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Original Article

Isolation, Screening and Characterization of Hyaluronidase Producing Bacteria

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Abstract

Hyaluronidase has a panoramic use in biotechnology processes and therapy due to its therapeutic, pathophysiological, physiological and biological importance. Since much of the preparations of hyaluronidases are from animal source (bovine and ovine testicular sources) with limited sources of microbial origin, that prompted the authors to screen and isolate a new promising bacterial strain with higher yield followed by its characterization employing detailed taxonomic studies. The newly isolated strain was identified based upon their micro- and macro-morphological, cultural, physiological and biochemical parameters. Twenty isolates from different pathological samples were primarily selected and further screened for their hyaluronidase producing capabilities by measuring reduction in turbidity and hydrolyzed zone of substrate hyaluronic acid. Four isolates showing marked reduction in turbidity (A_{600} nm) and hydrolyzed zones were selected and subjected to secondary screening by shake flask fermentation. Isolate $S_{II}9$ (Dental caries specimen) exhibited maximum hyaluronidase activity (117 U/ml) when compared to the reference *Streptococcus mitis* MTCC*2695 (106 U/ml). A close scrutiny of the literature revealed that the characteristics of our isolate $S_{II}9$ are mostly identical to *S. equi* subsp. *equisimilis* with few differences and thus designated as *S. equi* SED 9.

Keywords: Hyaluronidase; Isolation; *Streptococcus equi; Streptococcus mitis* Turbidity reduction assay.
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1. Introduction

Hyaluronidase (hyase) is a general term initially introduced by Karl Meyer in 1940 to describe enzymes that are able to break down primarily hyaluronic acid (HA) [1]. The

degradation of HA produces a disaccharide product. The 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 [2], for hypodermoclysis and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents [3]. The enzyme is used as a spreading factor in several medical fields viz. orthopaedia, surgery, ophthalmology [4], dermatology, dentistry [5], oncology [6], gynecology [7] and internal medicine [8]. Bacterial hyaluronate lyases were reported to be virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronan [9]. Hyase facilitates diffusion of antiviral drugs, dyes and toxins [10]. Based upon the medical, physiological, biological and commercial importance of hyases, authors have screened and isolated a newly promising bacterial strain with higher yield followed by its characterization employing detailed taxonomic studies [11]. Turbidity reduction assay [12, 13] method and determination of hydrolyzed zone of HA were employed for isolation and screening of hyase producing microorganisms.

2. Materials and methods

2.1. Primary screening

The pathological specimens were collected from different pathological laboratories at Bhubaneswar, Orissa, India. The pathological samples were collected and transferred into sterile screw capped culture tubes containing Stuart's transport medium [14]. Each specimen collected (swab, sputum and blood) was immediately spread on sterile blood agar plates containing an antifungal agent (fluconazole 25 µg/ml) to prevent fungal contamination and incubated at 37 °C for 24 h. A total of 20 isolates selected on the basis of colony size, shape and extent of hemolysis were screened for hyase activity. The isolates exhibiting good hemolytic zones were aseptically transferred into different 100 ml EM flasks containing 25 ml sterile nutrient broth and incubated at 37 °C for 24 h. Five ml culture broth was then withdrawn, centrifuged at 8000 rpm for 20 min. at 4 °C and clear supernatant collected was subjected to turbidity reduction assay [12, 13] (A_{600} nm by UV-visible spectrophotometer; Systronics, Model-118) and determination of zone of hydrolysis.

2.2. Secondary screening

The four isolates showing good hyase production were further screened along with reference strain *Streptococcus mitis* MTCC*2695 (IMTECH, Culture Collection and Gene Bank, Chandigarh, India) by shake flask fermentation method. The selected isolates were sub-cultured onto nutrient agar slants, incubated at 37 °C for 24 h. The growth contents of each slant was transferred into 50 ml of nutrient broth in 250 ml Erlenmeyer flask. The flasks were incubated at 37 °C for 48 h on rotary shaker (Ilshin Lab Co., Korea, Model BBT-1) (150 rpm). The fermentation broth of each flask was centrifuged at 4 °C (8000 rpm) and the clear supernatant was used for the enzyme assay. The experiments were conducted in triplicate and the average values were taken into consideration. The results are recorded in Table 2.

2.3. Hyaluronidase assay

Hyaluronidase activity was measured spectrophotometrically by turbidity reduction assay using HA sodium salt (Sigma Aldrich, USA) as a substrate. The enzymatic assay is based on Dorfmans method [15] 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 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

Table 1. Screening of selected isolates for their hyaluronidase production.

Isolate no.		ΔT^* (A_{600} nm)	Hydrolyzed zone (mm)
	1	0.018	29
	2	0.049	33
	3	0.040	17
S_I	4	0.045	12
	5	0.058	11
	6	0.023	13
	7	**0.239**	**36**
	8	0.029	22
	9	**0.324**	**38**
S_{II}	10	0.035	21
	11	0.031	18
	12	0.026	21
	13	**0.164**	**22**
	14	0.054	21
S_{III}	15	0.128	17
	16	0.057	27
	17	0.041	28
	18	0.050	24
S_{IV}	19	**0.173**	**29**
	20	0.049	20
S. mitis MTCC*2695		0.318	32

ΔT^*-Reduction in turbidity of broth cultures after 30 min. at 37 °C (A_{600} nm) (Uninoculated culture broth as blank). -Increase in turbidity (A_{600} nm) after 30 min. at 37 °C.

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 37 ºC 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 ºC, at pH 7.0 under specified assay conditions similar to that caused by one unit of an international standard.

2.4. Turbidity reduction assay

To one ml of substrate [containing 0.25 ml (0.04 %) hyaluronic acid, 0.5 ml distilled water and 0.25 ml of acidified bovine serum albumin (BSA) fraction V (1% w/v) in 0.5 M sodium acetate buffer (pH 3.1)] 0.5 ml of the supernatant (diluted 1:2 in saline) of an 18-24 h broth culture of isolated microorganisms was added, mixed and incubated at 37 °C for 30 min. At the end of incubation time the tubes were cooled in ice bath. To the above mixture 0.1 ml of acetic acid (2 N) was added to precipitate the remaining HA [12, 13]. Tubes containing sterile broth or broth from inactive cultures became turbid while tube containing broth from hyaluronidase producing organism remained clear on addition of the acid. The isolates exhibiting reduction in turbidity is given in Table 1.

2.5. Determination of hydrolyzed zone of hyaluronidase

Each isolate from slant was transferred into conical flask containing 25 ml sterile nutrient broth, incubated at 37 °C for 24 h. Five ml broth was then drawn, centrifuged at 8000 rpm for 20 min. at 4 °C and clear supernatant was collected. Then 25 µl of clear supernatant of each selected (20) isolates were aseptically added into sterile wells of 20 different molten nutrient agar plates containing 1 ml of substrate hyaluronic acid at a concentration of 10 mg/ml. The plates were then incubated at 37 °C for 24 h. The hydrolyzed zone (diameter in mm) of isolates was recorded and is given in Table 1. Thus four isolates showing good hydrolyzed zone were subjected for secondary screening.

Table 2. Hyaluronidase production of selected isolates by shake flask method.

Isolate no.	Hyase activity (U/ml)
S_{I7}	79
$S_{II}9$	**117**
$S_{III}13$	63
$S_{IV}19$	73
***S. mitis* MTCC*2695**	**106**

The isolate showing maximum hyase activity was subjected for identification following detailed taxonomic studies [11].

2.6. Identification of the promising isolate

The micromorphology studies include the shape of the cells, formation of spores, test for motility and gram staining were done. The cultural characters were studied by inoculating the organisms in different media viz. nutrient agar medium, blood agar medium, trypticase soy agar medium, *Streptococcus* selective agar medium and recording the growth pattern. The physiological and biochemical tests [11, 16, 17] were carried out by inoculating the isolate into the prescribed media. The tests included growth in air, growth under anaerobic condition, growth at different temperatures, growth at pH 9.6 (pH tolerance), sodium chloride tolerance, bile esculin agar, optocin test, alpha haemolysis, beta haemolysis, arginine dihydrolase test, decarboxylase test (Moeller's method), carbohydrate metabolism (acid-gas production) test, ONPG, lysine decarboxylase, ornithine decarboxylase, phenylalanine deamination, methyl red, indole, malonate, hydrogen sulphide production test, β-galactosidase production test, urease test, indole production test, nitrate reduction test, citrate utilization test, test for acid from esculin, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, lactose and Voges-Proskauer (acetoin production) test (VP) for identification of *Streptococcus* species.

3. Results

The strains $S_{II}9$ (0.324 OD, 38 mm) followed by S_I7 (0.239 OD, 36 mm), $S_{IV}19$ (0.173 OD, 29 mm) and $S_{III}13$ (0.164 OD, 22 mm) showed highest reduction in turbidity (A_{600} nm) and hydrolyzed zone, respectively, were selected for secondary screening by shake flask fermentation method.

Four promising isolates which showed good hyase activity were further screened for their enzymatic activity by shake flask method and the increase in enzyme yield was compared to the reference strain *S. mitis* MTCC*2695. The data recorded in Table 2, indicated that isolate $S_{II}9$ (Dental caries specimen (patient with bleeding gums and inflammation) from P.G. Dept. Microbiology, OUAT, Bhubaneswar) exhibited maximum hyase activity (117 U/ml) while the reference *S. mitis* MTCC*2695 exhibited (106 U/ml) after 48 h. The promising isolate $S_{II}9$ (117 U/ml) was subjected to detailed taxonomic studies.

The morphological and cultural characteristics of the isolate $S_{II}9$ are appended herewith. The isolate grew as small spheres or ovoids, occuring in pairs or chains. It was nonmotile, nonsporing and gram positive. The growth of the isolate was restricted at 10 ºC while growth observed at 45 ºC (Optimum 37 ºC). The isolate showed no growth at pH 9.6 and 6.5% sodium chloride. An abundant, smooth, scanty circular, semitransparent growth was observed on nutrient agar medium. In case of nutrient broth, moderate turbid growth was observed.

Results of the biochemical studies revealed that the isolate $S_{II}9$ utilized different carbon sources and showed acid and gas production as indicated in Table 3. The isolate SII9 also utilized inulin, ribose, salicin, trehalose, glucose, sucrose and malonate and produced acid while lactose, mannitol, raffinose,

Table 3. Physiological and biochemical tests of the isolate $S_{II}9$.

Biochemical test	Response	Result
Growth in air	Growth	Positive
Growth anaerobically	Growth	Positive
Growth at 10 °C	No growth	Negative
Growth at 45 °C	Growth found	Positive
pH (9.6)	No growth	Negative
Sodium chloride (6.5%)	No growth	Negative
40% bile	Growth not found	Negative
Esculin	Blackening of medium	Negative
0.25% optocin	Growth not found	Positive
Alpha-hemolysis	Greenish discoloration (1-2 mm zone)	Negative
Beta-hemolysis	Clear zone of hemolysis (2.5 mm)	Positive
Hydrolysis:		
Arginine	Yellow-purple coloration	Positive
Lysine decaroxylase	Yellow-purple coloration	Positive
Ornithine decaroxylase	Yellow-purple coloration	Positive
Acid from		
Inulin	Acid and gas production	Positive
Lactose	No acid and gas produced	Negative
Mannitol	No acid and gas produced	Negative
Raffinose	No acid and gas produced	Negative
Ribose	Acid and gas production	Positive
Salicin	Acid and gas production	Positive
Sorbitol	No acid and gas produced	Negative
Trehalose	Acid and gas production	Positive
Glucose	Acid and gas production	Positive
Sucrose	Acid and gas production	Positive
Arabinose	No acid and gas produced	Negative
Adonitol	No acid and gas produced	Negative
Rhamnose	No acid and gas produced	Negative
Cellobiose	No acid and gas produced	Negative
Melibiose	No acid and gas produced	Negative
Saccharose	No acid and gas produced	Negative
Production of		
β-Galactosidase	Yellow product	Negative
H_2S production	Blackening of the strips	Negative
Urease	Purplish pink colour	Negative
Indole	Pink ring	Negative
Nitrate reduction	Deep pink colour appeared	Positive
Citrate utilization	Green to blue medium	Positive
Voges-Proskauer test	Crimson red colour medium	Negative

sorbitol, arabinose, xylose, adonitol, rhamnose, cellobiose, mellibiose and saccharose showed no acid production. The isolate utilized arginine, lysine and ornithine as nitrogen sources. The strain could reduce nitrate and also utilized citrate, glucose, lactose, trehalose and malonate during its growth. The isolate was H_2S, ONPG, indole, esculine and VP negative. The isolate did not show production of urease, phenylalanine deamination, β-galactosidase as indicated in Table 3.

From the above morphological, cultural and biochemical tests, it is proposed that our isolate $S_{II}9$ can be characterized as a strain of *S. equi* with few characters differentiated from *S. equi* subsp. *equisimilis* and thus it was designated as *S. equi* SED 9.

4. Discussion

The isolated strain $S_{II}9$ showed good hyase producing activity in comparison to the reference strain *S. mitis* MTCC*2695. Similar results for screening of hyase producing isolates employing turbidity reduction assay were reported by many workers. McClean *et al*. (1943) reported that *Clostridium perfringens* growing in tissues produced hyase

employong turbidity reduction assay [18]. Group B Streptococci with good hyase activity was isolated by many workers [12, 19, 20]. Some workers also reported the production of hyaluronidase (hyaluronate lyase) by *Corynebacterium acnes* [21, 22].

A detailed survey of literature indicated that the characteristics of our isolate $S_{II}9$ is mostly related to *S. equi* subsp. *equisimilis* [11]. A detailed comparative study was done employing our isolate $S_{II}9$ to that of reported characteristics of *S. equi* subsp. *equisimilis*. A detailed comparison was done to establish the novelty of our isolate. There are a large number of similarities of our isolate with *S. equi* subsp. *equisimilis* [11]. But some significant qualitative and quantitative differences could also be observed. Our isolate $S_{II}9$ differs from *S. equi*. Our isolate $S_{II}9$ showed growth at 45 ºC while *S. equi* showed no growth. Carbohydrate metabolism pattern of our isolate $S_{II}9$ indicated utilization of inulin and salicin and produced acid and gas while it showed negative results with lactose. The utilization of salicin was not determined for *S. equi* and only 21-79% strains are lactose positive. The reported strain *S. equi* could not ferment inulin. The other physiological and biochemical parameters when compared were found to be similar to that of our isolate.

5. Conclusion

The future studies are aimed at the optimization of growth conditions of the isolate by selecting a suitable production medium along with its bioparametric studies including nutritional and physical parameters, perform strain improvement studies and then subjected to purification and characterization of the enzyme from the newly isolated strain.

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