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EFFECT OF pH, AGITATION AND AERATION ON HYALURONIC ACID PRODUCTION BY *STREPTOCOCCUS ZOOEPIDEMICUS*.

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Summary

The optimum pH for both the rate of production and yield of hyaluronic acid (HA) by *Streptococcus zooepidemicus* from glucose medium was 6.7 $\pm$ 0.2 under anaerobic conditions. High agitation rates (600 rpm) gave superior results compared to 300 rpm. Aeration of the culture (0.3 VVM) improved the HA yield, but not the rate of production and lead to some acetate and CO_2 being formed, in addition to lactate and HA.

INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight, linear polysaccharide which is produced commercially for a wide range of uses, principally as an ophthalmic surgery aid. The traditional method of production involves extraction of HA from rooster combs (Van Brunt, 1986). More recently, HA production by certain Streptococci has become an alternative route, since the bacterial polymer is identical to the eucaryotic HA.

Relatively little has been published concerning optimal conditions for the production of HA by fermentation, although the initial discovery was made by Kendall *et al.* (1937), nearly 55 years ago. Medium pH is known to be important. Hyaluronate synthase, which catalyses the polymerisation of HA, was reported to have maximal activity at pH 7.1 in cell free extracts (Stoolmiller and Dorfman, 1969) and this value is widely used in patent literature. Akasaka *et al.* (1988) reported that pH 7.4 gave the highest viscosity compared to pH 6.0 and 7.9. These authors also studied the effect of agitation rate (up to 450 rpm) on HA yield and found that higher rates gave better yields.

The effect of aeration on HA production is unclear. A recent patent (Biotech Gen. Corp, 1986) reported higher HA yield and molecular weight when the broth was aerated, compared to anaerobic fermentation. However, the metabolism of the bacteria remains fermentative under either condition, since Streptococci lack a complete TCA cycle.

The objective of the experiments reported in this paper was to examine the effect of pH, agitation rate and aeration on HA production by *Streptococcus zooepidemicus.*

MATERIALS AND METHODS

Organism. Streptococcus equi sub-species zooepidemicus ATCC 35246 HA^+ Lac^+ Em^S was obtained from the ATCC (Rockville, MD, USA) as freeze-dried culture and maintained as freeze-dried ampoules in the dark at 8 °C.

Inoculum and media. The contents of a freeze-dried culture were suspended in sterile RO water, mixed thoroughly and streaked onto SBA plates. The agar plates were incubated at 37°C for 30 h. Colonies of HA-producing cells were characteristically white, large and mucoid on this medium. Mucoid colonies were aseptically inoculated into three 10 ml McCartney bottles of M17-glucose broth (Terzaghi and Sandine, 1975) and incubated at 37°C for 3 h. The contents were then added to 70 ml of VIG broth and after identical incubation period, the culture was added to 250 ml of VIG broth in a 500 ml measuring cylinder. After 3 h incubation at 37°C, the optical density obtained a value between 0.6-0.9, and the top 200 ml of solution was used to inoculate the fermenter.

Sheep blood agar comprised Tryptic Soy broth (Difco) containing 5% (v/v) fresh sheep blood. VIG medium comprised Veal Infusion broth (VIB) supplemented with 8 g/l glucose. Solid medium contained 1.8 % (w/v) agar (Difco).

Growth medium A was composed of (in g/l): glucose, 20; yeast extract (Difco), 10; $MgSO_4.7H_20$, 0.5; $Na_2HPO_4.12H_2O$, 2.5; guanine, adenine, uracil, 0.1 mg/l each. Media were sterilised at 121°C for 20 min. Glucose solutions were autoclaved separately and added aseptically to the sterilised medium. Stock solutions (100 mg/l) of adenine, uracil and guanine (BDH Chemicals, Kilsyth, Vic) were sterilised by filtration through a 0.2 μm cellulose acetate membrane filter (Sartorius GmBH, Gottingen, Germany) and was kept at 4 °C. All chemicals and medium components were analytical grade and used with RO (Reverse Osmosis) water.

Cultivation. Batch cultures (2-l) were performed in a 3-l BIOFLO III fermenter system (New Brunswick Scientific Co Inc., Edison, NJ) with agitation provided by three, 6-blade disk turbines at 600 rpm, unless stated otherwise. Automatic temperature (37°C) and pH control was performed, the latter using a steam-sterilisable glass pH probe (Ingold AG, Urdorf, Switzerland) and a sterile solution of 5 M NaOH. The pH probe was calibrated at pH 4 and 7 using standard pH buffer solutions before autoclaving. Dissolved oxygen tension was measured using a steam sterilisable, polarographic oxygen electrode (Ingold AG). The fermenter and medium were sterilised at 121°C for 20 min and allowed to cool before inoculation (25% v/v). For anaerobic fermentations, the sterile medium was sparged with sterile nitrogen gas for approximately 1 h prior to inoculation.

Analysis. Fermentation samples (20 ml) were collected aseptically, after discarding the dead volume. Cell concentration was measured at 530 nm by spectrophotometer (Hitachi Ltd., Tokyo, Japan). Cell dry weight was determined from a calibration curve.

The concentration of glucose, lactate and acetate was determined by HPLC (Johns and Stuart, 1991). Samples were first diluted with Milli Q water (1:6) and filtered through a 0.2 μm cellulose acetate membrane filter (Sartorius). Sample peak area was compared with that of a standard solution, which was injected between every 5 samples.

Hyaluronic acid concentration was determined by a modification of the method of Kery *et al.* (1992). A sample (5 ml) was filtered through a 0.2 μm membrane filter and 20 μl was injected onto an HPLC (Waters Associates) equipped with a Bio-gel TSK 60-XL (7.8 x 300 mm) gel exclusion column (BioRad Lab., Richmond, Calif., USA), operated at ambient temperature with an UV absorbance detector (Waters, Milford, Mass., USA) set at 206 nm. The mobile phase consisted of 50 mM sodium phosphate, (pH 7.8) at an isocratic flow rate of either 1.0 or 1.5 ml/min, depending on the viscosity of the sample. Standard HA was obtained from Pharmacia (Uppsala, Sweden) and was a highly purified aqueous solution with a molecular weight of 3.8×10^6 Da. A standard curve was developed comparing HA concentration with peak height.

The CO_2 concentration in the broth was measured by an off-line carbon dioxide electrode (Orion Research Inc, Cambridge, MA) using a digital pH/mV meter (LH Fermentation Ltd., Stoke Poges, UK). The CO_2 measurement range was between 10^{-4} to 10^{-2} M. Calibration of the probe was against $NaHCO_3$ solutions containing CO_2 buffer.

RESULTS AND DISCUSSION

Effect of pH

The medium pH had a considerable effect on the maximum HA concentration, yield and volumetric rate of HA production in anaerobic, batch cultures of *S. zooepidemicus* (Fig. 1). The optimum HA yield and the highest volumetric rate was obtained at pH 6.7 $\pm$ 0.2. This observation agrees with the results of Akasaka *et al.* (1988) who found a similar decline of HA yield at pH 6 and pH 7.9. By contrast, there was little effect of pH on the final lactate (14 - 16.1 g/l) or glucose (0 - 0.8 g/l) concentrations.

Other than hyaluronic acid and lactate, no other extracellular products of metabolism were detected and these products and cell mass accounted for all the carbon contained in the glucose initially added. No carbon dioxide production was detected, which is consistent with homofermentative metabolism. Whereas lactate production was strongly related to cell growth and ceased once glucose exhaustion occurred, up to 20% of the final HA concentration in solution appeared after glucose was exhausted. It is probable that this is due to the release of capsular HA from cells into solution once glucose exhaustion occurs.

Maximum values of rate data and cell yield ($Y_{X/S}$) from these fermentations are presented in Table 1. The volumetric rate of glucose consumption (r_S) and lactate production (r_L) was maximal at a pH of 6.7 $\pm$ 0.2, and declined sharply at higher pH. The maximum volumetric (r) and specific (q) rates of HA production and glucose consumption occurred within 1 hour of each other. Both specific rates peaked early in the fermentation, typically around 4 $\pm$ 1 h after inoculation.

Table 1. Effect of pH on cell and lactate yield ($Y_{L/S}$) and rates of lactate production and glucose consumption.

pH	$Y_{L/S}$ (g/g)	$Y_{X/S}$ (g/g)	r_{Lmax} (g/lh)	r_{Smax} (g/lh)	q_{Lmax} (g/gh)	q_{Smax} (g/gh)
6.0	0.64	0.18	4.4	6.1	1.4	2.4
6.5	0.73	0.19	6.4	8.4	2.2	3.4
6.7	0.67	0.19	5.8	8.0	1.9	3.0
6.9	0.66	0.19	6.2	9.0	2.4	3.2
7.1	0.64	0.18	3.6	5.3	1.5	2.8
7.3	0.76	0.11	1.9	2.6	1.3	2.1
7.5	0.74	0.13	2.4	2.8	1.3	2.7

The maximum specific growth rate of *S. zooepidemicus* was estimated for each fermentation. In each case, a semi-log plot of the ratio X_t/X_o against time was linear. These data are presented in Figure 2. The value was strongly affected by medium pH, with a maximum at a pH of 6.5. At pH values of 7.1 or more, much lower growth rates were obtained and the fermentation times required were longer.

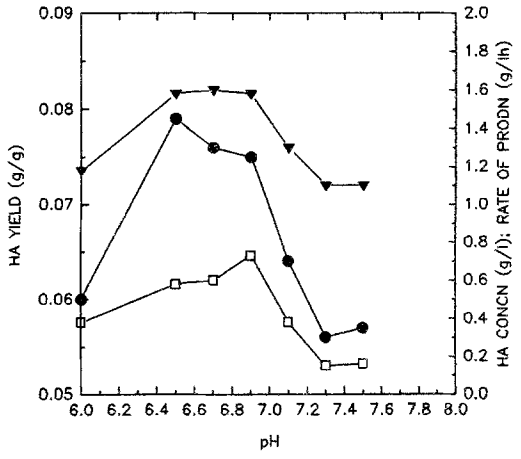

Figure 1. Effect of pH on yield (●), final concentration (▼) and rate of production (□) of HA.

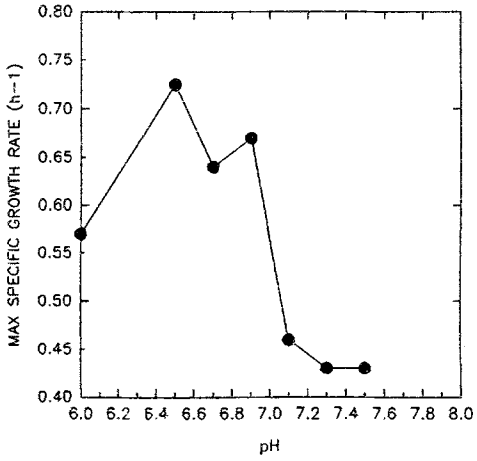

Figure 2. Effect of pH on specific growth rate of *S. zooepidemicus*.

Effect of agitation rate

Agitation rate had a profound effect on both the rate and yield of HA produced in anaerobic fermentations controlled at a pH of 7.1 (Figure 3). In all fermentations, lactate

and hyaluronic acid were the only products detected. In contrast, there was no effect of agitation rate on the growth rate or yield ($Y_{x/s}$) of the bacterium, which were 0.5 h^{-1} and 0.18 $\pm$ 0.02, respectively. The decrease in the rate of HA synthesis at low agitation rate was counterbalanced by an increased rate of lactate synthesis from glucose.

These results are not unexpected, since the production of HA increases significantly the viscosity of the broth. This may impair mass transport of nutrients to the cell and the removal of lactate from the local cell environment. The pseudoplastic nature of hyaluronic acid would lead to reduced solution viscosity at higher shear rates (i.e. agitation speed), relieving these effects. Alternatively, a higher shear rate may be required to release the HA capsule from the bacterial cell into the medium. Very high agitation speeds may be deleterious to HA quality, since a high shear rate may also damage the polymer. The effect of shear rate on the molecular weight distribution of HA produced by microbial fermentation has not been reported.

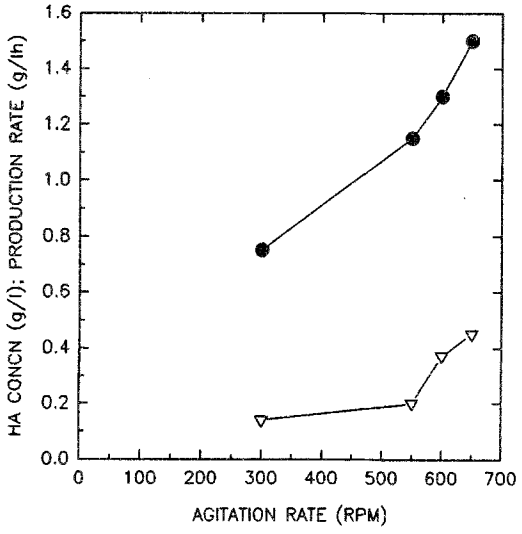

Figure 3. Effect of agitation rate on final concentration (●) and production rate (▽) of of HA.

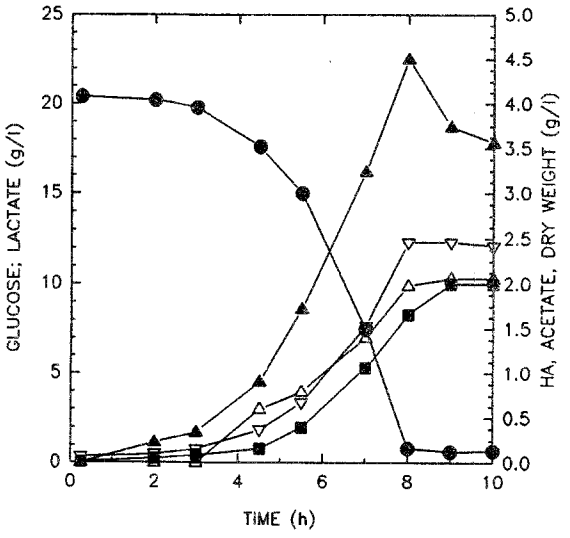

Figure 4. Timecourse of aerated, batch S. zooepidemicus culture. Glucose (●), lactate (▽), HA (■), acetate (△) and dry weight (▲).

Effect of aeration

The results of an aerated culture performed at 0.3 VVM and pH 6.7 are given in Figure 4. In addition to hyaluronic acid and lactate, a significant quantity of acetate was produced late in the exponential growth phase with concomitant carbon dioxide formation. This corresponded to the period of lowest dissolved oxygen tension and appeared to be at the expense of lactate formation. The production of acetate from pyruvate permits a

superior ATP yield from glucose (Atlas, 1988). Streptococci lack an intact TCA cycle, but are able to use molecular oxygen to oxidise nicotinamide metabolites. However, it would appear that this ability is limited, since large quantities of lactate were still produced.

The aerated culture gave higher HA concentration (2.1 g/l) and yield (0.11 g/g) than the equivalent anaerobic fermentation, but the volumetric rate of production was similar. The higher HA concentration in aerated culture is probably due to the superior energy yield obtained by the use of molecular oxygen and the diversion of pyruvate to acetate, rather than lactate. Many patents report the use of aeration in HA fermentations to increase the yield of HA, although the aeration and agitation conditions are usually vague. However, whether the increase in yield is worth the additional cost of aerating the broth is uncertain.

CONCLUSIONS

All three variables, pH, agitation and aeration have a major effect on the production of HA by *S. zooepidemicus*. The optimal pH is 6.7 $\pm$ 0.2 for HA production in batch culture. Performance falls off significantly either side of this range. Agitation rate also lower than 600 rpm in the fermenter used, led to a marked decline in both the yield and rate of HA produced. Aeration improved HA yield from glucose, probably due to improved energy yields. Acetate was produced in addition to lactate under these conditions.

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