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Optimization And Kinetic Analysis On The Production Of Hyaluronic Acid By *Streptococcus Zooepidemicus* In A Batch System

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Abstract: In this study, the optimization and kinetic analysis on the effect of process parameters for the production of hyaluronic acid production by *S. zooepidemicus* were performed in batch system. The fermentation condition such as inoculum size (2 to 20 %), pH (4 to 10), temperature (30 to 45) and agitation (50 to 500) were optimized using “one-factor-at-a-time” method. It was found that the inoculum size of 10 % had the greatest effect on the fermentation process which gave the highest value of specific growth rate, cell biomass and HA production of 0.72 h⁻¹, 1.96 g l⁻¹ and 0.82 g l⁻¹, respectively. Furthermore, the other process parameters such as pH, temperature and agitation speed were also found to improve the cell biomass, HA production and kinetic analysis. The highest cell biomass (0.17 g_{cell} g_{glucose}⁻¹) and HA yield (0.096 g_{HA} g_{glucose}⁻¹) were obtained at pH 7.0, 37 °C and agitation speed 300 rpm for 12 h. The overall kinetics analysis of maximum specific growth rate, volumetric cell biomass productivity and volumetric HA productivity obtained at optimum parameters were, 0.81 h⁻¹, 0.183 g_{cell} l⁻¹ h⁻¹ and 0.111 g_{HA} l⁻¹ h⁻¹, respectively.

1. Introduction

The optimization of fermentation conditions is an important step in the development of economically feasible bioprocess systems. The success of the fermentation depends on the existence of the defined environmental condition for cell growth and product formation. These goals can be achieved by optimizing media composition, fermentation conditions and bioreactor design as well as by developing superior strains by mutation. Many factors involved in optimization of Hyaluronic acid (HA) production by *S. zooepidemicus*. As reported by previous researchers, temperature, pH [1], nutrient [2-4], agitation and aeration [5, 6] substrate concentrations [7] were the most typical factors to be considered. In view of the demand for HA products in pharmaceutical and cosmetic industries by microbial fermentation, this study presents an investigation on the production of HA by *S. zooepidemicus* ATCC 39920 in batch fermentation system at different parameters conditions. One-factor-at-a-time method and kinetic analysis were used to investigate and optimized the effect of fermentation conditions such as inoculum size, agitation, pH, and temperature.

2. Methodology



2.1 Microorganism

The bacteria, *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920 was obtained from American Type Culture Collection (Rockville, Md) as a freeze-dried culture in ampoules. The strains were maintained by weekly transfer on sheep blood agar (SBA) and stored at 4°C after incubated at 37°C for 24 hr. Monthly subculture ensured the availability of sufficient stock cultures for experimental processes.

2.2 Inoculum

Seed culture or inoculum was prepared by inoculating 15 ml of cell suspension into a 500 ml Erlenmeyer flask containing 135 ml of the fermentation medium. The flask was then incubated in a rotary shaker at 37°C, 300 rpm for 8 h. The inoculum was standardized by measuring the absorbance (optical density) at 600 nm using a spectrophotometer (Thermo Spectronic, Genesys 20). Inoculum with optical density within 0.6-0.9 was used to inoculate the fermentation medium [7].

2.3 Fermentation medium

The composition of the medium used comprised of (gl⁻¹): glucose 30 (unless otherwise specified) yeast extract 10, KH₂PO₄ 0.5, Na₂HPO₄.12 H₂O 1.5 and MgSO₄.7H₂O 0.5, respectively (Mashitah, 2006). All the media, unless otherwise stated was sterilized in an autoclave at 121°C for 20 minute. Glucose was autoclaved separately to avoid caramelisation and mixed aseptically with other components prior to inoculation.

2.4 Fermentation Process

Fermentation process was carried out using 500ml Erlenmeyer-flask in a rotary shaker incubator from B.Braun Biotech International GmbH, Germany. The effect of inoculum sizes on HA fermentation by *S. zooepidemicus* was carried out at different (v/v) ratio; 2%, 5%, 10% and 20%. The flask with 200 ml working volume was incubated in a rotary incubator shaker at pH 7.0, temperature at 37°C and agitation speed at 250 rpm for 12 h.. The other parameters were setup at pH ranges from 2 to 9, temperature ranges from 30 to 45 and agitation ranges 50 to 500 rpm, respectively.

2.5 Analytical methods

At appropriate intervals, culture samples were collected for analysis of cell dry weight. Growth was measured by optical density at 600nm and converted to grams of cell dry weight per volume (g_{CDW}/L).The concentration of glucose and HA were determined by high-pressure liquid chromatography (HPLC).The HA concentration was measured by HPLC as previously and streptococcal HA (Sigma H-9390) was used to prepare standard curve of peak area against concentration [7].

3. Results and Discussion

3.1 Effect of Inoculum

In order to promote success in fermentation, an appropriate volume or concentration of inoculum should be available in a healthy and active state, in a suitable morphological form, free of contaminants, and capable of forming the desired products [8]. In this study, various inoculum sizes (2%, 5%, 10% and 20% (v/v)) were used as seed culture for the fermentation of HA in shake-flask culture. As can be seen from Figure 1 (a), lower the inoculum size, the time taken for the lag phase would be longer. According to [9], a long lag phase would be disadvantageous in that not only was the time wasted but also much medium would be consumed in maintaining a viable culture prior to growth. [10] stressed out that bacterial inoculum should be transferred in the logarithmic phase of growth, when the cells were still metabolically active. In this study, the inoculum was standardized by incubating the *S. zooepidemicus*

cells for 8 h prior to transferring them into the production medium. In fact, the optical density was also measured within 0.6-0.9 to ensure that the cell growth was in the logarithmic phase. The effect of inoculum sizes on the specific growth rate (μ) and HA productions are shown in Figures 1 (b) and (c). It is important to provide an optimal inoculum size in the fermentation process, as lower inoculum density may give insufficient biomass, whereas a higher inoculum density may produce too much biomass which depletes the nutrients, necessary for the product formation. An inoculum size of 10% (v/v) was found to be the optimum for cell biomass (1.96 g l^{-1}), specific growth rate (0.72 h^{-1}) and HA production (0.82 g l^{-1}), respectively.

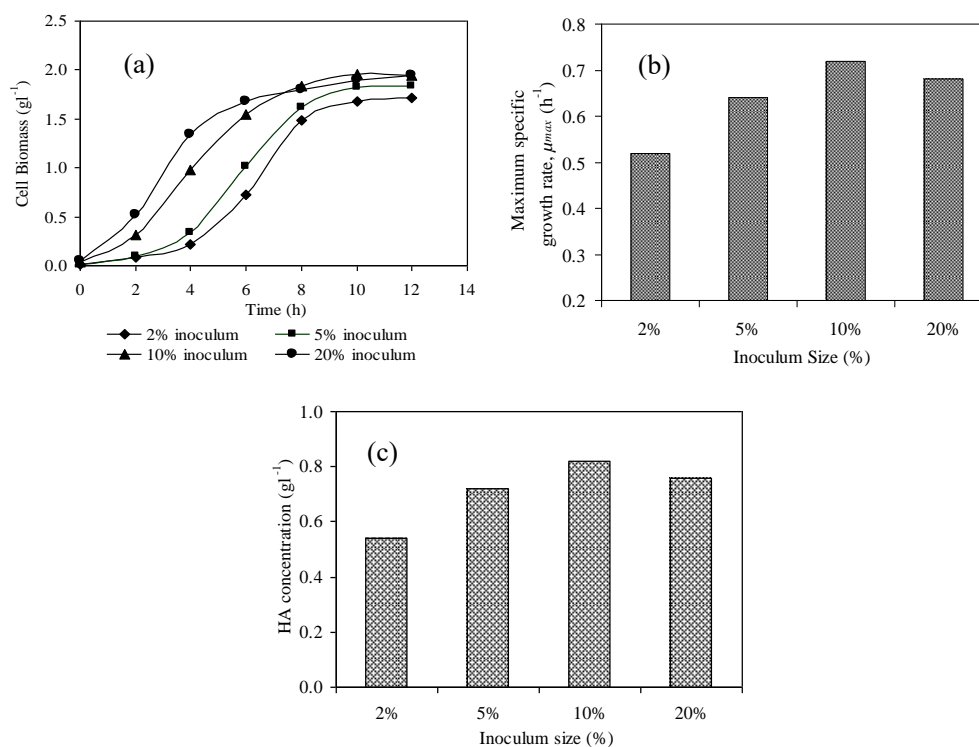


Figure 1. Effect of inoculum sizes on (a) cells biomass, (b) kinetic growth rate, and (c) HA concentration produced by *S. zooepidemicus* in batch fermentation

3.2 Effect of pH

The pH of the media is one of the essential parameters to be controlled in most industrial fermentation processes. The pH was found to have more influence on the polysaccharide production than on the cell growth, and the specific pH directly affected the synthesis of those enzymes responsible for extracellular polysaccharide production [11]. In a shake-flask culture however, the control of the pH of the medium is difficult to achieve. In this study, the pH was adjusted using either 3.0 M HCl or 3.0 M NaOH. The effect of pH on the cell growth and HA production by *S. zooepidemicus* are shown in Figure 2 (a) and (b). The kinetic analyses of this study are presented in Table 1. From the results obtained, the initial pH 7.0 was found to be the optimum for the cell growth and HA production by *S. zooepidemicus*. The higher volumetric cell biomass and HA productivity obtained at pH 7 were $0.172 \text{ g}_{\text{cell}} \text{ l}^{-1} \text{ h}^{-1}$ and $0.101 \text{ g}_{\text{HA}} \text{ l}^{-1} \text{ h}^{-1}$. Similar results were also obtained by [12] where HA production was peaked at pH 7.0 for the same organism. In fact, hyaluronate synthase, an enzyme that catalysed the polymerization of HA was reported to have a maximal activity at pH 7.1 in the cell free extracts [5]. After 12 h of the fermentation period, flask with an initial pH 4.0 and 10.0 showed lower cell biomass and HA production. According to [13], HA was more stable at neutral pH, but under extreme acidic or alkaline condition, it became highly susceptible to hydrolytic degradation, and its rate of depolymerisation was effectively increased by at least an order of magnitude. In fact, a greater degree

of HA degradation was achieved at pH 3.0 than at pH 11.0, and both types of hydrolysis followed a first order reaction.

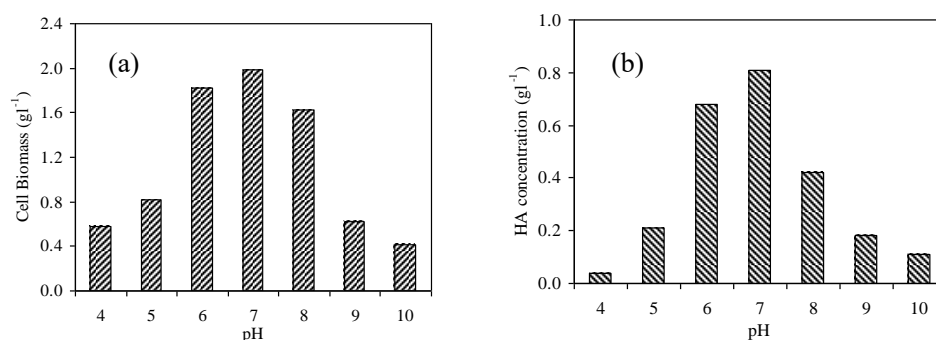


Figure 2. Effect of pH on (a) cells biomass, (b) HA concentration produced by *S. zooepidemicus* in batch fermentation

Table 1. Kinetic analysis on HA production by *S. zooepidemicus* in batch fermentation at different pH condition

Parameter	pH						
	4	5	6	7	8	9	10
Maximum specific growth rate, μ_{max} (h ⁻¹)	0.18	0.42	0.68	0.74	0.59	0.32	0.21
Cell biomass yield (g _{cell} g _{glucose} ⁻¹)	0.029	0.044	0.118	0.121	0.091	0.034	0.021
HA yield (g _{HA} g _{glucose} ⁻¹)	0.002	0.013	0.059	0.067	0.051	0.009	0.0054
Volumetric cell biomass productivity (g _{cell} l ⁻¹ h ⁻¹)	0.048	0.067	0.152	0.172	0.138	0.057	0.034
Volumetric HA productivity (g _{HA} l ⁻¹ h ⁻¹)	0.005	0.024	0.088	0.101	0.052	0.023	0.017

3.3 Effect of Temperature

Incubation temperature is often a critical factor in polysaccharide biosynthesis. Temperature affected the rate of biochemical reactions, the activity of extracellular enzyme, the generation time and activity of the microorganism involved. [8] has reported that the rate of reactions for microorganisms increased with increasing temperature (doubling with every 10 °C rise) until a limiting temperature is reached, after which the growth rate decreased. The optimum temperature for polysaccharide production depends on the type of microorganism. In this study, the temperatures tested were between 30°C to 45°C. The influence of temperature between 30 °C to 45 °C on cell biomass and HA production by *S. zooepidemicus* is shown in Figures 3 (a) and (b). Kinetic analysis for the effect of temperature is shown in Table 2. Results showed that the temperature tested was within the growth temperature range and no cell death was observed. However, the cell growth and HA production were significantly lower at 30°C and 45°C compared to that at 37°C. Thus, fermentation temperature of 37°C was found to be the optimal temperature for HA production by *S. zooepidemicus*, since high HA concentration and cell biomass were obtained at this particular condition. The results obtained were agreed fairly well with [2] suggesting that 37°C is a healthy compromise for an efficient HA production.

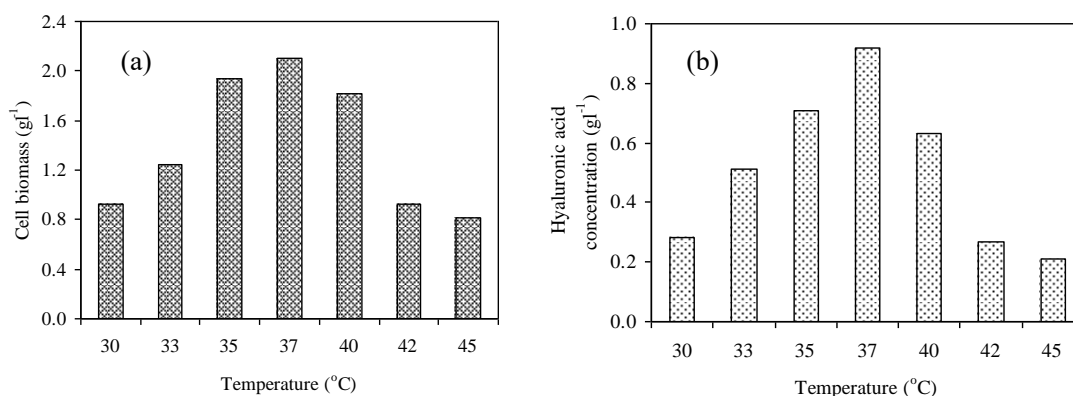


Figure 3. Effect of temperature on (a) cells biomass, (b) HA concentration produced by *S. zooepidemicus* in batch fermentation

Table 2. Kinetic analysis on HA production by *S. zooepidemicus* in batch fermentation at different temperature

Parameter	Temperature (°C)						
	30	33	35	37	40	42	45
Maximum specific growth rate, μ_{max} (h^{-1})	0.27	0.41	0.68	0.75	0.59	0.38	0.21
Cell biomass yield ($g_{cell} g_{glucose}^{-1}$)	0.046	0.068	0.122	0.131	0.091	0.048	0.041
HA yield ($g_{HA} g_{glucose}^{-1}$)	0.023	0.042	0.071	0.076	0.068	0.041	0.040
Volumetric cell biomass productivity ($g_{cell} l^{-1} h^{-1}$)	0.077	0.103	0.162	0.186	0.152	0.072	0.062
Volumetric HA productivity ($g_{HA} l^{-1} h^{-1}$)	0.023	0.062	0.062	0.073	0.048	0.043	0.021

3.4 Effect of Agitation

In order to study the effect of agitation on the cell growth and HA production by *S. zooepidemicus*, a series of experiments were conducted at different agitation speeds using rotary shaker incubator. The agitator speed was varied between 50 to 500 rpm. The effect of this agitation speed on cell biomass and HA production is shown in Figures 4 (a) and (b). The kinetics analysis of this study is presented in Table 3. Cell biomass and HA production were significantly increased with the increase in agitation speed up to 300 rpm, and gave maximum cell biomass at $2.20 g l^{-1}$ and HA concentration at $1.23 g l^{-1}$ when the culture was agitated at 300 rpm. This may be explained by the fact that at 300 rpm brings about a greater subdivision of bubbles, resulted in a larger surface area for gas-liquid mass transfer to occur and thus, bringing a reduction in the thickness of the gas and liquid films responsible for the resistance to the mass transport. Thereafter, the HA yield and cell growth were decreased as the agitation speed increased from 400 to 500 rpm. At a higher agitation speed (500 rpm), the decrease in cell growth was not drastically observed compared to that of the HA production. This indicated that HA concentration is more sensitive to agitator speed than the cell growth. [5] reported that, a higher shear rate may be required in order to release the HA capsule from the bacterial into the medium but very high agitation speed might be deleterious to HA quality, and therefore damaged the biopolymer.

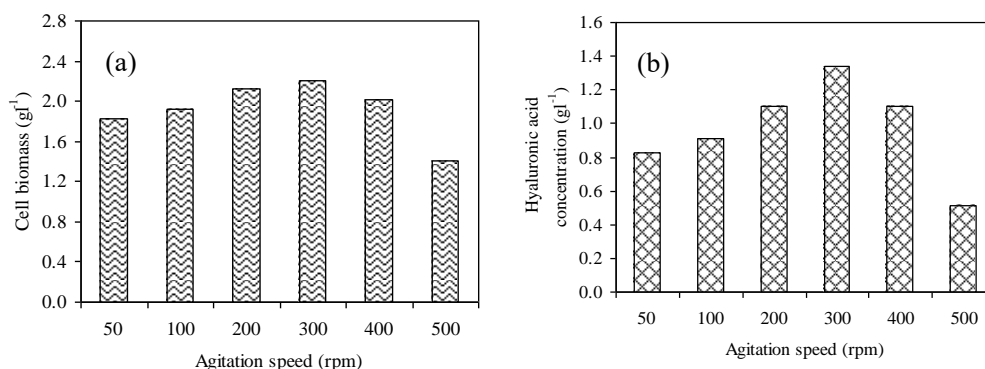


Figure 4. Effect of agitation speed on (a) cells biomass, (b) HA concentration produced by *S. zooepidemicus* in batch fermentation

Table 3. Kinetics analysis on HA production by *S. zooepidemicus* in batch fermentation at different agitation speed

Parameter	Agitation speed (rpm)					
	50	100	200	300	400	500
Maximum specific growth rate, μ_{max} (h^{-1})	0.64	0.66	0.78	0.81	0.61	0.54
Cell biomass yield ($g_{cell} g_{glucose}^{-1}$)	0.091	0.098	0.14	0.17	0.12	0.071
HA yield ($g_{HA} g_{glucose}^{-1}$)	0.041	0.048	0.058	0.096	0.054	0.026
Volumetric cell biomass productivity ($g_{cell} l^{-1} h^{-1}$)	0.151	0.178	0.183	0.183	0.168	0.118
Volumetric HA productivity ($g_{HA} l^{-1} h^{-1}$)	0.069	0.078	0.092	0.111	0.091	0.045

4. Conclusion

This study presents the fermentation results of HA by *S. zooepidemicus* in batch system. Process parameter optimization and kinetic analysis of HA by *S. zooepidemicus* were performed using “one-factor-at-a-time” approach. It can be concluded that, an inoculum size of 10% (v/v), pH of 7.0, temperature of 37 °C and agitation speed of 300 rpm showed the highest cell biomass of $0.17 g_{cell} g_{glucose}^{-1}$, HA yield of $0.096 g_{HA} g_{glucose}^{-1}$ with the maximum specific growth rate, volumetric cell biomass productivity and obtained at optimum parameters were, μ_{max} ($0.81 h^{-1}$, $0.183 g_{cell} l^{-1} h^{-1}$ and $0.111 g_{HA} l^{-1} h^{-1}$, respectively.

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