increase in HA yield and also significantly lowered the molecular weight of HA. The low molecular weight may be clarified by the increased competition for a foxed pool of UDP- monomers leading to sub-optimal levels of UDP-Glc UA and UDP-GlcNAc **(Chen *et al*., 2009).**

Effect of Inducer on sAE Growth

The sAE strain was selected to grow on the optimum medium with glucose as a sole carbon source incubated at 37°C. The growth curve of the sAE strain cultivated in the presence and absence of IPTG **(Figure 2)** clearly shows that once the inducer IPTG is added, it will cause the cell growth inhibition. In contrast, the cell grew well in without IPTG addition fermentation. The IPTG inducer was added at 2 h of fermentation when OD_{600} reached about 0.6. Both growth patterns of cells were same before IPTG is added, the cell growth started to maintain once IPTG is added after 2 h, however, when the fermentation was carried out without induction, the cell growth was still increased exponentially until 10 h. The cell could grow to 1.113 OD_{600} in the absence of IPTG. In contrast, in the presence of IPTG, cell growth was inhibited the cell decreased from 1.113 to 0.748 OD_{600} after 12 h cultivation. This indicates the strain sAE is susceptible to IPTG. The advantage of IPTG for in vivo studies is that it cannot be metabolized by *E. coli*, therefore the growth rate of cells (usually maintained with glycerol as the carbon and energy source), is not a variable in the experiment and since cells do not metabolize IPTG, its concentration does not change during the course of an experiment. Besides IPTG, **Yu and Stephanopoulos (2008)**, used *L*-Arabinose as inducer to induce the expression of HAS genes in recombinant *E. coli*. Lactose has been used as a cheap alternative to IPTG to induce *lac*, but lactose has a much lower induction potential **(Kweon *et al*., 2001)** and is consumed whereas IPTG is not consumed.

Although *E. coli* is a gram-negative bacteria with the cell wall structure composed of lipopolysaccharide, peptidoglycan and lipoprotein, its metabolic pathway for HA synthesis is very similar to that of *Streptococcus* spp.; the only enzyme of *E. coli* lacks is the hyaluronan synthase. Double expression of *hasA* with *hasE* gene in a single plasmid in the recombinant *E. coli* (sAE) significantly improved the HA biosynthesis from 21.2 mg/L at 10 h (without IPTG induction) to 110.5 mg/L (with IPTG induction) at 4 h of fermentation in shake-flask culture. This indicated that the IPTG as an inducer is required to induce *hasA* and *hasE* genes. This result was significantly higher than **Yu and Stephanopoulos, (2008)** who only managed to produce 95.9 mg/L HA by using triple expression sp ABC recombinant *E. coli* strain.

The hyaluronan synthase enzyme involved in HA synthesis in *Streptococcus* and mammals has shown to prefer magnesium ions while Chorella virus HASs prefers manganese ions to stimulate the synthesis HA **(Yamada and Kawasaki, 2005).** The use of magnesium sulphate in HA biosynthesis likewise, has been preferred by recombinant *E. coli*. Large number of ATP molecules are consumed in various enzymatic reactions involved in HA biosynthesis pathway, hence source of phosphate is essential to synthesize ATPs. In this study, salt dipotassium hydrogen and potassium dihygrogen phosphate used as a source of inorganic phosphate in recombinant *E. coli* fermentation.

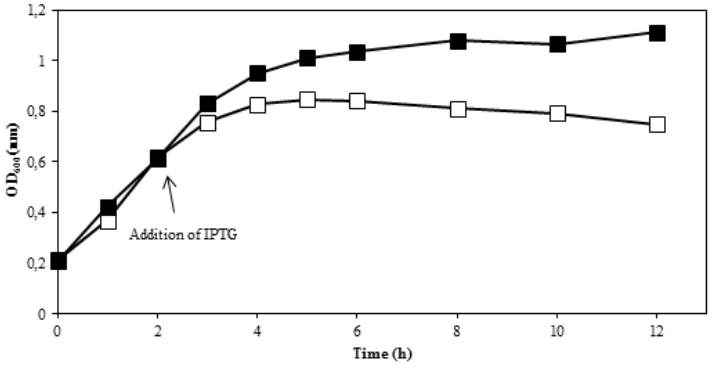

Figure 2 The growth curves of sAE strain cultivated in the shake-flask culture with the presence and absence of IPTG incubated at 37°C. (■) – without IPTG addition ; (□) – with IPTG addition

Effect of IPTG Induction Time on sAE Growth

In order to determine the time course of induction, two shake-flask cultures experiments were performed. The first culture was grown at 37 °C until the sAE growth, OD_{600} reached 0.6 and then 0.5 mM IPTG was added to the medium. The same amount of IPTG was also added to the second culture experiment when its OD_{600} reached 1.0. After the addition of IPTG to the cultures, the incubation was continued and samples were taken every hour to determine the cell growth, glucose consumption and HA concentration. As a result, the growth curve is shown in **Figure 3**. The cell growth was higher (OD_{600} is 1.088) with the addition of 0.5 mM IPTG at OD = 1.0 if compared to OD = 0.6. The maximum of HA biosynthesis was found to be attained 4 h after the addition of IPTG to the medium, which is 115.4 mg/L (OD=0.6) and 90.8 mg/L (OD=1.0). The induction times are considered to be important parameters in the production of the recombinant strains and they need to be carefully optimized to increase the yield of the product. The addition of IPTG in the middle of exponential phase able to achieve the higher HA production compared to addition of IPTG at late exponential phase. The strain starts goes into the stationary phase. Induction in the stationary phase reduces culture viability and can lead to production of proteases that can breakdown the desired recombinant protein **(Corchero *et al*., 2001).** On the other hand, if induction too early can slow down the doubling time of bacterial cells. The timing of induction of new recombinants need to be empirically determined for each new clone, due to the cellular responses depend on a number of interacting factors including the host/vector system **(Cserjan-Puschmann *et al*., 1999).**

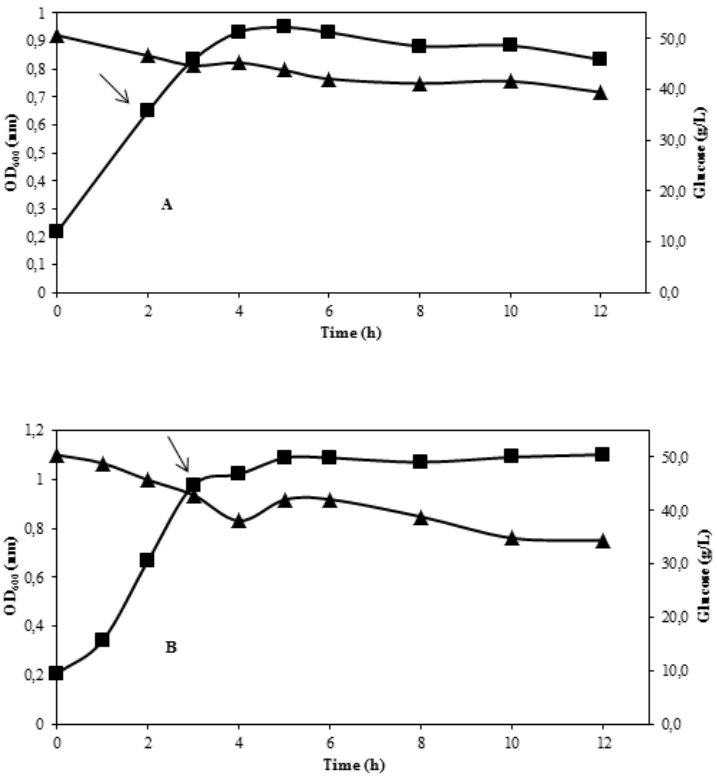

Figure 3 The growth curves of the strain sAE cultivated in the presence of IPTG with different induction time. The arrow indicated that IPTG is added. (A) IPTG added when OD_{600} is 0.6; (B) IPTG added when OD_{600} is 1.0

Effect of IPTG Concentration on HA biosynthesis by sAE

In order to determine the effect of different concentrations of IPTG on HA biosynthesis, addition of 0.1, 0.5 and 1 mM IPTG were examined. All cultures of strain sAE were grown until the OD_{600} reached 0.6 in the middle of exponential phase, 0.1, 0.5 and 1 mM IPTG was added to each culture flask respectively, and incubated till 12 hours. A control experiment was also performed in which the addition of IPTG was omitted. From **Table 2**, it can be seen that in the control experiment, the cells also grew well, however there is no HA production. The IPTG induction resulted in growth of sAE and HA production. An increment of IPTG concentration has meaningful effect on cell and HA production.

From **Table 2**, the cell growth was higher with no IPTG addition compared to 0.1 mM of IPTG addition (OD =1.113 and 1.1, respectively). The cell growth was reduced when the IPTG concentration was increased from 0.1 to 0.5 (OD= 0.949) and 1.0 mM (OD= 0.849). On the other hand, the HA concentration was the highest when the cell growth was at the lowest when 1 mM IPTG was added. This result proved the statement of there is a stiff competition for metabolites between HA production and cellular growth **(Mao *et al*., 2009).** From the **Table 2**, the HA concentration obtained are comparable, however, there is a significant difference when we compare the molecular weight of HA measured after the fermentations. Although HA with a relatively low molecular weight is broadly used in cosmetics fields, high molecular weight HA is more appealing to be used in medical applications (**Liu *et al*., 2008)**. High molecular weight HA is also highly viscous which makes it ideal for some uses such as reducing the adhesion formation of post-surgery **(Takeda *et al*. 2011)**. The highest HA molecular weight can be achieved at 40928 Dalton using 1mM of IPTG, which is about 3 folds higher than other IPTG concentration. At a concentration of 1 mM the *lac* promoter appeared to be fully induced. The concentration of IPTG (between 0.1

mM and 2mM) required for complete induction is known to vary widely with clones **(Li *et al*., 1999; Madurawe *et al*., 2000).** The presence of excessive IPTG reduced the final cell density. These results concur with **Yee and Blance (1993)** who observed a reduced growth rate in *E. coli* when IPTG concentration exceeds 1 mM.

Table 2 Effect of IPTG concentration on maximum cell growth, HA biosynthesis and molecular weight by strain sAE using shake-flask culture

IPTG (mM)	OD_{600} (nm)	HA (mg/L)	MW HA (Da)
0	1.113	0	0
0.1	1.1	99.6	14263
0.5	0.949	103.5	14641
1	0.849	109.6	40928

Fermentation in 2 L Stirred Tank Bioreactor

Batch Cultivation

Batch bioreactor studies were conducted with both the recombinant strains (sAE and sAE-E) initially with controlled DO at 20 %. With both recombinant strains, it was observed that glucose was only consumed 8 to 9 g/L throughout the 10 h fermentation (as shown at **Table 3**). Cell using glucose as carbon source can theoretically be thought of as used for four different purposes in recombinant protein producing cells, i.e. for: growth, maintenance, respiration and product formation. If the concentration is very high glucose will further be used for formation of overflow metabolite formation. Accumulation of acetate is a common obstacle to achieving high levels of recombinant protein and other fermentation products in *E. coli* **(Chen and Bailey, 1993).** With excess glucose in the medium, *E. coli* cells tend to synthesize high levels of acetate and other organic acids, such as lactic acid, usually resulting in cell growth inhibition and reduced HA production due to the reduced pH of the medium **(Liu *et al*., 2011).**

The growth curve of recombinant *E. coli* strains is shown in **Figure 4**. The cell growth curve in sAE and sAE-E cultures are same, the maximum OD_{600} in strain sAE is 1.022, and 1.075 for strain sAE-E cultures. On the other hand, strain sAE produced maximum of 50.7 mg/L of HA, while strain sAE-E produced nearly 5 times lower HA, which is only 10.3 mg/L. Since HA production increased approximately 2.5 fold over the non-aerated condition **(Prasad *et al*., 2010)**, HA production also further improved by controlled DO from 20 % to 30 % and to 50 %.

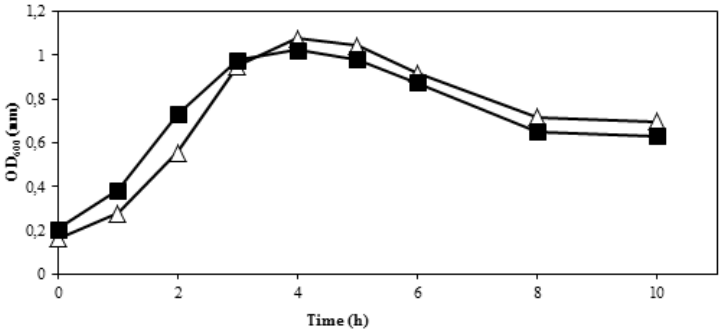

Figure 4 Growth curves of strains sAE and sAE-E in batch cultivation using 2 L of stirred tank bioreactor. (■)- strain sAE; (Δ)- strain sAE-E

The operation conditions of the process greatly affect the metabolic behavior of the production strain. The genetic manipulation themselves are not enough to make a strain achieve maximum theoretical yield. HA synthesis is a demanding process, requiring large amounts of energy. Four molecules ATP are needed for a single molecule of disaccharide repeating unit. Aerobic fermentation is therefore favorable to HA biosynthesis. A common challenge in metabolic engineering is to ensure adequate supply of precursors for target molecules. This is particularly challenging since HA synthesis tightly couples with the cell growth. Consequently, cell growth inevitably competes with the HA synthesis for the same precursor molecules. The ability to decouple these two processes will not only increase the efficiency of the processes but also allow synthesis process to be controlled independent of cell growth such that any conditions limiting cell growth may not affect the synthesis **(Mao *et al*., 2009).**

Table 3 shows the cell and HA production with different DO levels, while the impeller speed control remained the same along with the standard fermentation conditions mentioned above. Under aerobic conditions, the final cell growth are not affected by various DO levels, indicating that cell growth is unaffected by presence of oxygen, regardless the DO levels. However, HA synthesis is affected by different DO levels. The highest HA synthesis attained when the DO is controlled at 30 % which is 127 mg/L. This result can be supported by **Huang *et al*. (2006)** found that DO played an important role as a stimulant in HA synthesis and there existed a critical DO level of 5 % during HA fermentation, meaning the capacity of HA synthesis was affected below this level. Furthermore, the yield coefficient $Y_{p/x}$ was investigated, and the results are summarized in **Table 3**. There were no distinct differences in the values of $Y_{p/x}$ under 30 % and 50 %, indicating that the efficiency of the HA synthesis was unaffected when DO level is higher than 30 %. However, if compared to DO at 20 %, the $Y_{p/x}$ is about two fold lower than DO above 30 %. The highest concentration of the inclusion body protein and the volumetric titres of *Eg95* were attained at 30% DO level **(Manderson *et al*., 2006). Chisti and Moo Young (1996)** stated that maintaining a high dissolved oxygen level in high cell density cultures requires intense agitation and aeration that can damage fragile recombinant cells.

From our results, it shown that HA biosynthesis in bioreactor under controlled pH conditions results in better glucose uptake and enhances the productivity of culture in comparison to shake-flask cultures. It is possible that the development of acid tolerance response **(Sriraman and Jayaraman, 2006)** in the shake-flask cultures due to acidification of the broth limits the energy and precursor availability for HA biosynthesis.

Molecular weight is one of key characteristic of HA product. **Chen *et al.* (2009)** have shown that in *S. zooepidemicus*, the overexpression of single genes involved in the UDP-glucuronic acid biosynthesis pathway decreased the HA molecular weight, whereas the overexpression of single genes involved in the UDP-N-acetylglucosamine biosynthesis pathway increased the HA molecular weight. In this study, the HA molecular weight was found to be sensitive to the DO level, and the results are shown in **Table 3**. At DO is 20 %, the HA molecular weight was only 54166 Da, yet it reached 70056 Da at a DO level of 30 %, and then decreased to 55041 Da at 50 %. This phenomenon was caused by the balance between HA synthesis **(Armstrong and Johns, 1997)** and oxygen-mediated degradation with various DO levels. As such, the HA molecular weight increased owing to the effect of the DO on the HA synthesis, and then decreased because of oxygen radical degradation. Thus, the HA molecular weight at 50 % DO was less than that at 30 % DO owing to oxygen radical degradation.

Cleary and Larkin, (1979) previously proposed a defense mechanism where HA capsules protect cells from oxygen in Group A, and the protective mechanism is activated in the presence of oxygen. In other words, an appropriate DO level could stimulate the synthesis of HA, and when DO level was higher than the critical level of 30 %, the stimulation effect was not so significant. Therefore, developing an efficient DO control approach was very important to improve HA productivity. However, there are no reports regarding the influence of DO control approach on the microbial HA production by recombinant *E. coli*.

Table 3 Comparison of kinetic parameters values HA biosynthesis at different controlled DO levels in a 2 L stirred tank bioreactor using strain sAE

Kinetic parameter values	20%	30%	50%
t(h)	12	8	8
OD_{600} (nm)	0.815	0.794	0.758
P_m (g/L)	0.057	0.127	0.118
S_0-S_i (g/L)	8.152	8.999	9.099
$Y_{OD/s}$ (OD/g)	0.100	0.088	0.083
$Y_{p/s}$ (g/g)	0.007	0.014	0.013
$Y_{p/OD}$ (g/OD)	0.070	0.160	0.155
P_r (g/L/h)	0.005	0.016	0.015
MW (Da)	54166	70056	55041

Fed-batch cultivation

Since HA biosynthesis of recombinant *E. coli* in our research is in simple batch culture in flask or bioreactor, it is reasonable that they are not very high. Therefore, we proceed to fed-batch cultivation by using sAE strain. In order to investigate the potential of the recombinant strain for industrial application and to probe possible limitation for HA synthesis in recombinant strains, a fed-batch fermentation process was developed. Fermentation processes of *E. coli* are commonly operated in fed-batch mode in order to prevent the accumulation of toxic substrates or products **(Lee *et al*., 1999),** thus allowing the achievement of higher product concentrations. The bacteria *E. coli* is usually grown under this kind of operation due to the well-known negative effect of acetate, which is produced when the substrate, glucose, is presented above certain concentrations **(van de Walle and Shiloach, 1998).**

In the constant feeding rate fed-batch cultivation, 10 g/L of glucose was fed into the bioreactor at a constant feeding rate of 2 ml/ min. The cell reached OD_{600} at 1.923, however the HA biosynthesis in the constant feeding rate fed-batch cultivation is only 40 mg/L, 31 % lower than batch cultivation. The comparison of batch and constant feeding rate fed-batch on HA production was provided in **Table 4**. Batch culture evidently showed the highest HA productivity on glucose, and fed-batch culture had a higher cell yield ($Y_{OD/s}$) on glucose. The average specific HA synthesis rate in fed-batch culture was 0.004 (g HA/OD/h) compared with 0.016 (g HA/OD/h) in batch culture. Therefore, it can be deduced that fed-

batch culture was more favorable for cell growth compared to batch culture, which was a more favorable way for the synthesis of HA. Fed-batch culture was chosen because of the desire to probe limitation under more practically useful conditions which lead to high titers in HA **(Mao *et al*., 2009).** The concentration of acetic acid was increased to 1.623 g/L compared with 1.407 g/L in batch culture. The average specific HA synthesis rate in fed-batch culture was 0.021(g HA/OD/h) compared with 0.16 (g HA/OD/h) in batch culture. The synthesis of acetic acid was coupled to cell growth, and thus the fed-batch culture evidenced a higher acetic acid yield as compared with that in the batch culture. In comparison of HA molecular weight in fed-batch cultivation (60881 Da) is 10 % lower than batch cultivation (70556 Da). In the fed-batch cultivation, the increased accumulation of acetic acid inhibited HA biosynthesis more severely, thus resulting in a reduced production and productivity of HA.

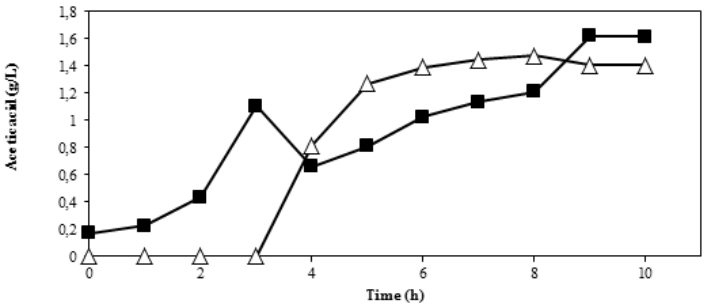

Figure 4 Acetic acid production at different mode of cultivation. (■)- fed-batch cultivation (Δ) - batch cultivation

The high cell growth in fed-batch cultivation resulted from the low inhibition of glucose in the cells. However it appeared that low glucose concentrations were not favorable for the synthesis of HA. It was previously reported that there was competition between HA synthesis and cell growth for the common precursors; UDP-glucuronic acid and UDP-N-Acetyl Glucosamine. It should be noted that cell wall is necessary for cell growth, while HA as a cell capsule is not a necessary component for cell growth. Carbon source was utilized preferably for cell growth and thus HA synthesis is inhibited, especially at low substrate concentration in fed-batch culture **(Chong *et al*., 2005).** Therefore, a lower HA productivity in fed-batch culture compared to batch culture was observed in the present study. The competition between cell growth and HA synthesis was also observed by other researchers **(Chong *et al*., 2005)**. Hence, the lower HA productivity in fed-batch culture resulted from the competition between cell growth and HA synthesis, not the decreased HA synthesis ability of *S. zooepidemicus*.
Other metabolites in the HA biosynthetic pathways such as glucose-6-phosphate and fructose-6-phosohate are consumed in the pentose phosphate pathway and glycolysis, respectively. Thus, there is stiff competition for the metabolites between HA production and cellular growth, and there is a close correlation between energy metabolism and precursor supply. As discussed above, fed-batch culture was favorable for cell growth, and cells in fed-batch culture can reach a concentration as high as that in batch culture but in a shorter time.

Table 4 Comparison of kinetic parameter values of batch and constant feeding rate fed-batch cultivation on HA biosynthesis by sAE strain

	Batch Fermentation	Fed-batch Fermentation
t(h)	8	9
OD_{600} (nm)	0.794	1.923
P_m (g/L)	0.127	0.040
AA_m (g/L)	1.407	1.623
$Y_{OD/s}$ (OD/g)	0.088	0.134
$Y_{p/OD}$ (g/OD)	0.160	0.021
P_r (g/L/h)	0.016	0.004
MW (Da)	70056	60881

CONCLUSION

In this study, the *hasA* and *hasE* genes expressed together in a plasmid in recombinant *E. coli* strains were successfully produced HA using glucose as a carbon source under aerobic condition. Production of 127 mg/L HA was observed in a batch fermentation process with the highest HA molecular weight (70056 Da) using strain sAE in 2 L stirred tank bioreactor with controlled DO at 30%. The fed-batch culture with constant feeding of glucose was favorable for cell growth and the highest OD_{600} achieved at 1.923. However, the HA biosynthesis and molecular weight in fed-batch culture were lower than that observed in the batch culture. Further optimization of fermentation process and control strategies should lead to an even higher yield.

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