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Bioparametric Investigation for the Production of Hyaluronidase by S*treptococcus mitis* MTCC *2695

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ABSTRACT

The effect of some physical and nutritional parameters were studied for the optimum production of extracellular enzyme hyaluronidase employing *Streptococcus mitis* MTCC *2695 by submerged fermentation. The effects of initial pH, incubation temperature, time, inoculum concentration and age of inoculum were studied. Maximum enzymatic activity was observed at initial medium pH 5.8, at 37°C, within 48 h and with 6% inoculum. The effect of different carbon and nitrogen sources and antibiotics were studied. Sucrose and ammonium chloride showed the highest enzymatic activity among different carbon and nitrogen sources studied. The antibiotic clarithromycin showed strong inhibitory effect on hyaluronidase production.

Keywords: enzyme activity, extracellular enzyme, inhibitory effect, nutritional parameters, submerged fermentation
Abbreviations: BSA, Bovine serum albumin fraction-V; **HA**, hyaluronic acid; **hyase**, hyaluronidases; **TSA**, Trypticase Soy Agar

INTRODUCTION

The therapeutical benefit of hyaluronidases (hyase) is based on the cleavage of hyaluronan in tissues resulting in increased membrane permeability, a reduced viscosity and a facilitated diffusion of injected fluids and referred as spreading effect of hyases. The recombinant enzyme acts as an adjuvant, accelerate and increase absorption and dispersion of injected drugs, e.g. antibiotics, to promote resorption of excess fluids and improve the effectiveness of local anaesthesia and to diminish pain due to subcutaneous or intramuscular injection of fluids (Csoka *et al*. 1996), for hypodermoclysis. Moreover it also acts as an adjunct in subcutaneous urography for improving resorption of radiopaque agents (Law and Rowen 1981). Bacterial hyaluronate lyases are considered as virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronan (Akhtar and Bhakuni 2004). Hyase facilitate diffusion of antiviral drugs, dyes and toxins (Duran-Raynals 1933). Hence, hyases, especially bovine testicular hyaluronidase (BTH) preparations, are widely used in many fields like orthopaedics, surgery, dentistry (Tam and Chan 1985), ophthalmology (Meyer and Palmer 1934) (vitrectomy), internal medicine, oncology (Muckenschnabel *et al*. 1998), dermatology and gynecology (Farr *et al*. 1997). Testicular hyases have significant homology with the protein pH-20 present on the posterior head and the acrosomal membrane of mammalian sperm that plays an essential role in fertilization (Primakoff *et al*. 1988). Based upon the medical, physiological, biological and commercial importance of hyases the present work was undertaken to optimize enzyme production parameters for hyases employing *Streptococcus mitis* MTCC *2695 strains under submerged fermentation conditions.

MATERIALS AND METHODS

Streptococcus mitis MTCC-2695 procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India (IMTECH) was used in the present study. It was rejuvenated by subculturing onto Trypticase Soy Agar (TSA) plates supplemented with 5% defibrinated sheep blood. Further, it was subcultured onto nutrient agar slants at 37°C for 24 h. The culture was washed with 5 ml of sterile distilled water and the optical density (OD) was measured at 675 nm resulting OD 0.580 (equivalent to 1.01×10^6 cells / ml) was used as inoculum. A 5% (v/v) level of inoculum was transferred into a 250 ml Erlenmeyer flask containing 50 ml of modified nutrient broth containing (g/l) peptic digest of animal tissue, 5; sodium chloride, 5; beef extract, 1.5; yeast extract, 1.5; casein enzyme hydrolysate type-1, 4; KH_2PO_4, 3; magnesium sulphate, 3; hyaluronic acid (HA), 0.001% with pH 5.8, was employed as production medium.

After inoculation, the flasks were incubated at 37°C on a rotary shaker (Ilshin Lab Co., Korea, Model BBT-1) at 150 rpm for 48 h. During fermentation, the microbial growth and hyase production were monitored. The microbial growth was monitored by measuring OD at 675 nm with UV-Visible spectrophotometer (Systronics, Model-118). At the end of fermentation 5 ml broth was aseptically withdrawn and centrifuged at 8000 × *g* for 30 minutes at 4°C. The clear supernatant was subjected to enzyme assay (Dorfman 1955).

Hyase activity was measured spectrophotometrically by turbidity reduction assay (Tam and Chan, 1983) using hyaluronic acid sodium salt (HA) from *Streptococcus equi* (Sigma Aldrich, USA) as a substrate. The enzymatic assay is based on Dorfman's method (Dorfman 1955) where enzymatic reduction in turbidity is recorded. To 1 ml of HA at 70 µg/ml was incubated with 1 ml of enzyme sample in the presence of 0.05 M sodium phosphate buffer with 0.05 M NaCl (pH 7.0). After incubation of the mixture for 30 min, 2.5 ml of acidified protein solution (1% w/v) BSA in 0.5 M sodium acetate buffer, (pH 3.1) was added and incubated at 37°C for 10 min and reduction in turbidity was read by measuring the absorbance at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that causes a reduction in turbidity, measured spectrophotometrically at 600 nm (A_{600}) in 30 min at 37°C, at pH 7.0 under specified assay conditions similar to that caused by one unit of an international standard.

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Original Research Paper

Optimization of physical parameters

Influence of initial pH

The production medium with composition stated above maintained at an initial pH 7.4 was considered as control and then the medium was adjusted to various levels of pH (4.0-9.0). Fermentation was conducted and samples were assayed for enzymatic activity as described earlier.

Effect of initial temperature and incubation period

To study the effect of initial temperature and incubation period on enzyme production and cell growth, the production medium was inoculated with 5% (v/v) of inoculum and incubated at various temperatures ranging from 20 to 50°C for 96 h at increments of 5°C. The production medium with composition stated above maintained at an initial temperature at 20°C was considered as control. The samples were withdrawn at regular interval of 12 h and assayed for biomass (mg/ml) (Sahoo *et al*. 2007) and enzymatic activity was assayed as described above. The optimal temperature and incubation period obtained at this level resulted optimum enzyme production.

Effect of inoculum size and age on enzyme production and cell mass

The flasks with the basal production medium were inoculated with inoculum age of 24 h old culture at 0.1, 1, 2, 4, 6 and 10% (v/v) level and incubated at 37°C for 96 h. Five ml samples were withdrawn at 12 h intervals and examined for biomass (mg/ml) (Sahoo *et al*. 2007) and enzyme activity as described above. The optimal level of inoculum obtained was used in further experiments. The production medium with composition as stated above inoculated with 5% inoculum level was considered as control.

Optimization of nutritional parameters

Effect of carbon sources

Various carbohydrates such as glucose, lactose, sucrose, mannitol, dextrin, dextrose, starch, sodium CMC and sodium alginate were studied by adding at a concentration of 5 mg/ml to the basal production medium. After fermentation enzyme activity was assayed as described above. A control set containing the basal production medium without inclusion of above carbohydrate source was considered as control.

Effect of inorganic nitrogen sources

Various inorganic nitrogen sources ammonium acetate, ammonium bicarbonate, ammonium chloride, ammonium sulphate, sodium nitrite and sodium nitrate were added (5 mg/ml) to the basal production medium. A control set containing the basal production medium without inclusion of above inorganic nitrogen source was considered as control.

Effect of antibiotics

Different antibiotics clarithromycin, azithromycin, penicillin, gentamicin, cefixime and cefuroxime were added (10 µg/ml) to the basal production medium and assayed for enzyme content as described above after fermentation. A control set without above antibiotics was run simultaneously.

Statistical analysis

All the experiments were conducted in triplicates and the activity and biomass values were expressed in mean ± S.D. of three replicate analysis and tests of significance of differences between the means was carried out by one way ANOVA followed by Dunnet's test (Sigma Stat 3.5). A p value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Effect of process parameters

The results on the effect of initial pH on enzyme at different pH values are shown in **Fig. 1**. The enzyme production significantly increased ($p<0.01$) with decrease in initial pH of the medium when compared with control. The highest enzyme yield was observed at pH 5.8 (181 ± 5.5 U/ml) while the lowest was recorded at pH 9.0 (16 ± 4.3 U/ml). There was a gradual decrease in enzyme yield from pH range 5.8 to 7.2, above and below this range, activity decreased sharply.

Similar results were reported for the production of hyaluronidases from *S. dysgalactiae* and *S. zooepidemicus* exhibiting maximal enzyme activities at pH 5.6 and 5.8 respectively (Sting *et al*. 1990).

The result of incubation period on the fermentation cycle is given in **Fig. 2**. The highest enzyme activity (185 ± 7.1 U/ml) and cell mass (3.6 ± 0.54 mg/ml) at 48 h while the pH changed from 5.8 to 6.1. The results on incubation temperature (**Fig. 3**) indicated significant increase in enzyme production ($p<0.01$) up to 37°C while a significant decrease in enzyme activity was observed above 37°C.

Tam and Chan (1985) reported similar findings for maximum hyase production with Peptostreptococcus species at 48 h, incubation temperature 37°C employing brain heart infusion broth.

The effect of inoculum size on hyase production is indicated in **Fig. 4**. The effect of enzyme activity (179 ± 6.8 U/ml) and cell mass (3.3 ± 0.13 mg/ml) was significantly increased ($p<0.01$) at 6% inoculum level when compared with control. There was a gradual decrease in yield beyond 6% inoculum.

Optimization of nutritional parameters

The effect of various carbohydrates on enzyme production is indicated in **Fig. 5**. All carbohydrates except lactose and

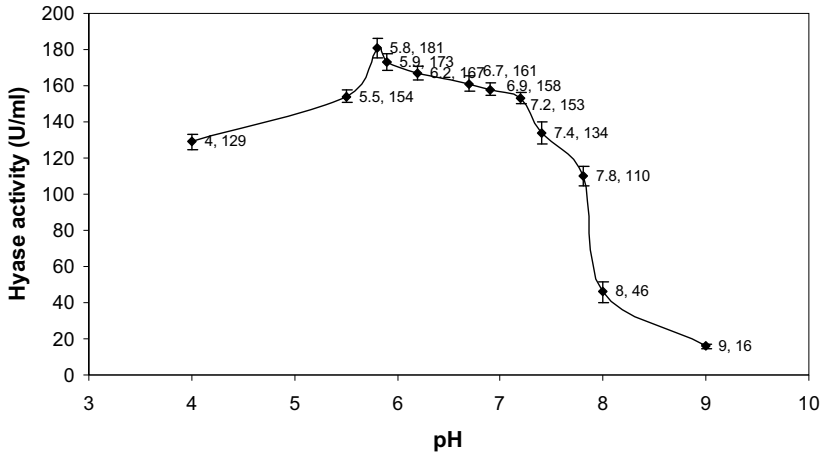

Fig. 1 Effect of initial pH on hyase production.

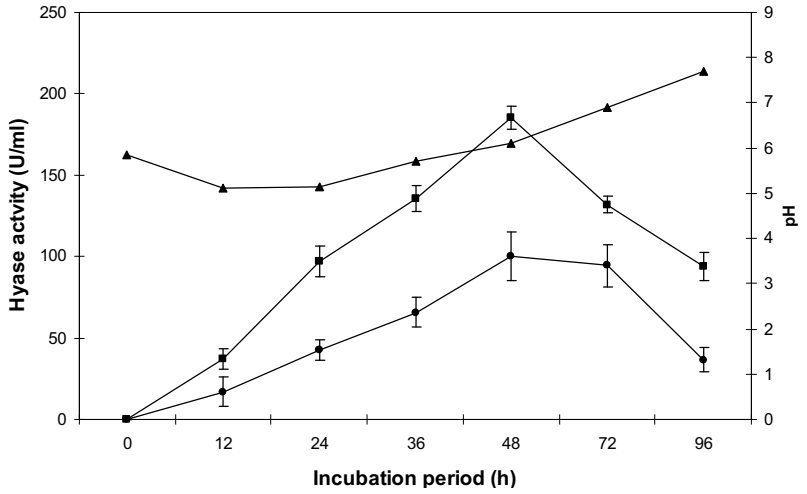

Fig. 2 Time course profiles of hyase production, cell mass (mg/ml) and pH by *S. mitis*. (■) enzyme activity, (●) cell mass, (▲) pH.

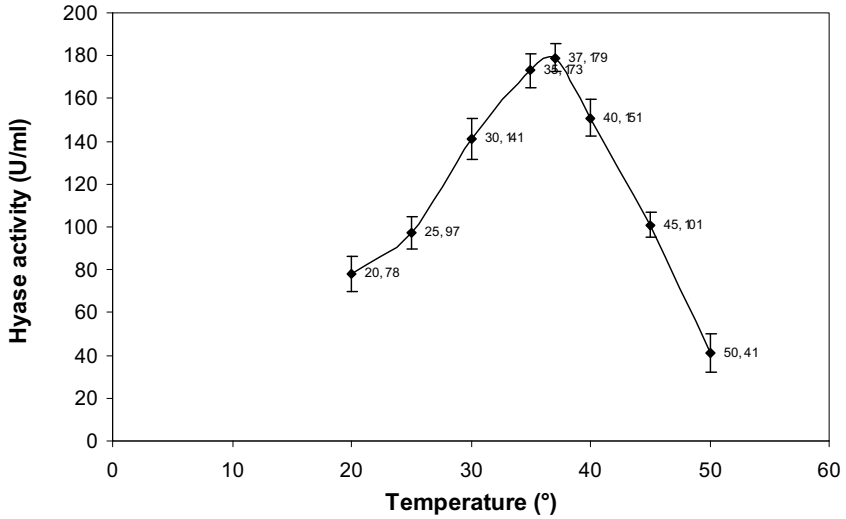

Fig. 3 Effect of initial temperature on hyase production by *S. mitis*.

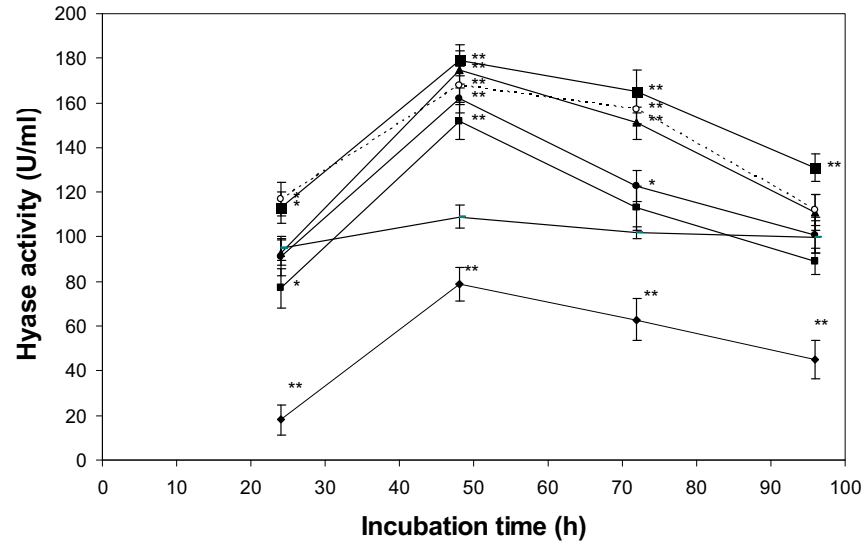

Fig. 4 Influence of inoculum level on hyase production by *S. mitis*. (♦) 0.1%, (■) 1%, (●) 2%, (▲) 4%, (■) 6%, (-○-) 10%, (-) control. **p<0.01, significantly different from control. *p<0.05, significantly different from control.

sodium CMC showed a significant increase in enzyme production compared to control ($p<0.01$). Sucrose exhibited highest enzyme production whereas mannitol, dextrin and sodium alginate decreased the enzyme yield ($p<0.01$)

Rogers (1944) reported the inclusion of glucose (0.5%) in Hedley-Wright broth and glucose, fructose, glycerin and starch as preferred carbon sources for optimum hyase production.

The effect of various inorganic nitrogen sources on enzyme production is shown in **Fig. 6**. Ammonium sulphate showed significant enzyme production (225 ± 6.9 U/ml) followed by ammonium chloride as compared to control ($p<0.01$). Sodium nitrate (103 ± 7.5 U/ml) did not show significant rise in enzyme production.

It was reported that the preferred sources of nitrogen are yeast extract, peptone, gluten meal, cottonseed meal, soybean meal and corn steep liquor as organic, ammonium salts (e.g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea as inorganic nitrogen compounds and amino acid stimulated hyase production (Yoshida *et al.* 1981; United States Patent 4258134).

Among the different antibiotics clarithromycin exhibited the significant inhibitory activity (91%) followed by azithromycin (87%), cefuroxime (79%), cefixime (67%),

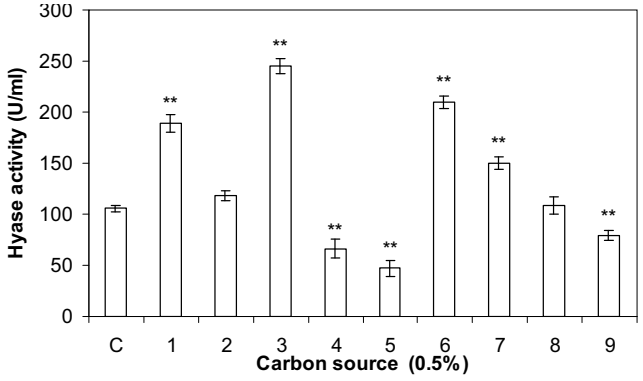

Fig. 5 Influence of carbon sources on hyase production. C. control, 1. glucose, 2. lactose, 3. sucrose, 4. mannitol, 5. dextrin, 6. dextrose, 7. starch, 8. sodium CMC, 9. sodium alginate. **p<0.01, significantly different from control.

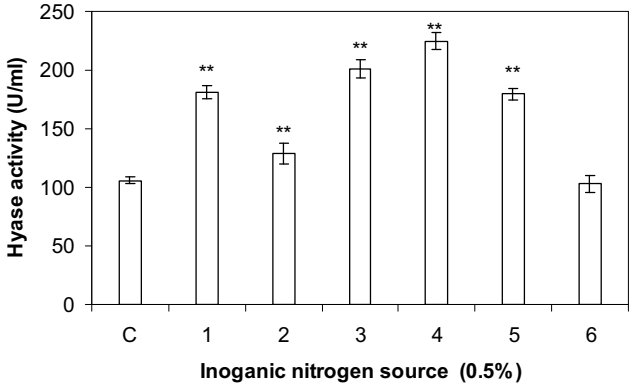

Fig. 6 Influence of inorganic nitrogen sources on hyase production. C. control, 1. ammonium acetate, 2. ammonium bicarbonate, 3. ammonium chloride, 4. ammonium sulphate, 5. sodium nitrite, 6. sodium nitrate. **p<0.01, significantly different from control.

gentamicin (51%) when compared to control where as penicillin (47 %) did not show significant results.

Future studies are aimed at screening and isolation of a promising bacterial isolate with optimum hyase activity with its subsequent purification.

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