

Original Research Article

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A Regulatory/ Signaling Molecule of Group A Streptococci (GAS) May be Responsible for Modulation of Bacterial Virulence

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ABSTRACT

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Pathogenic microbes secrete various enzymes to facilitate their survival within the host which may have direct/ indirect role to play in bacterial virulence. In the present work, extracellular amylase producing bacteria were isolated from air, water and soil samples by serial dilution and plating method. Twenty three bacterial isolates obtained were screened for amyolytic activity, out of which only 5 isolates showed amyolytic activity viz: *Streptococcus sp.*, *Staphylococcus sp.*, two *Bacillus sp.* and *Veillonella sp.*, which were identified by phenotypic characterization. The results revealed that *Streptococcus sp.* exhibit beta haemolysis on blood agar and had the potential to produce 120 EU extracellular amylase after 48 hours. This is reported here for the first time for production of extracellular amylase. This enzyme might be a regulatory/ signaling molecule and thus be an attractive drug target in the treatment of bacterial infections.

Introduction

Human pathogenic bacteria produce various extracellular factors including enterotoxin, hemolysin, cytotoxin, protease, collagenase, phospholipase, siderophore, and hemagglutinin. Of these factors, enterotoxin, hemolysin and cytotoxin are directly related to the clinical symptoms, however siderophore and hemagglutinin may have a role to play in the establishment of the infection (Mitchell, 2003; Janda, *et al.*, 1988). Also fungal plant pathogens are known to produce extracellular enzymes that cleave virtually every glycosidic linkages in plant cell wall polysaccharides.

The polysaccharides present in the environment of the pathogen regulate the growth of the microorganisms and the enzymes produced (Shambe, 1982). Extracellular enzymes, including serine protease, chitinase and collagenase have been reported to be involved in the infectious process as virulence factors in nematodes (Huang, *et al.*, 2004). Extracellular lipases and phospholipases have evidences in contribution to the virulence of pathogenic fungi like *Candida albicans* and *Cryptococcus neoformans*.

They have been also shown to influence growth, morphology, adherence and dissemination of fungal cells across the host (Park *et al.*, 2013).

Group A Streptococci (GAS) have been reported to cause various diseases like pharyngitis (Wannamaker *et al.*, 1951) scarlet fever (rash), impetigo (infection of the superficial layers of the skin) (Bisno and Stevens, 1996) or cellulitis (infection of the deep layers of the skin) (Stevens *et al.*, 1994). Invasive, toxigenic infections can result in necrotizing fasciitis, myositis and streptococcal toxic shock syndrome (Stevens, 1995; Cunningham, 2000). However the pattern changes with time and varies from place to place (Mitchell, 2003). Virulence factors of *S. pyogenes* Group A (GAS) potentially cause a large number of diseases because of variety of virulence factors present in them which include M protein, fibronectin-binding protein (Protein F) and lipoteichoic acid for adherence, invasins such as streptokinase, streptodornase (DNase B), hyaluronidase, and streptolysins, exotoxins such as pyrogenic (erythrogenic) toxin (Cunningham, 2000; Metzgar and Zampolli, 2011). The *S. pyogenes* Group A (GAS) capsule is composed of a polymer of hyaluronic acid containing repeating units of glucuronic acid and *N*-acetylglucosamine and is required for resistance to phagocytosis (Wessels and Bronze 1994). Terao Y (2012) has reported that *S. pyogenes* is equipped with a wide variety of virulence factors which includes surface-located fibronectin-binding proteins (FbaA and FbaB), major invasion molecule, bacterial cysteine protease (SpeB) and a C6-binding protein in cell-surface protein fraction.

In the present study, we report the production of extracellular amylase by *S.*

pyogenes and propose its function in the utilization of carbohydrates as well as establishing infection.

Materials and Methods

Soil, air and water were screened for bacterial isolates secreting extracellular enzyme amylase for which 23 colonies were picked and isolated. Total five bacterial isolates could produce amylase on starch agar plate assay (Fig 1). These isolates were characterized on the basis of colony morphology, microscopic characters, endospore formation, presence of capsule and biochemical tests viz: Indole, methyl red, Voges-Proskauer, Simmons citrate test, gelatin hydrolysis test, oxidase test and catalase test (Holt *et al.*, 1994; Koneman *et al.*, 1997).

Results and Discussion

Isolate 1 (BT1) showed circular, small, translucent, dark creamy to light orange coloured colonies with entire margin without pigmentation. It was gram-negative cocci, present singly or as diplococci and was non motile. It did not have endospore, was non-capsulated, VP and catalase positive. It was indole, MR and citrate negative and unable to ferment glucose, sucrose, lactose and mannitol showing very unusual metabolism. As reported earlier, *Veillonella* were characterized by their unusual metabolism as they are unable to ferment carbohydrates including glucose but grow well anaerobically on lactate, pyruvate, malate or fumarate (Kolenbrander, 2006).

Isolates BT4 and BT5 were large, wide, gram-positive rods, facultative anaerobe, often occur singly or in pairs and can produce endospores. They were found to be catalase-positive, indole positive, VP

positive, utilized citrate as source of carbon, urease positive, showed acid production from glucose and sucrose. They were motile with sub terminal endospore and did not produce capsule suggesting to be considered as *Bacillus* spp. Colony morphology of *Bacillus* spp. was highly variable, often growing as round opaque colonies on nutrient agar medium with sticky consistency.

Isolate BT3 was gram-positive cocci found in clusters, was not motile, did not produce endospores and capsule. *Staphylococcus* sp. was catalase-positive, oxidase negative, VP positive, utilized citrate as source of carbon, showed acid production from glucose and sucrose. Colony morphology of *Staphylococcus* sp. grew as pinpoint creamy white with entire margin and opaque colonies on nutrient agar medium with no pigmentation. When grown on blood agar it did not show haemolysis. As known, biochemically *Staphylococcus* produced catalase that degrade hydrogen peroxide whereas *Streptococci* did not (Holt *et al.*, 1994).

Isolate BT2 was gram-positive, non-motile, non-spore forming coccus that occur in chains or in pairs of cells. Individual cells were round-to-ovoid cocci. Streptococci divided in one plane and occurred in pairs or in chains of varying lengths. The metabolism of BT2 was fermentative, the organism was catalase-negative. It was found to be aero tolerant anaerobe (facultative anaerobe) and was capsulated. Based on Bergey's Manual of Determinative Bacteriology the isolates were identified as *Veillonella* sp.(BT1), *Streptococcus* sp.(BT2), *Staphylococcus* sp. (BT3) and two *Bacillus* spp.(BT 4, BT 5) (Holt *et al.*, 1994; Koneman *et al.*, 1997).

The strain showing maximum zone of

inhibition in starch hydrolysis test was found to be *Streptococcus* sp. and therefore further characterized using blood agar (enriched medium). It exhibited beta (clear) hemolysis on blood agar showing complete lysis of red blood cells in the medium (Fig 2). The complete haemolysis on blood agar and other bio chemicals suggested it to be *S. pyogenes* Group A (GAS).

Soil, air and water samples contained different microorganisms and found to be potential sources of various useful enzymes. Among different types of enzymes obtained from microbial sources, amylases are the most widely used in industry. In the previous studies, dental caries by *S. mutans* arose as a consequence of metabolism of dietary carbohydrates (Mitchell, 2003) and also utilization of starch by *S. mutans* was dependent on the addition of exogenous α -amylase (Simpson and Russell, 1998). It had been reported that *Bacillus* sp. as the potent source of amylases (Roy *et al.*, 2012; Najafi *et al.*, 2005), however, in our results we got pathogenic *Streptococcus* spp. more potent amylase producer than the isolated *Bacillus* spp. The results showed that *S. pyogenes* Group A (GAS) had the potential to produce 120 EU extracellular amylase in 48 hours by DNSA method of quantification. Other carbohydrates like 2% maltose and 2% sucrose were also used instead of starch (Freer, 1993) but they did not induced extracellular amylase production. One unit of amylase (EU) EC 3.2.1.2 was defined as the amount of enzyme that releases 1 μ mole of reducing sugar as glucose per min under the assay conditions (Plummer, 1978). This enzyme production might be attributed for obtaining nutrients via digestion of carbohydrate substrate. Further the role of extracellular enzymes secretion in modulation of bacterial virulence cannot be neglected.

In previous studies, Walker GJ (1965) had isolated the cell-bound alpha-amylase of *S. bovis* by chromatography on DEAE-cellulose. Christine L. Simpson and Roy R. B. Russell (1998) deduced amino acid sequence by showed a strong similarity (60% amino acid identity) to the intracellular α -amylase of *Streptococcus bovis*. Whitehead TR and Cotta MA (1995) identified intracellular amylase activity in *S. bovis* and *S. salivarius*. SN Freer (1993) purified and characterized extracellular alpha-amylase from *S. bovis* JB1. However, the extracellular amylases have not been reported till now by *S. pyogenes* Group A (GAS).

secrete extracellular enzymes which were involved in the infectious process as virulence factors. *S. pyogenes* Group A (GAS) infections were caused using a wide variety of virulence factors which includes bacterial cysteine protease, SpeB and C6-binding protein, a cell-surface protein and FbaA and FbaB surface located proteins as major invasion molecules (Terao,2012). Since various regulatory and signaling molecules were attractive drug targets in the treatment of bacterial infections thus understanding signaling responses of *S. pyogenes* Group A (GAS) was essential for elucidation of pathogenesis of *S. pyogenes* Group A (GAS) infection and for the identification of novel therapeutic agents.

It has been reported that certain pathogens

Fig.1 Zone of Clearance due to the Hydrolysis of Starch

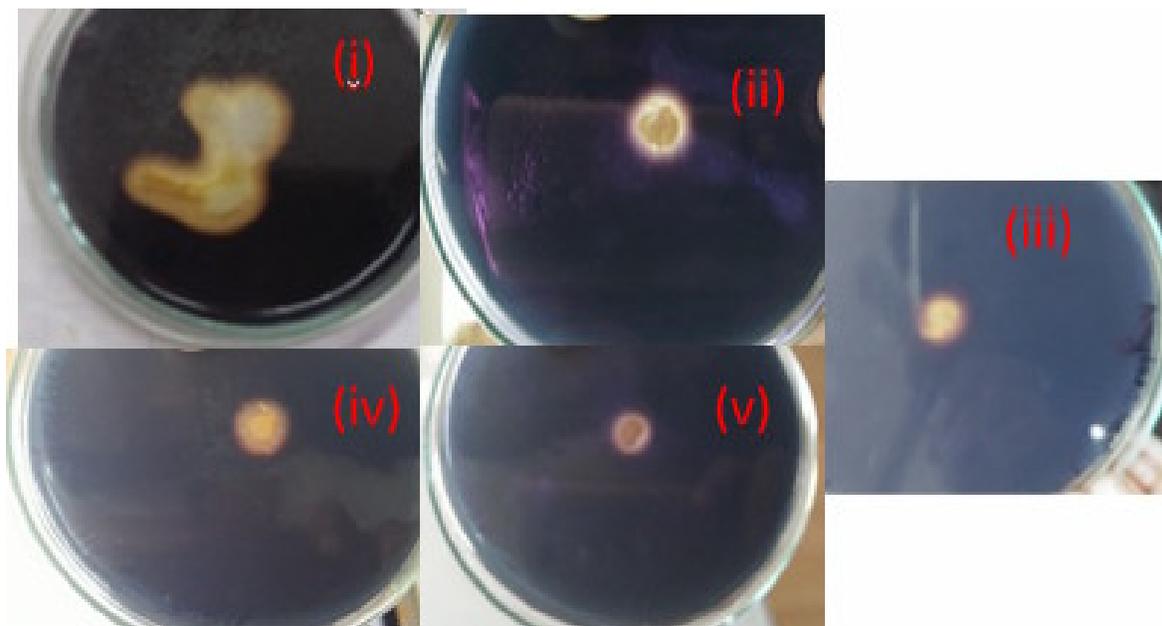


Fig.1 Zone of Clearance due to the Hydrolysis of Starch. Screening of five isolates (i to v) for capability of amylase production (plate assay method). Isolates were spot inoculated/ streaked in the center on the starch agar plate and plates were incubated at 37°C for 24 h - 48 h. After incubation iodine solution was flooded on the starch agar plate for 30 seconds. Presence of clear zone of hydrolysis around the bacterial growth indicates positive result and isolates were considered as amylase producers.

Fig.2 Blood Agar Test



Fig.2 Blood Agar Test: When streaked on Blood Agar, *Streptococcus* sp. cause hemolysis i.e., destruction of the erythrocytes (and hemoglobin) in the medium. The colony is surrounded by a white or clear zone in which few or no intact erythrocytes are found. Zone of haemolysis observed after 24 hours (i) and after 48 hours (ii).

The amylase production by *S. pyogenes* Group A in considerable amounts with considerable certainty may have links with its pathogenicity (stress survival) or nutrition or both which is further an indication that this extracellular protein may have potential to be developed as a vaccine target.

The findings of the paper has possibility of facilitating the discovery of new compound (regulatory/ signaling molecule) which may have a greater role to play in modulation of bacterial virulence and thus may pave the path for further research leading to attractive drug targets in the treatment of bacterial infections.

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Compliance with Ethical Standards

Authors declare that they have no conflict of interest. This article does not contain any

studies with human participants or animals performed by any of the authors.

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