PRODUCTION OF HYALURONIC ACID BY *STREPTOCOCCUS ZOOEPIDEMICUS* ATCC 39920

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PRODUCTION OF HYALURONIC ACID BY *STREPTOCOCCUS ZOOEPIDEMICUS* ATCC 39920

by

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LIST OF SYMBOLS

A	First factor or input variable- agitation	rpm
B	Second factor or input variable-pH	dimensionless
b_o	The offset term	dimensionless
b_i	The linear effect	dimensionless
b_{ii}	The first order interaction effect	dimensionless
b_{ij}	The squared effect	dimensionless
C	Third factor of input variable- temperature	^oC
Δt	Time delay	h^{-1}
K_La	Volumetric mass transfer coefficient	s^{-1}
K_s	Monod saturation constant	dimensionless
K_I	Inhibition constant	dimensionless
m_s	maintenance coefficient	$g_{glucose}.g_{biomass}^{-1}h^{-1}$
R^2	Squared regression correlation coefficient	dimensionless
S	substrate concentration	$g\ l^{-1}$
T	Temperature	^oC
Δt	time delay	h
t	time	h
μ_o	specific growth rate	h^{-1}
μ_{max}	maximum specific growth rate	h^{-1}
X	biomass concentration	$g\ l^{-1}$
X_o	maximum biomass concentration	$g\ l^{-1}$
Y	The Predicted response	dimensionless
Y_1	The first response	dimensionless
Y_2	The second response	dimensionless
Y_3	The third response	dimensionless
$Y_{HA/glu}$	HA yield per glucose consumed	$g_{HA}\ g_{glucose}^{-1}$
$Y_{X/glu}$	Biomass yield per glucose consumed	$g_{cell}\ g_{glucose}^{-1}$

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CCD	Central Composite Design
DoE	Design of Experiment
CV	Coefficient of Variation
DF	Degree of Freedom
OD	Optical density
O_2	Oxygen
HA	Hyaluronic acid
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
mM	Milimolar
P	Probability
pH	Measure of acid or alkali
rpm	Rotation per minute
RSM	Response Surface Methodology
ROS	Reactive Oxygen Species
SBA	Sheep Blood Agar
SOD	Superoxide dismustase

PENGHASILAN ASID HYALURONIK OLEH *STREPTOCOCCUS ZOOEPIDEMICUS* ATCC 39920

ABSTRAK

Memandangkan permintaan yang semakin meningkat bagi produk asid hyaluronik (HA) sebagai bahan permulaan bagi penyediaan polimer bioserasi dan biopenurunan baru yang telah digunakan dalam kebanyakan industri perubatan dan kosmetik, kajian fermentasi mikrob bagi penghasilan HA oleh *S. zooepidemicus* ATCC 39920 telah dijalankan dalam sistem kelompok dan suapan selanjar. Pengoptimuman keadaan fermentasi menggunakan teknik "satu-faktor-pada-satu-masa" telah dijalankan di dalam kultur kelalang goncang supaya julat dan keadaan yang sesuai untuk digunakan dalam kajian ini boleh dianggarkan. Saiz inokulum 10% (v/v), pH 7.0, suhu 37^{o}C dan kelajuan pengaduk 300 rpm menunjukkan kadar pertumbuhan spesifik yang maksimum, biojisim dan penghasilan HA yang tinggi pada 0.72 j^{-1}, 1.96 gl^{-1} and 0.82 gl^{-1}, masing-masing.

Untuk mempertingkatkan penghasilan sel dan produk, pengoptimuman bagi parameter untuk proses ini telah dijalankan secara mendalam di dalam kultur kelalang goncang dengan menggunakan kaedah sambutan permukaan (RSM) bersama dengan rekabentuk komposit berpusat (CCD). Keadaan optimum yang diperolehi adalah seperti berikut: suhu 37^{o}C, pH 6.96 and kelajuan pengaduk 300 rpm, dengan penghasilan HA per glukosa yang digunakan ($Y_{HA/glukosa}$) sebanyak 0.11 $g_{HA}\ g_{glukosa}^{-1}$ dan penghasilan biojisim per glukosa yang digunakan ($Y_{X/glukosa}$) sebanyak 0.19 $g_{biojisim}\ g_{glukosa}^{-1}$. Suhu dan pH menunjukkan kesan positif terhadap pengumpulan Hydrogen peroksida (H_2O_2), tetapi tidak sensitif terhadap kelajuan

pengaduk. H_2O_2 membantu pertumbuhan dan penghasilan HA pada kadar yang terhad (< 10 mM), meskipun H_2O_2 dirembeskan oleh sel-selnya sendiri.

Bagi kajian di dalam bioreaktor, rekabentuk komposit berpusat (CCD) telah digunakan untuk mengkaji keadaan yang optimum bagi pengudaraan, kelajuan pengaduk dan kepekatan awal glukosa supaya penghasilan HA oleh *S. zooepidemicus* dapat dipertingkatkan. Faktor-faktor ini telah memberi pengaruh yang kuat terhadap penghasilan sel dan HA. Keadaan kultur yang optimum bagi pengkulturan tersebut adalah seperti berikut: pengudaraan 4.5 $lmin^{-1}$, kelajuan pengaduk 330 rpm dan kepekatan awal glukosa 45.0 gl^{-1}. Pada keadaan optimum ini, kadar pertumbuhan spesifik yang maksimum (μ_{maks}) adalah 1.18 j^{-1}, penghasilan HA dan biojisim per glukosa digunakan 0.218 $g_{HA}\ g_{glukosa}^{-1}$ dan 0.37 $g_{biojisim}\ g_{glukosa}^{-1}$, pekali isipadu pemindahan jisim k_La dan kepekatan H_2O_2 adalah 0.0323 s^{-1} and 6.94 mM, masing-masing.

Model kinetik tidak berstruktur dikenali sebagai Logistik, persamaan Logistik digabungkan bersama Leudeking-Piret dan Logistik digabungkan bersama persamaan Leudeking-Piret dengan masa lengah, Δt telah dicadang dan disahkan, dan keputusan menunjukkan ketiga-tiga model yang digunakan ini sesuai untuk menerangkan pertumbuhan sel, penggunaan glukosa dan penghasilan HA pada setiap kepekatan awal glukosa yang berbeza di dalam keadaan berkelompok. Kerencatan bagi substrat terhadap pertumbuhan sel bagi strain ini turut juga dikaji menggunakan pelbagai jenis model. Model Hans dan Levenspiel dan Teissier menunjukkan padanan yang sesuai bagi semua sistem yang dikaji dengan R^2=0.997 dan 0.985, masing-masing.

S. zooepidemicus dikenali sebagai perangsang negatif dan menghasilkan H_2O_2 yang boleh memberi kesan terhadap pertumbuhan sel, penghasilan HA dan penggunaan glukosa. Bagi mengenalpasti daya kerintangan sel terhadap agen pengoksidaan H_2O_2, kesan penambahan H_2O_2 daripada luar terhadap pelbagai fasa pertumbuhan kelompok telah dikaji. Keputusan menunjukkan semakin tinggi kepekatan H_2O_2 ke dalam kultur media semakin tinggi kesan perencatan berlaku. Sel daripada fasa pegun telah menunjukkan ketahanan yang lebih terhadap keadaan beroksida berbanding dengan sel di dalam keadaan fasa eksponen dan fasa susulan. Sel di fasa pegun lebih tahan kerencatan sehingga mencecah 50 mM kepekatan H_2O_2 di dalam kaldu pertumbuhan.

Untuk mempertingkatkan penghasilan biojisim dan HA, fermentasi secara suapan selanjar dengan menggunakan teknik kemostat telah digunakan. Kesan pengoksidaan daripada agen pengoksidaan, H_2O_2 pada keadaan mantap juga turut dikaji. Lebih tinggi kadar pencairan lebih kurang biojisim sel, walaupun substrat pertumbuhan terhad glukosa menunjukkan peningkatan. Produktiviti biojisim dan HA yang tertinggi iaitu 0.41 gl^{-1} j^{-1} dan 0.30 gl^{-1} j^{-1} diperolehi pada julat kadar pencairan iaitu 0.24 j^{-1}. Beberapa pemalar kinetik turut juga ditentukan; kadar pertumbuhan spesifik yang maximum, μ_{maks}, 1.09 j^{-1}, pemalar ketepuan pertumbuhan sel Monod, K_s 54.87 gl^{-1} dan kadar julat yang kritikal, D_c 0.504 j^{-1}, masing-masing. Model tidak berstruktur berasaskan persamaan Monod dan Leudeking Piret telah dicadangkan, dan keputusan menunjukkan model-model tersebut sesuai digunakan untuk menerangkan pertumbuhan sel, penggunaan glukosa dan penghasilan HA di dalam keadaan mantap.

PRODUCTION OF HYALURONIC ACID BY *STREPTOCOCCUS ZOOEPIDEMICUS* ATCC 39920

ABSTRACT

In view of the demand for hyaluronic acid (HA) products as a starting material for the preparation of new biocompatible and biodegradable polymers that have applications in many medical and cosmetics domains, research on microbial fermentation of HA by *S. zooepidemicus* ATCC 39920 was undertaken in batch and continuous system. Optimization of fermentation conditions using "one-factor-at-a-time" technique was investigated in shake-flask culture in order to determine the suitable ranges and conditions for use in the study. An inoculum size 10% (v/v), pH 7.0, temperature 37^{o}C, and agitation speed 300 rpm gave the highest specific growth rate, cell biomass and HA production at 0.72 h^{-1} 1.96 gl^{-1} and 0.82 gl^{-1}, respectively.

In order to improve the cell and product yield, optimization of the process parameters were further studied in shake-flask culture using response surface methodology (RSM) coupled with the central composite design (CCD). The optimum conditions obtained from this method were as follows: temperature 37^{o}C, pH 6.96 and agitation 300 rpm, with HA yield per glucose consumed ($Y_{HA/glucose}$) 0.11 $g_{HA}\ g_{glucose}^{-1}$ and biomass yield per glucose consume ($Y_{X/glucose}$) 0.19 $g_{cellbiomass}\ g_{glucose}^{-1}$, respectively**.** Temperature and pH have positive effect on accumulation of hydrogen peroxide (H_2O_2) but insensitive against agitation. H_2O_2 supported growth and HA production at limited levels (< 10 mM) regardless of H_2O_2 being released by the cells itself.

For bioreactor studies, a central composite design (CCD) was also employed to evaluate the optimal aeration, agitation rates and initial glucose concentration so as to improve HA production by the *S. zooepidemicus* cells. The cell biomass and HA production were greatly influenced by these factors. The optimal culture conditions were at aeration 4.5 $lmin^{-1}$, agitation speed 330 rpm, and initial glucose concentration 45.0 gl^{-1}. Under these conditions, maximum specific growth rate (μ_{max}) obtained was 1.18 h^{-1}, HA and biomass yield per glucose consumed were 0.218 $g_{HA}\ g_{glucose}^{-1}$ and 0.37 $g_{cell}\ g_{glucose}^{-1}$, volumetric mass transfer coefficient and H_2O_2 concentration were 0.0323 s^{-1} and 6.94 mM, respectively.

The unstructured kinetic models namely the Logistic, the Logistic incorporated Leudeking-Piret-like equation, and Logistic incorporated Leudeking-Piret equation with time delay, Δt were proposed and validated. It is found that all the models were suitable to describe bacterial growth, substrate utilization and HA production at different initial glucose concentration ranging from 10-60 gl^{-1} in batch culture. The inhibition of substrate on the growth of the tested strain was also studied using different type of models. The Han and Levenspiel model, and the Teissier-type model gave the best fit for all the systems studied with R^2=0.997 and 0.985.

S. zooepidemicus is known to be catalase negative and produced H_2O_2 which may affect cell growth, HA production and glucose utilization. In order to determine the resistances of the cell against this oxidizing agent, H_2O_2, the effect of external addition of H_2O_2 were determined at different batch growth phase. The results showed that higher the concentration of H_2O_2 in the medium, the greater was the inhibition. Cells from stationary phase were shown to be more oxidative tolerant

compared to the exponential and lag phase. Stationary phase cells were resistant to the oxidative agent up to a concentration of 50 mM in the culture broth.

In order to improve the cell biomass and HA production, a continuous culture using a chemostat technique also applied. The oxidative response by the oxidizing agent, H_2O_2 at steady state condition also studied. The higher the dilution rate, the lower was the cell biomass although the growth limiting substrate glucose increased. The highest cell biomass and HA productivity of 0.41 $gl^{-1}h^{-1}$ and 0.30 $gl^{-1}h^{-1}$ were obtained at a dilution rate of 0.24 h^{-1}. Several kinetic parameters were also determined; maximum specific growth rate, μ_{max} 1.09 h^{-1}, Monod cell growth saturation, K_s 54.87 gl^{-1} and critical dilution rate, D_C 0.504 h^{-1}, respectively. An unstructured model based on Monod and Leudeking Piret equation was proposed and found to be suitable to describe the cell growth, HA production and glucose consumption by *S. zooepidemicus* at steady state conditions.

CHAPTER ONE

INTRODUCTION

1.1 Background

Polysaccharides are renewable resources which offer a wide variety of potentially useful products to mankind. They offer a wide variety of glycosidically linked structures based on 40 different monosaccharides (Sutherland, 2004). Polysaccharide comprised of a distinct class of biopolymer, produced universally among living organisms. Polysaccharides form major structural components of the walls of marine crustaceans, plants, algae and microorganism. In fact, they exhibit a large variety of unique and rather complex chemical structures, different physiological functions, and wide range of applications (Steinbüchel and Rhee, 2005). The importance of polysaccharides in industrial and cosmetic applications is enormous with the world market biopolymer estimated to be around US$ 5 to 10 billion per annum (Goh, 1998). Some of the major commercial polysaccharides and their sources are shown in Table 1.1 (Linton *et al.,* 1991).

Table 1.1: Commercial polysaccharides and their sources (De Philippis *et al.,* 2001; Sutherland, 1998)

Type of polysaccharides	Sources
Alginate	Marine algae (Phaeophyceae)
Carrageenan	Marine algae (Rhodophyceae)
Curdlan	Bacteria (*Agrobacterium*)
Dextran	Bacteria (*Leuconostac*)
Gellan	Bacteria (*Sphingomonas*)
Arabic Gum	Plants (*Acacia*)
Hyaluronic acid (HA)	Bacteria (*Streptococcus zooepidemicus*)
Xanthan	Bacteria (*Xanthomonas campestris*)
Starch	Plants
Pullulan	Fungi (*Aureobasidium pullulans*)
Pectin	Plants (citrus fruits and other sources)

During the second half of the 20th century, many new and useful polysaccharides of scientific and commercial interests have been discovered which can be obtained from microbial fermentation (Sutherland, 1998). The microbial cell generally contained various polysaccharide structures contributing its shape and rigidity. In prokaryotes, according to Sutherland (2002), these polysaccharides include peptidoglycan (composed of repeated units of N-acetyl-D-glucosamine and N-acetyl-D-glucuronic acid, found in almost all Eubacteria), lipopolysaccharides (in gram-negative bacteria) and teichoic and teichuronic acid (in gram-positive bacteria). Microorganisms such as bacteria and fungi produced three distinct types of carbohydrate polymers: (1) extracellular polysaccharides (EPS), which can be found either as a capsule that enveloped the microbial cell or as an amorphous mass secreted into the surrounding medium, (2) structural polysaccharides, which can be part of the cell wall and (3) intracellular storage polysaccharides (Kumar and Mody, 2009). Some of the EPS's that have been successfully commercialized are listed in Table 1.2.

Table 1.2: Industrially important microbial extracellular polysaccharides, their sources and uses (Goh, 1998; Lapasin and Pricl, 1999)

Polysaccharide	Microbial source	Applications
Alginate	*Pseudomonas aeruginosa* *Azotobacter vinelandii*	Textile, food and separation media
Curdlan	*Alcaligenes faecalis*	Food
Dextran	*Leuconostoc mesenteroides*	Therapeutic and separation media
Gellan (S-60)	*Aereomonas elodea* *Sphingomonas paucimobilis*	Food and reagent
Hyaluronic acid	*Streptococcus zooepidemicus*	Biomedical and cosmetic
Lentinan	*Lentinus edodes*	Anti-cancer
Pullulan	*Aereobasidium pullulans*	Food packaging industrial
Succinoglycan	*Pseudomonas* *Rhizobium meliloti*	Oil field and industrial
Welan (S-130)	*Alcaligenes*	Oil field and industrial
Xanthan	*Xanthomonas campestris*	Food, oil field, paint and industrial

The scientific and industrial success of polysaccharides of microbial origin was due to several factors. First, they can be produced under controlled conditions with selected species; second, they usually present a high structural regularity and third, different microorganisms can synthesize a wide range of very specific ionic and neutral polysaccharides with widely varying compositions and properties (Lapasin and Pricl, 1999). Such a variety is not found among plant polysaccharides and perhaps more importantly, it cannot be imitated by means of synthetic chemistry (Sutherland, 1999).

One of the important polysaccharides which can be derived from animal sources and also produced by microbial fermentation is hyaluronic acid (HA) (Marcellin *et al.,* 2009). In Yuzurlhara, a small Japanese village outside Tokyo, ten times as many people live the age of 85 than anywhere else in the United States. The inhabitants were also healthier and enjoyed great joint mobility, healthy vision and displayed usually smooth and well toned skin, even after spending decades farming under the sun. Scientist and researchers have examined the reasons for this selective longetivity and have determined that HA is the key component: the native lives on a diet of sweet potatoes and sticky vegetables that promoted the synthesis of HA (Petrella *et al.,* 2007; Price *et al.,* 2007).

HA was discovered by Meyer and Palmer in 1934. This biopolymer was first isolated from the vitreous of bovine eyes (Kogan *et al.,* 2007). It was special mucopolysaccharides that exist naturally in all living organisms. It occurred primarily in the extracellular matrix (ECM) and pericellular matrix (Almond, 2007). The largest content of HA so far is found in rooster combs (Kogan *et al.,* 2007). A

brief listing of the occurrence of HA in different animal tissues and their contents is provided in Table 1.3. Besides vertebrates, HA is also present in the capsules of some bacteria (e.g strains of *Streptococci*), which is absent in fungi, plants and insect (Sutherland, 2004)

Table 1.3: Occurrence of hyaluronic acid (HA) in different animal tissues and its concentration (Kogan *et al.*, 2007)

Tissues or body fluid	Concentration (μgml^{-1})	Remarks
Rooster comb	7500	The animal tissue with by far the highest HA content
Human umbilical cord	4100	Contains primarily HA with a relatively high molar mass
Human joint (synovial) fluid	1400-3600	The volume of the synovial fluid increases under inflammatory conditions. This leads to a decreased HA concentration
Bovine nasal cartilage	1200	Often used as a cartilage model in experimental studies
Human vitreous body	140-340	HA concentration increases upon the maturation of this tissue
Human dermis	200-500	Suggested as a 'rejuvenating' agent in cosmetic dermatology
Human epidermis	100	HA concentration is much higher around the cells that synthesize HA
Rabbit brain	65	HA is supposed to reduce the probability of occurrence of brain tumors
Human thoracic lymph	0.2-50	The low molar mass of this HA is explained by the preferential uptake of the larger molecules by the liver endothelial cells
Human urine	0.1-0.3	Urine is also an important source of hyaluronidase
Human serum	0.01-0.1	HA concentrations increase in serum from elderly people as well as in patients with rheumatoid arthritis and liver cirrhosis.

Swan and Kuo (1991) reported that HA was an essential functional component of almost all tissues in vertebrate organisms and various animal tissues; for instant, rooster combs, shark fin and bovine eyeballs have been used as sources of isolation and production of higher molecular weight of HA traditionally. However, it was difficult to isolate such a high molecular weight of HA economically from these sources because it formed a complex with proteoglycans (O'Regan *et al.*, 1994). It is presently impractical to control the molecular weight of the biopolymer while it is synthesized in animal tissue (Vert, 2001). Subsequently, extraction and purification processes result in an inherent molecular weight reduction. From a social viewpoint, the use of animal-derived biochemical for human therapeutics is being met with growing resistance, besides ethic arguments, because of the risk of viral infection (Goh, 1998). Besides that, extraction from animal sources also showed higher risks of contamination with protein and other glycosaminoglycans (GAGs), which required extensive purification (Poli *et al.*, 1996). Therefore, most industries have instead turned into bacterial fermentation processes, hoping to obtain commercially viable biopolymers (Goh, 1998).

In the nineties, HA from microbial sources through fermentation process has provoked great scientific and industrial biopolymer interest especially using gram-positive bacteria *Streptococcus zooepidemicus* (Sutherland, 2004). This interest is due to an extraordinary properties of HA as well as the increasing demand of HA used as a starting materials for the preparation of new biocompatible and biodegradable polymers that have applications in many medical and cosmetics domains (Jagur-Grodzinski, 1999). For the medicinal purposes, HA is used as an anti-adhesive component for a variety of clinical applications such as ophthalmic

viscosurgery, viscosupplementation for arthritis (Prestwich *et al.*, 1998; Volpi *et al.*, 2009), wound healing and plastic surgery (Alaish *et al.*, 1994), supplementation of the synovial fluid in patients with osteoarthritis, as a membrane for postsurgical separation of tissues (Kogan *et al.*, 2008), and as drug delivery systems (Prestwich and Vercruysse, 1998; Luo *et al.*, 2000). Many cosmetics contained HA as an ingredient, because it is believed to keep skin young and fresh by preventing dryness as a results of its water binding capacity (Baumann, 2009).

Commercially available HA is produced with the molecular weight ranging from less than $1x10^6$ Daltons to as high as $8x10^6$ Daltons (Sutherland, 2004). The world market of HA is difficult to estimate, but many companies produced it for medical and cosmetic applications. The current world market for HA-based products and therapies is worth at least US$ 500,000 per annum and this is rapidly expanding in view of the emerging applications (Kogan *et al.*, 1997). The current US market of $157 million for viscosupplementation in osteoarthritis is driven by two products, Genzyme and Hyalgan from Fidia Pharmaceutical (Sutherland, 1990). The details of the major pharmaceutical products are listed in Table 1.4. Although, microbes have been exploited for the production of HA by fermentation especially from *S. zooepidemicus*, yet an economically viable biosynthetic process for HA production is still to be developed and explored.

Table 1.4: Some commercially pharmaceuticals products containing hyaluronic acid (HA) (Lerner,1997)

No	Products	Concentration (mg ml^{-1})	Size (ml)	Company	Application	Price (USD)
1	AMO Vitrax Syringe	30	65	Allergen	Ophthalmology	138
2	Amvisc Plus Syringe	16	5	Chiron	Ophthalmology	145
3	Amvisc Plus Syringe	16	8	Chiron	Ophthalmology	190
4	Amcisc Syringe	12	5	Chiron	Ophthalmology	112
5	Healon GV Syringe	14	55	Kabi	Ophthalmology	101
6	Healon GV Syringe	14	85	Kabi	Ophthalmology	131
7	Healon Syringe	10	55	Kabi	Ophthalmology	94
8	Hyalgan SDV	10	2	Sanofi	Osteoarthritis	166
9	Hyalgan Syringe	10	2	Sanofi	Osteoarthritis	166
10	Provisc Syringe	10	4	Alcon	Ophthalmology	117
11	Provisc Syringe	10	55	Alcon	Ophthalmology	142
12	Provisc Syringe	10	85	Alcon	Ophthalmology	178
13	Synvisc Syringe	8	3	Wyeth	Osteoarthritis	705
14	Viscous Syringe	40	5	Alcon	Ophthalmology	151

1.2 Problem Statement

HA has a great potential for medical and pharmaceutical applications owing to its ability to retain large volumes of water and its rheological properties (Holmstrom, 1967). It is well known that HA and its salts can be obtained from animal sources such as human umbilical cord, rooster combs, and from bacterial streptococci culture (A and C hemolytic groups) (Ogrodowski *et al.*, 2005). However, some disadvantages are

associated with the animal sources, such as relatively low yields, contamination, and labor-intensive processing during the purification steps (Chong *et al.,* 2005).

Nowadays, HA from microbial sources through fermentation process has received increased attention especially using the gram-positive bacterium *S. zooepidemicus*. Production of HA by this species has been advocated for several reasons, technical and economical as well as ethical (Akasaka *et al.,* 1989). Furthermore, the biopolymer produced is identical to the eukaryotic HA (Ogrodowski *et al.,* 2005). The development of large scale processes had overcome many obstacles, including growth in chemically defined media (Van De Rijn and Kessler, 1980; Armstrong and John, 1997; Armstrong *et al.,* 1997; Cooney *et al.,* 1999), the production of high molecular-weight material (Kim *et al.,* 1996), the elimination of toxic impurity such as streptomycin and increasing the yield (Kumar *et al.,* 2008; Rangaswamy and Jain, 2008). This bacterial reaction not only gives an opportunity to optimize the product yield and quality through genetic engineering and control of culture conditions, but can also reduce the manufacturing cost (Armstrong *et al.,* 1997; Goh, 1998; Marcellin *et al.,* 2009).

Fermentations can be operated in batch, fed-batch or continuous process. In most fermentation process, fed-batch process offers many advantages over batch and continuous culture (Bibila and Robinson, 1995; Sanchez and Cardona, 2008). A cyclic fed batch culture has an advantage where the productive phase of a process may be extended under controlled conditions (Arpornwichanop and Shomchoam, 2007). However, the controlled periodic shifts in growth rate provide an opportunity to optimize product synthesis, particularly if the product of interest is a secondary

metabolite whereby maximum production takes place during stationary phase (Stanbury *et al.,* 1995). Since the HA is a primary metabolite (Gaudin and Meulenberg, 2006) and with respect to studies by Ellwood *et al.* (1996) on production of HA by continuous fermentation of *S. zooepidemicus* in a chemostat culture which gave high yields of HA and uncontaminated by toxic impurities, it was reasonable to focus this study on batch and continuous system.

During the fermentation processes, microorganisms is often exposed to multi environmental stresses such as low and high temperature, low pH, high osmotic pressure, nutrient starvation, and oxidation that can cause loss or reduction of bacterial viability, reproducibility as well as organoleptic and fermentative qualities. Among these stress factors, oxidation can be considered as one of the most deleterious to the cells, causing cellular damage at both molecular and metabolic levels, which is known as an oxidative stress (Moodie *et al.,* 2004). Although, several studies have been conducted regarding the optimal culture condition for HA production, there are still a considerable divergence on the optimization and design of culture conditions especially in HA batch culture.

For HA production, it seemed that the agitation and aeration rates were additional factors that influenced cell growth and HA production, and it has been a subject for many investigations (John *et al.,* 1994; Kim *et al.,* 1996; Armstrong and John, 1997; Hasegawa *et al.,* 1999; Chong and Nielsen, 2003; Mashitah *et al.,* 2005). Based on the work carried out by John *et al.* (1994) on the effect of pH, agitation and aeration on the production of HA by *S. zooepidemicus*, HA yield from glucose could be improved by improvement of agitation and aeration rates during the fermentation

process. Huang *et al.* (2006) reported that the dissolved oxygen (DO) saturation acted as a stimulant to HA synthesis; for scaling up, the DO that was above the critical level (5% air saturation) need to be maintained, and a mild agitation was needed for homogeneity in the fermenter. Nevertheless, there were some drawbacks related to the *Streptococcal* HA production routes. According to Goh (1998) under aerated conditions, HA production was found to be suppressed as a result of growth inhibition by the reactive oxygen species which is hydrogen peroxide (H_2O_2) in shake flask culture. Halliwell and Gutteridge (2007) and Mashitah *et al.* (2005) reported that H_2O_2 though chemically less reactive, was nevertheless a threat to the structure and functions of proteins, nucleic acids, lipids and membranes, whether it was added externally or produced intracellularly. Little attention has been paid and very little published information is available on the effect of oxygen demand and its relationship to the oxidative stress responses derived from reactive oxygen species on the production of HA by *S. zooepidemicus*. In fact, optimization of the aeration and agitation rate during fermentation using glucose as a carbon source with respect to HA and H_2O_2 production by this strain has not yet been investigated.

As in lactic acid bacteria, *S. zooepidemicus* lack in catalases and cytochrome system in their respiratory chain (Goh, 1998). As a result, the reactive oxygen species (H_2O_2), can accumulate to a level that is autoinhibitory or inhibitory to other metabolites or bacteria (Condon, 1987). Although, Mashitah *et al.* (2005) reported that in aerobic batch fermentation, a protective response could have been created by *S. zooepidemicus* cells up to a certain level of H_2O_2, but at high levels the protective mechanism became fully saturated. Previously, Halliwell and Gutteridge (1990) also

reported that most living organisms may evolve defense mechanisms or oxidative stress response when exposed to sub-lethal levels of H_2O_2.

Different concentrations of H_2O_2 were believed to have different influence on the different types of microorganisms (Roundy, 1958). For example, for *Escherichia coli*, the concentration of H_2O_2 that lied between 0.1 and 1.0 mM have shown to have killing or toxic effects and the killing was maximal between the concentration of 1.0 and 3.0 mM; whereas, killing occurred at higher concentrations of H_2O_2 (up to at least 50 mM) has showed to occur at slower rate (Farr and Kogoma, 1991). For *S. zooepidemicus*, a systematic study of how H_2O_2 effects streptococcal HA concentration in a bioreactor has not been reported, nor has there been any description of how the interaction between H_2O_2 and HA capsule and its defense mechanism are related over batch and continuous time courses.

From the above discussion, it is apparent that the *S. zooepidemicus* cells have significant effect on the oxidative stress by the cell itself as well by the addition of exogenous H_2O_2. Consequently, fermentation kinetic analyses over a broad range of environmental conditions need to be carried out for the development of better strategies for the optimization of the fermentation process. It is anticipated that such an approach would provide a means of assessing the importance of such effects to substrate limitation, product inhibition, endogenous metabolism, etc. and will allow an estimation of the values of the kinetic constants involved in each of them.

1.3 Research Objectives

In view of the above observations, this study was carried out with the following objectives:

i. To determine the optimum fermentation conditions of HA production by *S. zooepidemicus* using "one-factor-at-a-time" technique and statistical design approach in shake-flask culture.

ii. To determine the optimum of initial glucose concentration, aeration and agitation speed in a bioreactor of batch mode using a statistical design approach in order to improve the HA production by *S. zooepidemicus* cells.

iii. To propose and validate the kinetics model for microbial growth, glucose consumption and inhibition and HA production by *S. zooepidemicus* in batch culture.

iv. To determine the sensitivity of *S. zooepidemicus* cells to an oxidizing agent, H_2O_2 added externally at different growth phases during HA production in batch culture.

v. To evaluate the effect of dilution rate, proposed and validate the kinetic models of cell growth, substrate utilization, HA production and accumulation of H_2O_2 by the *S. zooepidemicus* cells in continuous mode.

1.4 Scope of Study

In view of the demand for HA products in pharmaceutical and cosmetic industries by microbial fermentation, this study presents an investigation on the fermentation of HA by *S. zooepidemicus* ATCC 39920 at various conditions of fermentation using optimization and kinetic analysis to increase the production of HA either in batch or continuous system. An oxidative stress response to the *S. zooepidemicus* cells due to accumulation of H_2O_2 in culture broth and external addition of H_2O_2 into the fermentation medium was studied at different phases of the fermentation processes.

Initially, process optimization of various inoculum sizes, temperature, pH, and agitation speed required for the production of HA by *S. zooepidemicus* using "one-factor-at-a-time" approach were evaluated in shake-flask cultures. After that, optimization using a statistical approach was carried out with a selected independent factor in a similar batch mode. At present, there is still no report on the use of statistical design in improving HA production from *S. zooepidemicus*. This method has been widely used in order to improve the product yield, reduce development time and overall process costs (Kammoun *et al.,* 2008; Guo *et al.,* 2009). In this study, process optimization was employed using response surface methodology (RSM) based on central composite design (CCD). The interaction between the factors that influenced the response of cell biomass yield, HA yield, and accumulation of H_2O_2 in culture broth has been discussed.

Since the initial glucose concentration, aeration, and agitation have significant impact on cell growth and HA yield, process optimization using DOE

coupled with RSM was further used to investigate the optimum condition in a bioreactor for improving the HA production. The focus of this study is not only to clarify the effect of oxygen demand to the cell growth and HA production by changing the agitation speed and aeration, but also to study the effect of various initial glucose concentrations so as to find the limitation and inhibition characteristics of the substrate utilization by the tested strain. Volumetric mass transfer coefficient (k_La) and accumulation of H_2O_2 in culture broth were also taken into account as a response during the process optimization.

Kinetic studies of cell growth, glucose uptake and HA productions were carried out to evaluate the fermentation characteristics. Three different models namely Monod and Logistic equations for cell growth, the Logistic incorporated Leudeking-Piret-like equation for glucose consumption and the Logistic incorporated Leudeking-Piret equation with time delay, Δt for HA production were proposed. The inhibition of substrate and toxic materials production on the growth of *S. zooepidemicus* in HA production using glucose as the major carbon source in a batch culture was also studied. Different kinetic models for inhibition studies were tested and the results obtained for each parameter were compared.

Oxidative stress in cell is usually studied by exposing the cell to high concentration of pro-oxidants for a short period of time by single addition of stressing chemicals, and comparing the enzymes activities obtained in stressed cultures and control cultures a few hours after the addition of the stressing compound such as H_2O_2. Studies on the effect of H_2O_2 are quite interesting as it is very reactive and a strong oxidizing agent. Since H_2O_2 is uncharged, it can readily diffuse across

membrane. H_2O_2 causes significant damage because it is not restricted to its point of synthesis in the cell and enter into numerous other reactions. Therefore, the fermentation products may be damaged and decreased under aerobic conditions as a result of the inhibition and the metabolism by H_2O_2. Due to that, the cultures response to oxidative stress produced either by the *S. zooepidemicus* cells itself or by the addition of exogenous H_2O_2 has been studied at different phases of fermentation, and various conditions of the fermentation processes.

Although traditional batch fermentation seemed to dominate the bacterial biopolymer fermentation today (Garcia-Ochoa *et al.*, 2000; Ogrodowski *et al.*, 2005), there is much interest in using fed batch and continuous culture for improving the biomass and HA productions. However, with respect to *Streptococci* cell, which have cell wall could be changed by growth under different nutrient limitations, in this study, a continuous fermentation using chemostat technique has been carried out at different dilution rate on the growth, substrate utilization, HA production, and accumulation of H_2O_2 in culture broth. Thus, fermentation kinetic model of microbial growth, substrate utilization and product formation for continuous mode were proposed and validated.

1.5 Organization of the Thesis

There are five chapters in this thesis and each chapter described the sequence of this study.

- Chapter 1: This introductory chapter emphasized the importance of HA production to its commercial end-used functions. Problem statements, research objectives, scope of research and thesis organization were also highlighted.

- Chapter 2: This chapter surveyed the history, chemical structure, application and properties of HA. Process fermentation of HA in batch and continuous system, oxidative stress phenomena, kinetic and modeling studies during fermentation process were also discussed in detail.

- Chapter 3: The material and methods used in this study were discussed in detail in this section.

- Chapter 4: This chapter presents the fermentation results of HA by *S. zooepidemicus* in batch and continuous system. Process parameter optimization of HA by *S. zooepidemicus* in shake flask culture and bioreactor were obtained from the design of experiment (DoE) coupled with response surface methodology (RSM). Sensitivity of the oxidizing agent (H_2O_2) in culture broth at every phase of the fermentation process was also discussed. The kinetics and modeling for fermentation process in batch and continuous systems were also presented.

- Chapter 5: This chapter summarized the overall findings based on the results obtained in the previous chapter. Recommendations for future research were also given in this section.

CHAPTER TWO

LITERATURE REVIEW

2.1 Hyaluronic acid (HA)

Hyaluronic acid (HA) is a naturally occurring biopolymer, which serves as an important biological function in bacteria and higher animals including humans. In 1934, Karl Meyer described a procedure for isolating a novel glycosaminoglycan from the vitreous humors of bovine eyes and named it hyaluronic acid (HA) (from the Greek hyalos=glassy, vitreous) (Luo *et al*., 2000). This substance contained uronic acid and an amino sugar, but no sulfoesters (Weigel *et al*., 1997). During the 1930s and 1940s, HA was isolated from many sources such as the vitreous body, synovial fluids, umbilical cord, skin, rooster comb and also from streptococci (Yamada and Kawasaki, 2005). The physical-chemical characterization of HA was carried out during 1950s and 1960s (Sutherland, 1994). Hardingham and Muir (1972) discovered that HA interacted with cartilage proteoglycan and served as a central structural backbone and cartilage. This was the first example of a specific interaction between HA and protein and many more such interactions were discovered during 90's. After 1980, the research has spread in many directions, mainly because it has been assumed that HA belonged to the proteoglycans and synthesis of HA occurred in Golgi body (Sutherland, 1994). The application of HA for medical purposes was first discovered by Balaz *et al.* in 1982 (Sutherland, 1994). A highly viscous and non-inflammatory preparation was produced and commercial scale for both ophthalmic surgery and as viscous-supplementation for synovial fluids in patients with osteoarthritis has been applied and well-known throughout the world (Kogan *et al.*, 2007).

2.2 Structure and Properties of HA

Naturally occurring HA could be found in the tissue of higher animals, in particular as intercellular space filler (Balazs *et al.,* 1991). It is found at higher concentrations in the vitreous humour of the eye and in the synovial fluid of articular joints (Fraser *et al.*, 1977). In gram positive streptococci, it appeared as a mucoid capsule surrounding the bacterium (Plate 2.1 and 2.2).

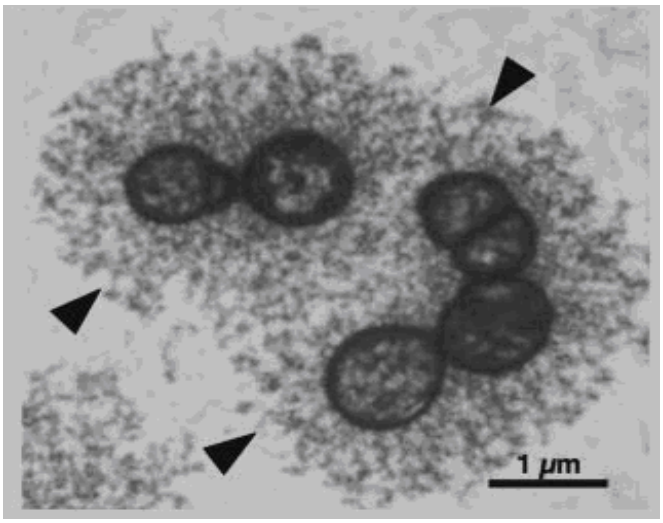

Plate 2.1: *S. zooepidemicus* cell with HA capsule from aerated culture (Goh, 1998)

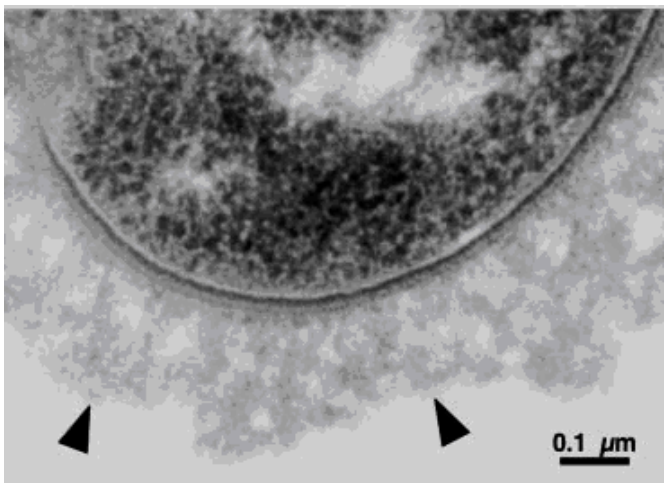

Plate 2.2: Cross-section of *S. zooepidemicus* cell with HA capsule from aerated culture (Goh, 1998)

The following description of the structures and properties of HA are based on that of Kogan *et al.* (2007). The utility of this biopolymer is derived from a remarkably simple construction. HA belongs to the family of glycosaminoglycan, also known as mucopolysaccharide. Glycosaminoglycan is a group of biopolymers, which include chondroitin sulphate, dermatin sulphate and heparan sulphate. This polymer comprised of D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) linked by β(1-3) glycosidic bond, with the disaccharide repeating units linked β(1-4) glycosidic bonds (Figure 2.1) (Ogrodowski *et al.,* 2005). However, the structure of HA was the most simple, the only one that is not covalently associated with the core protein, not synthesized in Golgi apparatus, and the only non-sulfate one (Chong *et al.,* 2005 and Kogan *et al.,* 2007).

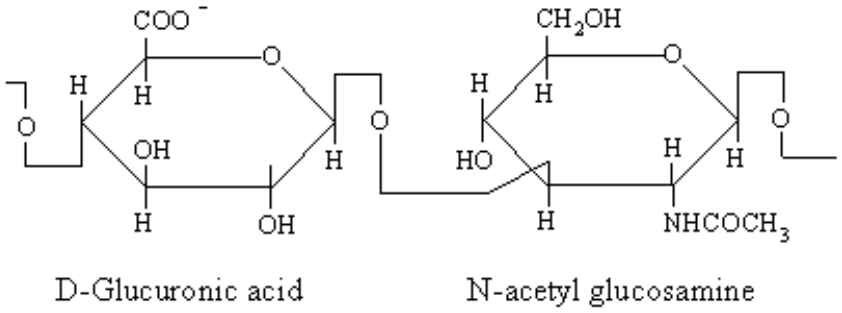

Figure 2.1: Structure (disaccharide repeating unit) of HA (Ogrodowski *et al.,* 2005)

When HA is incorporated into a neutral aqueous solution, hydrogen bond formation occurred between the water molecules and adjacent carboxyl and N-acetyl groups. This imparted a conformational stiffness to the polymer, which limited its flexibility (Fraser *et al.,* 1997). The hydrogen bond formation resulted in a unique water-binding and retention capacity of the polymer. It also followed that the water-binding capacity was directly related to the molecular weight of the molecule (Laurent and Fraser, 1992). Sutherland (1998) reported that up to six liters of water

may be bound in one gram of HA. The molecular mass of HA can reach as high as $1x10^3$ kDa. Such high molecular mass and its associated unique viscouselastic and rheological properties predispose HA to play an important physiological roles in living organisms and made it an attractive biomaterial for various medical applications. The extrusion of HA through the cell membrane as it is produced permitted unconstrained polymer elongation, and hence, molecule with a very high molecular weight (Scott *et al.,* 1991).

2.3 Biosynthesis of HA

HA is produced naturally in all eukaryotic cells (Sutherland, 2001). It is synthesized by a class of integral membrane proteins called hyaluronan synthases (HAS), of which vertebrates have three types: has*A*, has*B*, and has*C* (Chong *et al.,* 2005). The has*A* gene product was a 42 kDa membrane protein which displayed similarity in amino acid sequence to chitin synthase (DeAngelis, 1993), whereas has*B* and has*C* encode UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase, respectively. Both of them were essential in the synthesis of UDP-GlcUA. In fact, the UDP-GlcNAc precursor is believed to be synthesized by the so-called housekeeping genes since it is required in the synthesis of peptidoglycan (O'Reagen *et al.,* 1994). These enzymes can be elongated by repeatedly adding glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) to the nascent polysaccharide as it is extruded through the cell membrane into the extracellular space (Chong *et al.,* 2005). Both monomers of HA are derived from intermediates of glycolysis – GlcUA from glucose-6-phosphate and GlcNAc from fructose-6-phosphate (Chong *et al.,* 2005). Biosynthetic pathway for HA is described and presented in Figure 2.2 (Goh, 1998; Chong *et al.,* 2005).

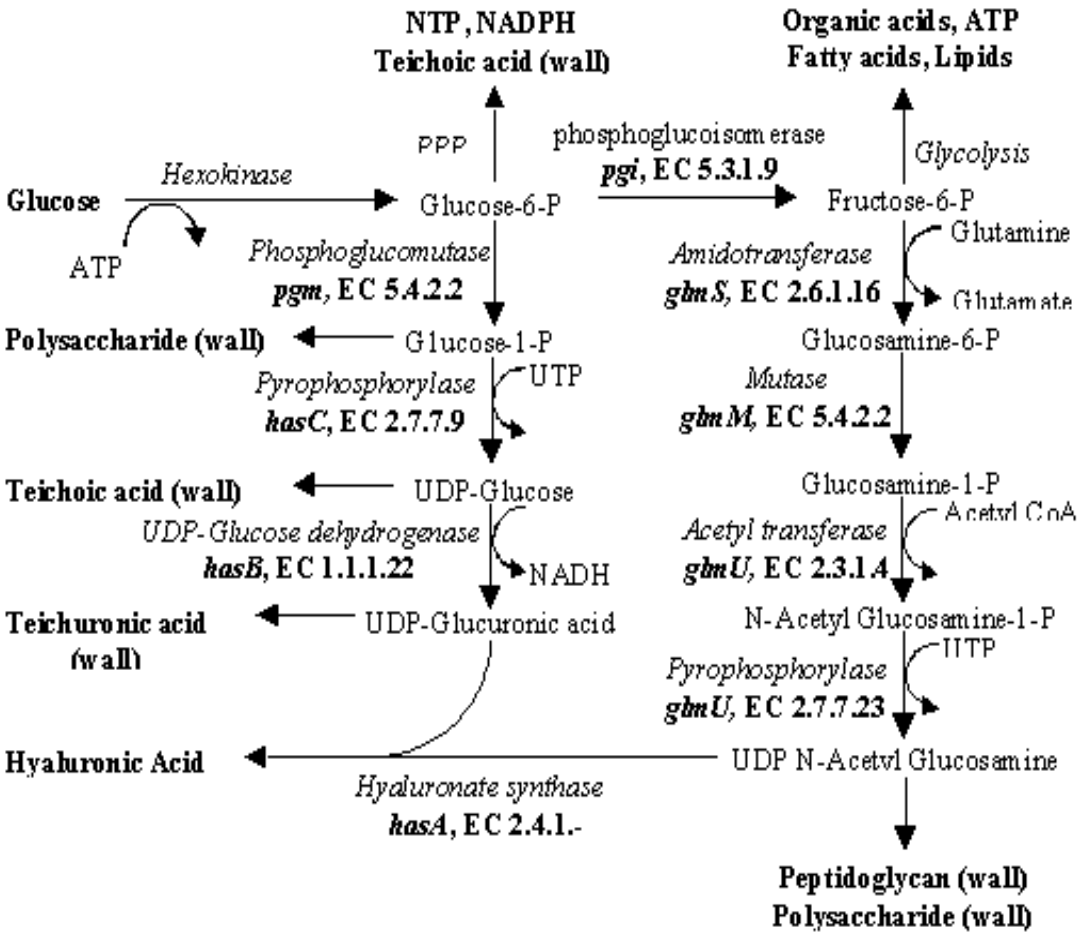

Figure 2.2: Biosynthetic pathway of HA (Goh, 1998)

The first reaction in the pathway leaving the glucose-6-phosphate node as a common step in the production of storage polysaccharides in many organisms. The α-phosphoglucomutase (EC 5.4.2.2) converts glucose-6-phosphate to glucose-1-phosphate in a reversible reaction. UDP-glucose pyrophosphorylase (EC 2.7.7.9), then catalyses the reaction of UTP and glucose-1-phosphate to produce the nucleotide sugar UDP-glucose. UDP-glucuronic acid is then obtained by a specific oxidation of the primary alcohol group of UDP-glucose through the action of UDP-glucose dehydrogenase (EC 1.1.1.22). The pathway originated from fructose-6-phosphate was involved in the production of amino sugars. Amino group transfer from glutamine to fructose-6-phosphate by an amidotransferase (EC 2.6.1.16)

yielded the glucosamine-6-phosphate. Acetyl group transfer by an acetyltransferase (EC 2.3.1.4) formed N-acetyl glucosamine-6-phosphate (Goh, 1998; Chong *et al.*, 2005).

Actually, this was an energy-consuming step since hydrolysis of the thioester bond in acetyl-CoA liberated the energy equivalent of ATP hydrolysis. Phosphate group rearrangement by a mutase (EC 5.4.2.3) generated N-acetyl glucosamine-1-phosphate from N-acetyl glucosamine-6-phosphate. Finally, a pyrophosphorylase (EC 2.7.7.23) added UDP to obtain UDP-N-acetylglucosamine. The participation of UTP in these reactions generates activated glycosyl donors that can be polymerised into HA by HA synthase.

A total of 4 mol ATP were consumed to produce 1 mol HA disaccharide repeating unit; 2 mol ATP were consumed in two glucokinase reactions to provide a phosphorylated hexose precursor for each branch of the HA pathway, and the other 2 mol ATP were utilized to regenerate the donor species UTP. The oxidation reaction catalysed by UDP-glucose dehydrogenase generated 2 mol of NADH for each 1 mol of HA synthesized. In the facultative microbes that naturally produced HA, these reduction equivalents cannot be utilized for energy generation (Chong *et al.*, 2005). Besides furnishing precursors for HA synthesis, these two pathways also supplied the structural constituents of the bacterial cell wall, specifically peptidoglycan, teichoic acids and antigenic wall polysaccharides. These three major wall components accounted for 20% (w/w) of the cell dry weight, and represented a significant drain on the precursor pool used to synthesis HA (Yamada and Kawasaki, 2005).

2.4 Applications of HA

Balaz et al., (1982) pioneered the application of HA for medical purposes which produced a highly viscous and non inflammatory process (Hargittai and Hargittai, 2008). The US Food and Drug Administration (FDA) have approved this drug for clinically utilizable material, such as in eye surgery, knee joint and the treatment of burns and skin ulcers (Yadav *et al.,* 2008). HA is widely needed by all range of people regardless of sexes and age. The basic area of the clinical applications of HA and its derivative are classified by Balaz *et al.* (1989) as shown in Table 2.1, and applications of HA in the biomedical field are summarized in Table 2.2.

Table 2.1: Classification and clinical application of HA (Garg and Hales, 2004)

Application	Description
Viscosurgery	To protect delicate tissue and provide space during surgical manipulations, as in ophthalmological surgeries.
Viscoargumentation	To fill and augment tissue spaces, as in skin, sphincter muscles, vocal and pharyngeal tissues.
Viscoseparation	To separate connective tissue surface traumatized by surgical procedure or injury, in order to prevent adhesions and excessive scar formation.
Viscosupplementation	To replace or supplement tissue fluids, such as replacement of synovial fluid in painful arthritis, and to relieve pain.
Viscoprotection	To protect healthy, wounded or injured tissue surfaces from dryness or noxious environmental agents and to promote the healing of such surfaces.

Table 2.2: Summary of the current and potential applications of HA and its derivatives.

Application of HA	Description	References
Ophthalmic	HA has been used as viscous gel which could be injected into the arterial chamber of the eye to protect tissues such as the cornel endothelium	Nishida *et al.*, 1991
Arthritis	HA has been used for several degenerative joint diseases as an alternative to the traditional steroid therapy	Wobig *et al*., 1999
Wound Healing and Scaring	HA has been used to foster the healing process, for burn and chronic ulcer patients	Goa and Benfield, 1994
Adhesion Prevention	HA preparations such as Separafilm from Genzyme can reduce adhesions and improve the surgical outcome	Beck, 1997
Drug Delivery	HA is an ideal molecule for use as carrier of drugs, particularly for local administration. Investigations are on-going for topical and intravenous drug delivery system using modified HA	Vercruysse and Prestwich, 1998

2.5 Production of HA

HA has been commercially produced by two methods: chemical extraction of animal tissue and microbial fermentation (Chong *et al.*, 2005). There are many HA producers worldwide as shown in Table 2.3. Today, there are still many products in the market that contained HA, which was isolated and extracted from animal tissue. This could be due to the high molecular weight, purity and non-inflammatory properties of HA (Kim *et al.*, 2006).