Screening of marine actinobacteria for amylase enzymes inhibitors

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Received: 20 June 2008 / Accepted: 22 October 2008

Abstract Amylase inhibitor producing actinobacteria were isolated and characterized from terrestrial environment and there is no much report found from marine environment, hence in the present study, 17 strains isolated from the rhizosphere sediments of mangroves were tested for their amylase inhibition ability. Seawater requirement test for the growth of actinobacteria found that the strains SSR-3, SSR-12 and SSR-16 requires at least 50% and SSR-6 requires at least 25% seawater for their growth. The inhibition activity of both prokaryotic and eukaryotic amylase was tested by using *Bacillus subtilis* and *Aspergillus niger*. The maximum amylase activity (40mm) produced by the *A. niger* was taken as positive control, when the test actinobacteria strains grown in the medium they inhibited amylase activity and was evidenced by the reduction in inhibition zone (14–37 mm) similarly the amylase produced by the *Bacillus subtilis* was also recorded maximum (35 mm) amylase activity and was taken as positive control, and the test actinobacterial strains reduced enzyme action (12–33 mm) it varied levels. This indicates that the actinobacteria strains were controlled amylase enzyme activity in both the cases. The strain SSR-10 was highly effective and SSR-8 was less effective in inhibiting eukaryotic amylase produced by *A. niger*. The strain SSR-2 was effective and SSR-6 showed very less effect in inhibiting the prokaryotic amylase produced by the *B. subtilis*.

Keywords Marine actinobacteria · Amylase enzyme · Amylase inhibitor

Introduction

Actinobacteria are representative of soil microorganisms very less attention was paid on its marine counterpart [1]. Actinobacteria in marine and estuarine sediments have not been investigated extensively although their ubiquitous presence in marine sediments [2, 3]. Studies on diversity, distribution and ecology of actinobacteria in the oceans are scarce [4]. But marine environment is considered to be the source of novel actinobacteria, many of which produce bioactive substances [5, 6].

In 1960's the potential use of microbial secondary metabolites were recognized which includes of amylase inhibitors. Amylase inhibitors were suggested for pharmaceutical or agricultural applications, eg. obesity, adipose, hyperlipimia (arteriosclerosis), diabetes, pre-diabetes, gastritis, gastric ulcer, duodenal ulcer and caries in man, or as insect controller in agriculture [7]. Amylase inhibitors are also known as starch blockers because they inhibit the activity of starch degrading enzymes like amylase and thereby reduce the starch conversion rate. The enzyme inhibitors of microbial origin have very low molecular weight when compared with that of animal and plant inhibitor because of its origin from the hydrolysis of macromolecules substances [1]. Considering the importance of amylase enzyme inhibitors especially from the microbial origin, the present study was attempted to evaluate the amylase inhibitory activity of marine actinobacteria.

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Material and methods

Sediment samples were collected from mangrove rhizosphere zone of the *Rhizophora mucronata* (Poir) in Vellar estuary, east coast of India. The sediment samples are shade dried, ground and mixed with equal weight of CaCO_3 (1:1) and incubated for ten days in an inverted petri plates, at room temperature [8]. Pre-treated samples (1 g) were serially diluted [9] and spreaded on Actinomycetes Isolation Agar (AIA, Hi-Media, Mumbai) and incubated at 30°C for 14 days. The colonies were picked and stock cultures maintained in AIA slants.

Bacillus subtilis and *Aspergillus niger* were isolated from sediment of mangrove environment and identified by biochemical and staining methods. The strains were screened for amylase production on starch agar plates with iodine solution [10]. They were confirmed as amylase producers by observing clear zone around the colony. Amylase crude enzyme stock solution was extracted by following the method described by Stamford *et al.* [11]. The amount of protein in the stock solution was estimated according to the method of Lowry *et al.* [12]. The isolated actinobacterial strains were screened for amylase inhibitor activity by the methods of Yokose *et al.* [13] and inhibitor stock solution was prepared by following Mahamud *et al.* [14] method.

The amylase inhibitory activity was estimated by reducing sugar method [15] with slight modification. Amylase inhibitor assay was made by using a reaction mixture (4.06 ml) consist of 0.05 ml of enzyme solution previously mixed with 0.01 ml of inhibitor solution and 1 ml of soluble starch in 0.990 ml phosphate buffer (pH 6.9). The mixture was incubated for 10 min at 30°C and the reaction was terminated by adding 2.0 ml of dinitrosalicylic acid. The reaction mixture without enzyme inhibitor was used as positive control and the reaction mixture without enzyme solution was used as negative control (enzyme solution replaced by distilled water). The amount of enzyme inhibitory activity was expressed in terms of units, One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 μM of glucose per min under the assay condition.

The dialysed and lyophilized amylase of both *Bacillus subtilis* and *Aspergillus niger* were prepared with the concentration of 10 mg/ml. Wells were made on agar plate and filled with amylase solution for positive control and amylase solution with various concentration of pre incubated (10 min) inhibitor solution (100 μl) in separate wells and kept at 30°C for 2 h. Inhibition of amylase activity were estimated by flooding the plate with iodine solution and zone of clearance around the wells were recorded.

Results and discussion

A total of 17 strains were isolated from the rhizosphere sediments of the *Rhizophora mucronata* (Poir) with a population density of 3.2×10^{-3} CFU/g of sediment. The colonies (SSR1-SSR17) selected for this study had white powdery aerial mass with substrate mycelia. Isolation of actinobacteria from marine and its adjacent sediments are well documented, yet it remains unclear that, what component of these bacteria represents indigenous marine microflora [9]. Isolation from marine areas are preponderantly reported from coastal or shelf regions. Watson and Williams [16] isolated 2×10^{-6} colonies per gram from dried sand samples and found that the colonies are more in mangrove rhizosphere region than that of the rhizoplane region. When compared with the earlier results from different mangrove environs of world $0\text{--}15 \times 10^{-4}$ CFU/g of actinobacteria from Cochin mangroves [17], 40×10^{-4} CFU/g from Mexico [18], 2.76×10^{-5} CFU/g from the *Suevada* zone of the Vellar estuary [19] and 4×10^{-4} CFU/g from Pichavaram mangroves [20], the rhizosphere of *R. mucronata* of Vellar estuary harboured, lower population density (3.2×10^{-3} CFU/g). Higher tannin and phenolic content due to the degradation of mangrove leaves might reduced the actinomycetes population in the rhizosphere.

It has been proposed, that most actinobacteria isolated from marine environments are of terrestrial origin and reside in the sea as spore or resting propagules [21, 22]. Seawater requirement test was carried out for all the actinobacterial strains and the results indicated that the strains SSR-3, SSR12 and SSR-16 required at least 50% seawater for their growth in the media, whereas the strain SSR-6 requires 25% seawater (Table 1). All the other strains showed their broad range of saline tolerance by growing in 0–100% seawater media. This indicates that these strains would have brought from terrestrial source and acclimatized to the marine environment. This is well supported by the finding of Goodfellow and Haynes [22] who had reported the actinobacteria strains isolated from North Atlantic sediments including *Streptomyces*, *Micromonospora* and *Rhodococcus* species not required any seawater and growing equally well on media prepared either distilled water or seawater. The actinobacteria are common soil bacteria, produce resistant spores and are known to be salt tolerant [23, 24]. Okami and Okazaki [25] concluded that many actinobacteria can survive for a period of time in the seawater and grow well. Therefore, a shallow sea site represents an interesting source of special actinomycetes which can survive under both terrestrial and marine environment. The actinobacteria reported in this study represent only a portion of the total microbial population. Because the isolation method used is

selective in nature and because of the criteria used for the selective isolation of actinobacteria colonies, certain marine genera producing fragmenting mycelia might have been excluded from the results [26].

B. subtilis and *A.niger* were tested positive for the amylase production. The activity was confirmed by the clear zone around the colonies in starch agar plate. Crude microbial amylase was extracted from the broth by precipitating with ammonium sulphate, the protein content of

the amylase was analyzed and found that the enzyme of *B. subtilis* contain 380 µg/ml while *A. niger* contain 550 µg/ml protein. Amylase enzyme activity of the stock solutions was 1200 units/ml for *B. subtilis* and 1500 units/ml for *A. niger*.

In general, amylase enzyme inhibitor of actinobacteria were effective against both prokaryotic (*B. subtilis*) and eukaryotic (*A. niger*) amylases. The strain SSR-8 was less effective, while the inhibition activity of the strain SSR-10 was high against amylase produced by eukaryotes (*A. niger*). In the case of prokaryotes, the strain SSR-2 was effective, whereas SSR-6 was less effective against the amylase of *Bacillus subtilis*. These inhibitor activities were depicted against the control amylase enzyme activity shown by the *B. subtilis* and *A. niger* with out any inhibitors. From the results, the strains SSR-2, SSR-3 and SSR-10 were found to be effective amylase enzyme inhibitor producers against both prokaryotic and eukaryotic amylases.

Amylase enzyme assay and inhibitory activity of the inhibitor were tested in plates containing soluble starch and the results were presented in Fig. 1. The results obtained from reducing sugar test (Fig. 2) was confirmed the minimum starch hydrolysis by the strains SSR-2, SSR-3 and SSR-10 in both *B. subtilis* and *A. niger* amylases indicating that higher enzyme inhibition activity of the strains. In contrast to this other strains showed maximum starch hydrolysing zone and there by showing the less effective inhibition activity of the respective strains.

Amylase inhibitors have received considerable attention in recent year because of their usefulness in determination of amylase isozyme activities and control of carbohydrate dependent disease such as, diabetes, obesity. The ability of *Streptomyces* strains to produce inhibitors in the presence of salt is already confirmed by Imada [1]. From the amylase

Table 1 Requirement of seawater for growth

S. No	Strains	Distilled water	Seawater		
			25%	50%	100%
1.	SSR-1	+	+	+	+
2.	SSR-2	+	+	+	+
3.	SSR-3	-	-	+	+
4.	SSR-4	+	+	+	+
5.	SSR-5	+	+	+	+
6.	SSR-6	-	+	+	+
7.	SSR-7	+	+	-	+
8.	SSR-8	+	+	+	+
9.	SSR-9	+	+	+	+
10.	SSR-10	+	+	+	+
11.	SSR-11	+	+	+	+
12.	SSR-12	-	-	+	+
13.	SSR-13	+	+	+	+
14.	SSR-14	+	+	+	+
15.	SSR-15	+	+	+	+
16.	SSR-16	-	-	+	+
17.	SSR-17	+	+	+	+

Fig. 1 Amylase enzyme inhibitor activity of actinobacterial strains recorded by agar plate method

Fig. 2 Amylase enzyme inhibitor activity of actinobacterial strains recorded by reduced sugar method

inhibitor study, we found that the prokaryotic amylase was more effectively inhibited by the actinobacterial strains when compared with the eukaryotic amylase. The strains SSR-2, SSR-3 and SSR-10 were more effectively inhibiting the starch hydrolysis and it is clearly conferring the production of amylase inhibiting components by these strains. Kim *et al.* [27] were in the opinion that the inhibition test carried out *in-vitro* using purified α - amylase, was not a suitable comparison for what occurs in the *in-vivo*. This statement cautions us to test the amylase inhibitor activity in terms of *in-vivo* before going for large scale production of amylase inhibitor compounds by using these actinobacterial strains. From the foregoing account it is confirmed that the actinobacteria strains SSR-2, SSR-3 and SSR-10 are the potential amylase inhibitor producers which can be explored further for isolation and characterization of amylase inhibitor compounds. Among The three strains SSR-3 requires at least 50% seawater for its growth which conform the marine nature of the strain.

Acknowledgements Authors thank Dr. T. Balasubramanian, Director of the Centre and authorities of Annamalai University for providing with necessary facilities.

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