

Optimization and Production of Hyaluronidase by *Streptococcus mitis* MTCC 2695

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Abstract

Hyaluronidase was produced by submerged fermentation from *Streptococcus mitis*. The possibility of using *Streptococcus mitis* for enzyme production has been recently investigated. In this study, the physical and nutritional parameters were optimized to improve the production of hyaluronidase by *Streptococcus mitis* and it was accessed. Maximum production of hyaluronidase was obtained when 5% starch supplemented as carbon source achieved by employing (98.7U/ml) and followed by ammonium chloride (140.4U/ml) incubation period about 48 hours showed (108.9U/ml) and temperature at 37°C showed (179.9U/ml). The maximum enzyme yield on pH 4 is (110.7U/ml). The production of hyaluronidase by means of immobilized *Streptococcus mitis* was evaluated and a maximum production was obtained with the medium was inoculated with 100 beads (591U/ml) which was more than that of mobilized cells.

Keywords: Hyaluronidase; *Streptococcus mitis*; Submerged fermentation; Hyaluronan; Immobilization

Introduction

Hyaluronidase is an enzyme which hydrolyses hyaluronic acid, a high molecular weight non-sulfated linear glycosaminoglycan, which is composed of repeating disaccharide units, D-glucuronic acid and N-acetyl glucosamine [1]. Hyaluronidase is naturally found in mammals, insects, leaches and bacteria [2]. Hyaluronidase can be produced by submerged fermentation from *Streptococcus mitis* [3-5]. It is a valuable enzyme because it can acts as an adjuvant, which accelerates and increases the absorption and dispersion of injected drugs [6].

The hyaluronidases from different sources vary in their molecular weight, based on substrate specificity, pH optima and catalytic mechanism [6]. Although widely distributed in nature, hyaluronidases are not well characterized and are a group of neglected enzymes owing to their difficult purification and lack of scientific interest over a large period of time [7]. However in recent years there is growing interest in the possible role of hyaluronan and hyaluronidase in numerous biological processes. Hyaluronidase was also useful in the direct reduction of hyaluronic acid that was improperly placed during injection [8-10].

The hyaluronate lyases, isolated from various microorganisms as e.g., strain of *Clostridium*, *Streptococcus*, *Micrococcus*, *Streptomyces*, *Staphylococcus*, *Peptostreptococcus* and *Propionibacterium* differ in substrate specificity [11-18].

Application of hyaluronidase

For many years, hyaluronidases, especially BTH preparation are widely used in many fields like orthopedic, surgery, ophthalmology, internal medicine, oncology, dermatology and gynecology [19].

The most common application is in ophthalmic surgery, in which it is used in combination with local anesthetics [20]. Sperm hyaluronidase is involved as a key play in successful fertilization in most mammalian, including human [10,21]. Hyaluronidase is also used for extravasation of hyperosmolar solution. Usually a 0.2 ml of the drugs is injected around the area of extravasation. Some bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Clostridium perfringens* [19,22-26] produce hyaluronidase as a means for greater mobility through the body's tissues [27] and as an antigenic distinguish that

prevents their being recognized by phagocytes of the immune system [11,28]. Hyaluronidase has been investigated as an additive to chemotherapeutic drugs for augmentation of the anticancer effect [29]. There is evidence that hyaluronidase may have intrinsic anticancer effects and can suppress tumor progression [8]. It has been used therapeutically due to their capacity to reduce biological fluid viscosity, increase vascular permeability and render tissues more accessible to certain drugs [30].

Materials and Methods

Procurement of microorganism

Streptococcus mitis MTCC 2695 were procured from Chandigarh and maintained on Mitis salivarius agar base medium slant. Sub culture was done at subsequent intervals.

Substrate for hyaluronidase production

The suitable substrate for hyaluronidase is hyaluronic acid which was procured from Sigma Aldrich (USA). The best substrate achieved by this step was fixed for subsequent experiments.

Submerged fermentation

Submerged fermentation was conducted in 250ml flask containing 100ml of the modified nutrient broth. 25µl of hyaluronic acid was added in the medium. After sterilization by autoclaving, flasks were cooled and inoculated with a 5%v/v inoculum level and incubated at 37°C on a rotary shaker at 150 rpm for 48 hours [31].

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Extraction and recovery of enzymes

The well grown culture was centrifuged at 8000g, for 30 minutes at 4°C. The supernatant, used as crude enzyme was assayed for hyaluronidase activity.

Analytical method

Enzyme assay: The method of assay is Dorfmans in which the enzymatic reduction in turbidity was determined by monitoring spectrophotometrically, at 600 nm and kept at 37°C. 1ml of hyaluronic acid was taken in a test tube. 1ml of enzyme sample solution was added in the presence of 0.05M sodium phosphate buffer with 0.05M NaCl. This mixture was incubated for 30 minutes at 37°C. To the above incubated mixture, 2.5ml of acidified protein solution (1%w/v) bovine serum albumin fraction – V (BSA) in 0.5M sodium acetate buffer was added. Then incubated at 37°C for 10 minutes and reduction in turbidity was read by measuring the absorbance at 600 nm [32].

Optimization of media for hyaluronidase production by *Streptococcus mitis*

Carbon source: Effect of addition of carbon source for maximal enzyme production was evaluated using various sugars which include maltose, fructose, lactose; starch and cellulose were studied by adding at a concentration of 0.5mg/ml to the basal production medium. Then hyaluronidase assay were performed as described earlier [32].

Nitrogen source: Effect of addition nitrogen source on hyaluronidase production was evaluated using ammonium acetate, ammonium nitrate, sodium nitrite, urea and ammonium chloride at a concentration of 0.25g each and hyaluronidase assay were done [32].

Temperature of the medium for hyaluronidase production: Suitable temperature of the medium that support maximal enzyme production was determined by keeping production conical flask at the temperature ranges as 20°, 37°, 45°, 50° and 60°C. Then hyaluronidase assay were completed [32].

Initial pH of the medium for hyaluronidase production: Suitable pH of the medium that support maximal enzyme production was determined by adjusting the pH to various levels ranges as pH 4, 5, 6, 8 and 9 using 1N HCl and 1N NaOH. Further hyaluronidase assay were done [32].

Incubation period of the medium for hyaluronidase production: Suitable incubation period of the medium that support maximal enzyme production was determined by adjusting the duration to various levels ranges from 24, 48, 72, 96 and 120 hours. Then hyaluronidase assay were performed [32].

Effect of immobilized cell

Cells were harvested after 48 hours centrifugation at 6000xg for 15 minutes, 1mg (dry cell weight) suspended in 2ml of sterile distilled water and added to 2 % (w/v) sterile sodium alginate solution to achieve the required cell/alginate ratio. The obtained mixture was then extruded drop wise through a 5ml syringe into a gently stirred 2% (w/v) CaCl₂ solution from about 5cm height and hardened in this solution for 1 hour. The beads were washed twice with sterile distilled water before being used as inoculum for hyaluronidase. Suitable cell immobilization of the medium that support maximal enzyme production was determined by adjusting the beads to various levels ranges from 50, 100, 150, 200 and 250 for 24 and 48 hours. Further hyaluronidase assay were performed as described earlier [32].

Results and Discussion

Effect of various carbon sources on hyaluronidase production

Various carbon sources were added to the fermentation medium for the production of hyaluronidase at 0.05% concentration. The sugars were tested glucose, sucrose, mannitol, dextrin, dextrose, starch, and sodium alginate. All these sugars were good enough for hyaluronidase production, but especially sucrose (245 U/ml) yields slightly higher amount of hyaluronidase [9].

A pathological isolate *Streptococcus equi* SED 9 was used for production of an extracellular enzyme hyaluronidase by using various carbon sources. Among them dextrose enhanced production and it was found to be 271 U/ml [4,25].

Various carbon sources such as maltose, fructose, starch, lactose and cellulose were used in present research. From this carbon source of starch gives maximum yields of hyaluronidase (98.7 U/ml) when compared to other sources (Figure 1).

Effect of various nitrogen sources

The effect of various nitrogen sources such as ammonium acetate, ammonium bicarbonate, ammonium chloride and ammonium sulphate. The maximum enzyme production was suggested that ammonium chloride (225 U/ml) gave the highest enzyme production [9].

The experiments were carried out for the production of various nitrogen sources on hyaluronidase production by using *Streptococcus equi* SED 9. Among them ammonium sulfate influenced the maximum yield of enzyme production and it was found to be 258 U/ml [4].

In the present research studied with various nitrogen sources were added to the fermentation medium for the production of hyaluronidase at 0.25g. The nitrogen sources were tested ammonium chloride, ammonium acetate, urea, sodium nitrite and ammonium nitrate. Maximum yield of hyaluronidase was obtained in the order of ammonium chloride (140.4 U/ml). From this result concluded that the nitrogen source variation changes due to the nutritional factors and environmental factors (Figure 2).

Effect of various temperatures

The effect of various temperatures such as 20°, 37°, 45°, 50° and 60°C and maximum yield (179 U/ml) was produced at 37°C [9].

The experiments were carried out for the production of various temperatures on hyaluronidase production by using *Streptococcus equi*

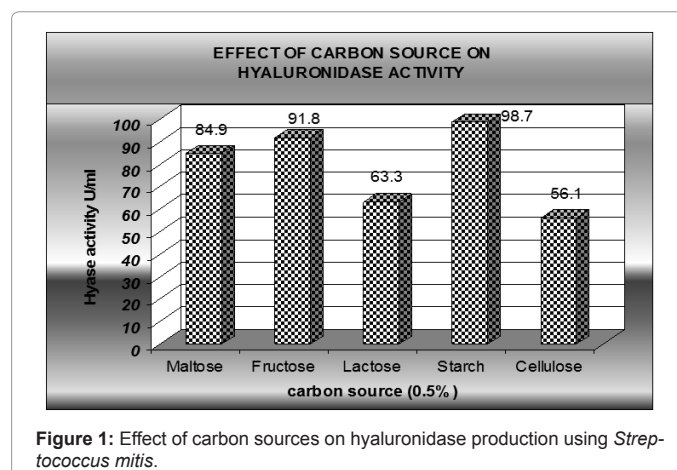


Figure 1: Effect of carbon sources on hyaluronidase production using *Streptococcus mitis*.

SED 9. Among them 37°C influenced the maximum yield of enzyme production and it was found to be 167 U/ml [24].

In the present research studied with fermentation by varied temperature of the medium 20, 37, 45, 50 and 60°C. The maximum yield is (174.9 U/ml) was produced at 37°C. Similarly in our investigation proves that 37°C optimal or maximal production (Figure 3).

Effect of various pHs

The effect of pH on hyaluronidase production was studied by varied the pH of the medium from 4-9. The production increased to 181 U/ml at pH 5.8. There was a gradual decrease in enzyme field from pH range from 5.8 to 7.2 above and below this range activity decreased sharply [9].

The experiments were carried out for the production of various pH on hyaluronidase production by using *Streptococcus equi* SED 9. Among them pH 5.5 influenced the maximum yield of enzyme production and it was found to be 165 U/ml [4,24].

In the present research studied at various pHs such as 4, 5, 6, 8 and 9. The maximum yield of hyaluronidase production up to (110.7 U/ml) produced at pH 4. These increased yields support the growth of organism and increase metabolic rate of organism. Due to the rapid metabolic process on the cell increase the product directly. This concludes that pH 4, supports organism metabolic rates actively. The rate of product was high when compared to Sahoo et al. (Figure 4) [4].

Effect of various incubation periods

The effect of fermentation process, and the incubation period of medium varied from 12-96 hours. Among them 48 hours enhanced the maximum yield of enzyme production and it was found to be 181 U/ml [9].

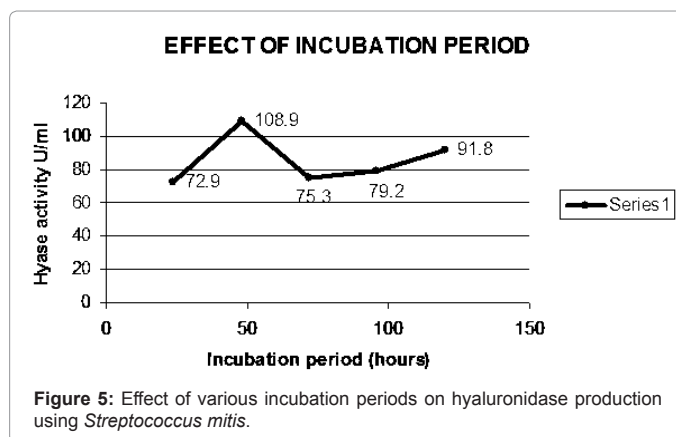
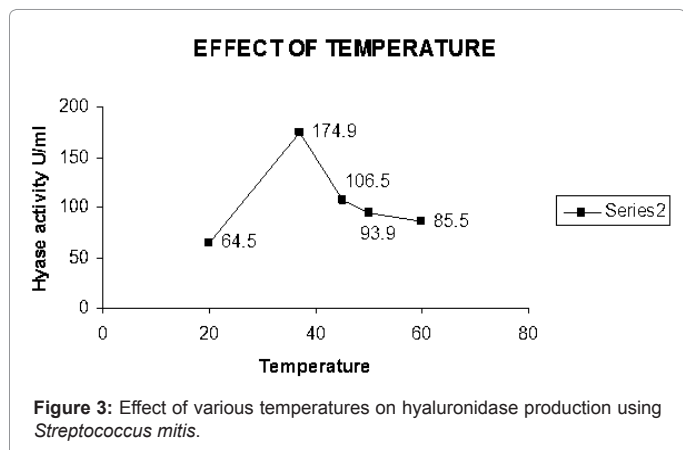
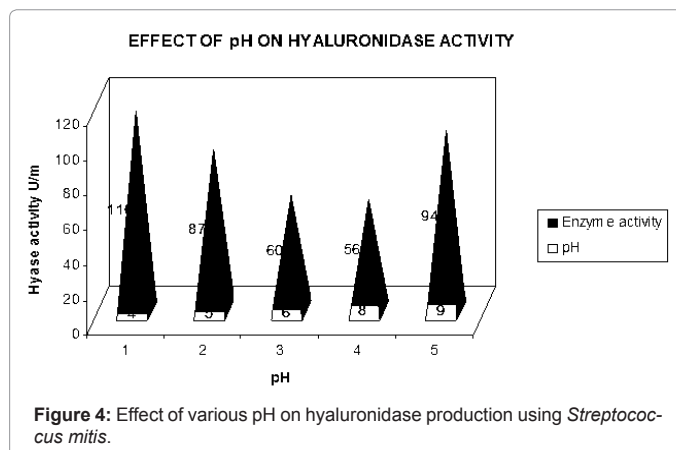
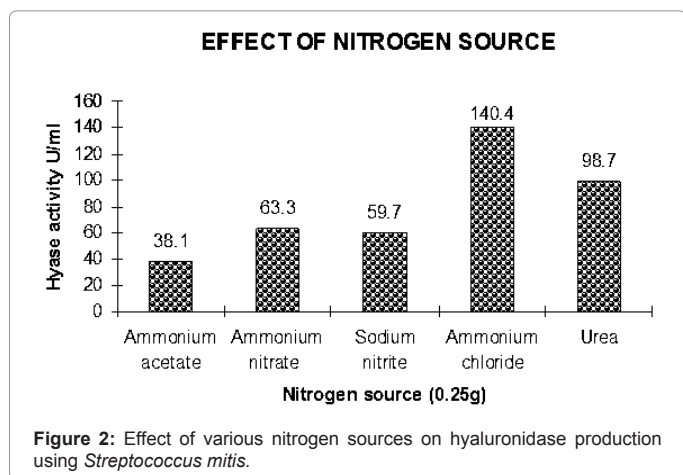
In the present research studied with fermentation of incubation period is 24, 48, 72, 96, 120 hours and maximum yield (108.9 U/ml) was produced within 48 hours. From, this result in concluded that the incubation period variation changes the metabolic pathways of organism to regulate the enzyme activity. When compared to Sahoo et al. report, it is slightly decreased but other parameters support for the maximum production (Figure 5) [24].

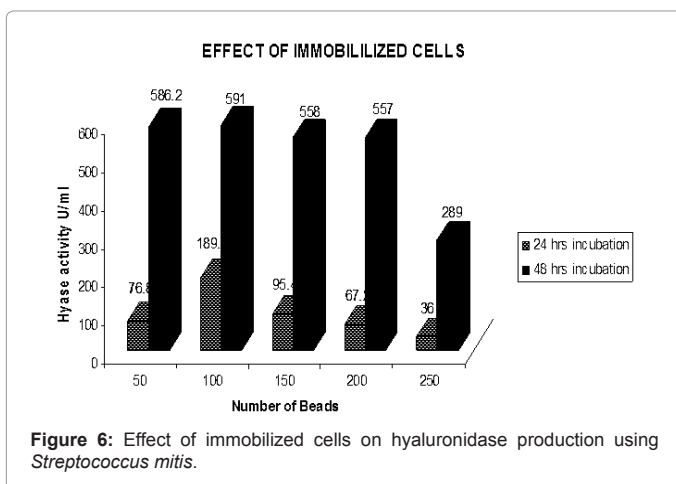
Effect of immobilized cells

In the present research studied with fermentation by varied number of beads of the medium such as 50, 100, 150, 200 and 250 for 24 and 48 hours. The maximum number of 100 bead yield is (591 U/ml) was produced for 48 hours. It is first report on immobilization that gives more amount of enzyme production (Figure 6).

Conclusion

The present research showed that higher levels of hyaluronidase produced at increasing agitation values and the time of fermentation with the maximum values are produced with its satisfactory yields. The production of hyaluronidase were influenced by the carbon source like starch, maltose, fructose, lactose and cellulose, nitrogen source like ammonium chloride, ammonium nitrate, sodium nitrite, ammonium





acetate and urea, pH-4, 5, 6, 8 and 9, temperature such as 20-60°C and incubation period such as 24-120 hours. Then the effect of immobilized cells in different beads such as 50, 100, 150, 200, and 250 incubated for 24 and 48 hours. Among all these, high amount hyaluronidase was produced by optimized source yield starch (98.7U/ml), ammonium chloride (140.4 U/ml), pH 4 (110.7 U/ml), temperature 37°C (174.9U/ml). The strain of *Streptococcus mitis* was evaluated and maximum production was obtained when the medium was inoculated with 100 beads (591 U/ml) for 48 hour which was more than that of immobilized cells. From the hyaluronidase production medium were measured. This present research concluded that hyaluronidase production gives boon for the medical and pharmaceutical fields.

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