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Optimization of some Physical and Nutritional Parameters for Production of Hyaluronidase from S*treptococcus equi* SED 9 by Submerged Fermentation.

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The effects of some physical and nutritional parameters were studied for the optimum production of an extracellular enzyme hyaluronidase employing *Streptococcus equi* subsp. *equisimilis* by submerged fermentation. The effects of initial pH, incubation temperature and period, inoculum age and level were studied. The maximum enzymatic activity was obtained with an initial pH of 5.5 at 37ºC when incubated for 48 h at 10% inoculum level with inoculum age 48 h. The effects of various antibiotics, growth hormones and purine base on hyaluronidase production were studied. The results indicated that meropenem showed strong inhibitory activity among antibiotics where as kinetin and adenine acted as stimulator among all the growth hormones and purine base on hyaluronidase production.

Keywords: Physical, Nutritional parameters, Hyaluronidase, *Streptococcus.* Fermentation

Hyaluronidase (Hyase) enzyme acts as an adjuvant, accelerate and increase absorption and dispersion of injected drugs, fluids[1], resorption of radiopaque agents[2] and facilitate diffusion of antiviral drugs, dyes and toxins[3]. Bacterial hyaluronate lyases are considered as virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronan[4]. Hyases, especially bovine testicular hyaluronidase (BTH) preparations, are widely used in many fields like orthopaedics, surgery, dentistry, ophthalmology (vitrectomy), internal medicine, oncology, dermatology and gynecology[5] and fertilization[6]. The present work was undertaken to optimize enzyme production parameters including effect of pH[7], temperature, incubation period, inoculum level and age of inoculum employing *Streptococcus equi*. The effect of antibiotics, growth hormones and purine base on enzyme production was also studied.

A pathological isolate identified as *Streptococcus equi* subsp. *equisimilis* producing an extracellular hyaluronidase enzyme within 48 h was used for subsequent experiments. The slants were maintained for subsequent experiments at 2% nutrient agar slants at 4ºC. The culture was grown in nutrient agar plates, incubated at 37ºC for 48 h. The culture was rejuvenated in medium containing with composition (g/l) peptic digest of animal tissue, 5; sodium chloride, 4; beef extract, 1.5; yeast extract, 1.5; casein enzyme hydrolysate type-1, 4; Na_2HPO_4, 3; magnesium sulphate, 3; sodium citrate, 1; glycine, 0.025; trehalose, 5; hyaluronic acid (HA), 0.001 % with

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pH 5.5. The growth content of each slant was suspended in 5 ml of sterile water and the suspension was measured at 600 nm resulting 0.621 OD (equivalent to 1.01 x 10^6 cfu / ml) that constitutes the inoculum. A 10% level of inoculum was transferred into 250 ml Erlenmeyer flask containing 50 ml of modified nutrient broth. After inoculation, the flasks were incubated at 37ºC on a rotary shaker at 150 rpm for 48 h. During fermentation, the microbial growth and hyase production were monitored. At the end of fermentation 5 ml broth was aseptically withdrawn and centrifuged at 8000 x g for 30 mins at 4ºC. The clear supernatant was subjected to enzyme assay.

Hyase activity was measured spectrophotometrically by turbidity reduction assay[8] using HA sodium salt (Sigma Aldrich, USA) as a substrate. The enzymatic assay is based on Dorfmans method[9] in which the enzymatic reduction in turbidity, resulting when 1 ml of HA at 70 µg/ml was incubated with 1 ml of enzyme sample solution. The mixture was allowed to stand for 30 mins. To the above incubated mixture, 2.5 ml of acidified protein solution (1% w/v) bovine serum albumin fraction-V (BSA) in 0.5 M sodium acetate buffer, (pH 3.1) was added and incubated at 37ºC 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 reduced the absorbance by 0.1 at 600 nm (A_{600}) in 30 min at 37ºC, pH 7.0 under assay conditions similar to that caused by one unit of an international standard.

To investigate the influence of initial pH, temperature, incubation period, cell growth, inoculum age and inoculum level on enzyme production, the production medium was adjusted to various levels of pH (4.0-9.0), incubated at temperatures ranging from 20ºC to 55ºC for 96 h, inoculum age of 48 h at 0.1 to 12 % and the samples were withdrawn at regular interval of 12 h, assayed for biomass (mg/ml) and enzymatic activity.

Different antibiotics including meropenem, cefaclor, cefotaxime, cefixime, ceftazidime, cefuroxime, ceftriaxone, amoxyllin, amoxyllin+clavulanic acid, clarithromycin, erythromycin, cotrimoxazole, few growth hormones kinetin, gibberlic acid, indole-3-acetic acid and purine base adenine were added (10 µg/ml) to the basal production medium and assayed for enzyme activity. The MIC values of the above antibiotics were also tested against *S. equi* by two fold serial dilution method[10]. MIC values of antibiotics and their inhibitory effect on hyaluronidase production by *S. equi* is given in Table 1.

The highest enzyme activity (165 U/ml) and cell mass (3.55 mg/ml) was observed at pH 5.5 at 37° when incubated for 48 h at 10 % inoculum level as indicated in fig 1. Among the different antibiotics meropenem exhibited MIC (0.03 µg/ml) acted as potent inhibitor of hyase activity with percentage inhibition (95%) where as kinetin (252 U/ml) and adenine (189 U/ml) were considered as stimulators of hyase production.

Table 1. MIC values of antibiotics and their inhibitory effect on Hyaluronidase production by *S. equi*.

Antibiotics (µg/ml)	*S. equi* subsp. *equisimilis* MIC(µg/ml)	PI
Mr (10)	0.03	95
Cj (30)	1.25	65
Ce (10)	≤1	69
Cfx (5)	0.8-1.25	73
Ca (30)	≤0.75	77
Cu (30)	0.75-1	79
Ci (10)	≤1	67
Am (10)	<1.75	48
Ac (10+20)	≤0.25	89
Cw (15)	<1	71
E (10)	1-1.25	63
Co (25)	<2.5	37

PI-indicates percentage inhibition of hyase activity.
Mr-meropenem, Cj-cefaclor, Ce-cefotaxime, Cfx-cefixime, Ca-ceftazidime, Cu-cefuroxime, Ci-ceftriaxone, Am-amoxyllin, Ac-amoxyllin+clavulanic acid, Cw-clarithromycin, E-erythromycin, Co-cotrimoxazole.

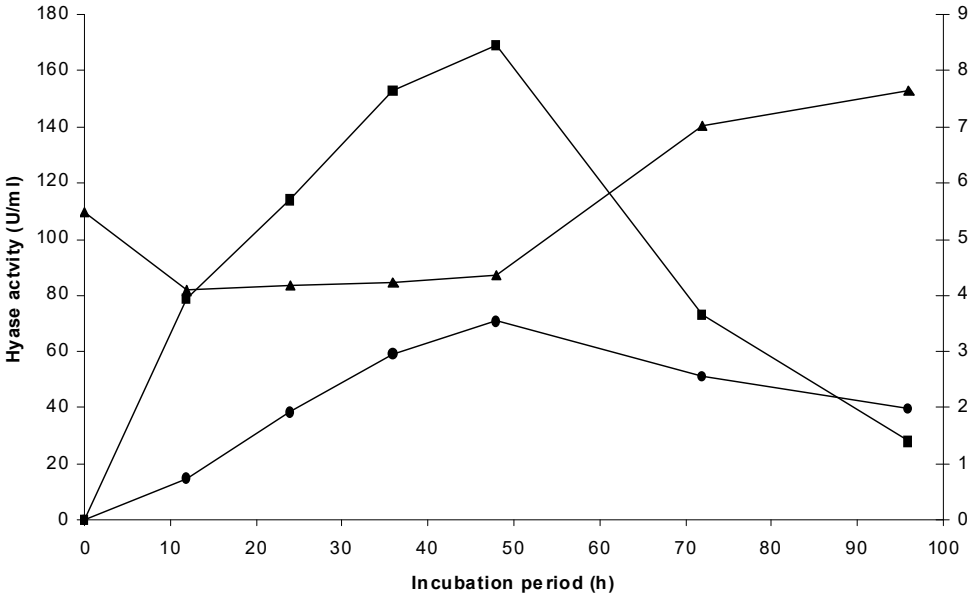

Fig. 1. Time course profiles of hyase production, cell mass (mg/ml) and pH by *S. equi*. (■) enzyme activity, (●) cell mass, (▲) pH.

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