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Screening, characterization and optimization of production parameters of alpha amylase inhibitor produced by *Paenibacillus lentimorbus*

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ABSTRACT

To screen amylase inhibitor producing marine microorganisms from sea, a bacterial sps *Paenibacillus lentimorbus* isolated from marine sediment produced an extracellular amylase inhibitor having activity against bacterial and fungal amylases. The optimal medium for the production of inhibitor was investigated by using shake flask method. Glucose as carbon source and soybean meal as a nitrogen source for better production of amylase inhibitor as well as the growth of the isolate. The maximum production of inhibitor was observed in a optimized medium consisting of 2.0 % glucose, 1.0% soybean flake extract, 0.3% NaCl, pH7.0 in 100% distilled water. Out of 50 marine bacterial isolates only one marine bacterial sps was able to produce amylase inhibitor. The yield of fungal alpha amylase and bacterial alpha amylase inhibitor was increased by optimizing the above mentioned medium by 38% and 34% respectively. The organism was designated as SS11/16 (sea sediment -11th sample 16th colony). This was the first report on amylase inhibitors produced from marine *Paenibacillus*

KEYWORDS

Paenibacillus lentimorbus;
Amylase inhibitor;
SS11/16;
Starch plate method;
Modified blue value method.

INTRODUCTION

Amylase inhibitors received increasing attention for the treatment of carbohydrate dependent disorders, weed control in cultivation and study of active sites of amylases. It is one of the enzymes important in controlling blood sugar levels in the body. Inhibiting α-amylase could be a beneficial treatment for insulin-dependent diabetes mellitus, obesity and hyperlipedeamia. Amylase inhibitors reduce the rate of digestion of starches in the small intestine. They are primarily proteins, derived from plants and micro-organisms, and inhibit α-amylase, a pancreatic enzyme that hydrolyzes starch[4,12]. This paper describes the results of screening, optimization parameters and identification of organism.

MATERIALS AND METHODS

Paenibacillus lentimorbus isolated from marine sediment by earlier workers of our laboratory. The strain was maintained on nutrient agar medium. Amylase

inhibitory activity was screened using Starch plate method[7], Starch agar plate method[2], TLC plate method[6]. Alpha amylase inhibitory activity was tested by fermentation in shake flask on rotary shaker at 120 rpm at 37^{O}C. The inhibitory activity was assayed by modified blue value method[2,314]. The production medium[15] comprises of Glucose 2.0 %, Soybean flake extract 1.0%, NaCl 0.3% Inoculum level 7.5%, pH 7.0, 100% distilled water.

Determination of type of amylase inhibitor produced by the isolate SS11/16

To determine whether the inhibitor produced by the isolate SS11/16 inhibits which type of amylase ex: Salivary, Pancreatic, *Bacillus*, fungal and other starch hydrolyzing enzymes, the following procedure was followed. Inhibitory activity of the harvested broth was assayed against different amylases and other starch hydrolyzing enzymes like glucosidase etc., by modified blue value method.

Time course of inhibitor formation in growing culture of SS11/16

The progressive inhibitor secretion into the medium was examined, when the isolate SS11/16 was cultivated aerobically in production medium IV at 28^{O}C, 120rpm in shake flask fermentation method in triplicate for 7days. Every day 5mL of sample was collected, centrifuged and tested for the percentage of inhibition of fungal and bacterial α-amylase by modified blue value method[2].

Effect of preincubation time on degree of inhibition[1]

1. (Cultured broth of SS11/16+bacterial amylase)
2. (Cultured broth of SS11/16+fungal amylase)

The time required for maximum enzyme-inhibitor (E-I) complex formation was tested by incubating the reaction mixture for different time periods. Reaction mixture consists of 0.5mL α-amylase; 2mL starch solution and 0.5mL broth containing inhibitor and the percentage of inhibition of fungal and bacterial amylase was determined by modified blue value method. It was conducted in triplicate and mean values were recorded.

Optimization studies of fungal and bacterial alpha amylase inhibitor production

Production of active metabolites depends on the nature of the strain, the composition of the medium and also on the cultural conditions. As such it is decided to investigate the optimum cultural conditions for inhibitor production. The following parameters were investigated. Effect of different production media, carbon sources, nitrogen sources, initial pH, and level of inoculum, aeration, agitation and sea water concentration were tested. Production medium IV was found to be better for production of alpha amylase inhibitor. The medium and cultural conditions were optimized to get further increase in the yield of the inhibitor. The optimized medium composition IV (modified medium IV-a for fungal amylase and medium-b for bacterial amylase inhibitor) and initial composition of production medium was compared and the results were depicted in the further sections.

TABLE 1 : Different types of production media used for production of alpha amylase inhibitor:

S.No	PM*	References
1	PM-I	(Koichi Katsuyama *et al*.,1992)
2	PM-II	(Laszlo Vertesy and Tripier, 1985)
3	PM-III	(Sawao Murao *et al*., 1981)
4	PM-IV	(Sawao Murao *et al*.,1983)
5	PM-V	(Chiaki Imada and Usio Simidu, 1992)
6	PM-VI	(Narimasa saito., 1982)
7	PM-VII	(Jin Hwan *et al*.,1985)
8	PM-VIII	(Volker Oeding *et al*.,1981)

***PM=production medium**

Taxonomical characterization of isolate SS11/16

The identification of a bacterial species is based on many factors, including cell and colony morphology, chemical composition of cell walls, biochemical activities, and nutritional requirements. The morphological characterization[5] and biochemical tests[8] were conducted for identification.

RESULTS AND DISCUSSIONS

Starch agar plate method

Morphology of SS11/16

Even though the isolate was screened from marine sediment, it shows better growth and production of alpha amylase inhibitor in presence of distilled water. The organism might be washed from fresh waters. SS11/

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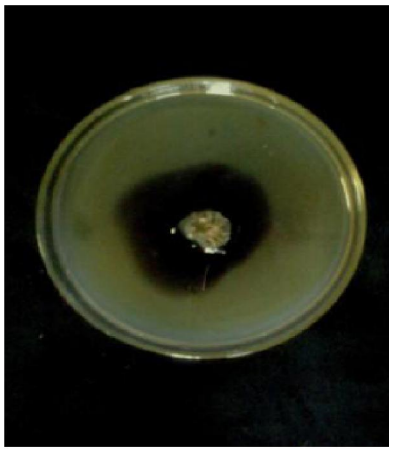

Figure 1 : Screening of fungal α-amylase inhibitor by starch agar plate- SS11/16. Blue color portion around the colony indicates the formation of fungal alpha amylase inhibitor

Figure 2 : Screening of bacterial α-amylase inhibitor producing- SS11/16 by starch agar plate: Blue color portion around the colony indicates the formation of (Bacterial Bacillus alpha amylase inhibitor.

TABLE 2 : Determination of type of amylase inhibitor produced by the isolate SS11/16

Type of amylase	Substrate	pH	SS11/16
Human salivary α-amylase	Soluble Starch	7.0	-
Bacillus.subtilis α- amylase	Soluble Starch	7.0	+
Fungal α-amylase	Soluble Starch	7.0	+
Glucosidase	Soluble Starch	7.0	-

+ = indicates inhibition - = indicates no inhibition; Isolate SS11/16 was able to produce bacterial and fungal amylase inhibitor in presence of starch as a substrate.

TABLE 3 : Estimation of amylase inhibitory activity by modified blue value method

S.No	Medium No	Percent inhibition of fungal α-amylase±S.D	*Number of inhibitor units /mL	Percen tinhibition of bacterial α-amylase	Number of inhibitor units /mL
1	I	26±0.1	1.04	20±0.3	0.80
2	II	28±0.2	1.12	21±0.05	0.84
3	III	24±0.3	0.964	23±0.2	0.92
4	*IV	33±0.1	1.32	29±0.3	1.16
5	V	22±0.2	0.88	20±0.3	0.80
6	VI	25±0.3	1.0	25±0.2	1.0
7	VII	24±0.4	0.96	23±0.3	0.92
8	VIII	24±0.22	0.964	22±0.1	0.88

***One unit of inhibitor (IU) was defined as the amount of inhibitor required to decrease the amylase activity by 50% under the above conditions; *IV ——Better production of amylase inhibitor was found in medium IV**

TABLE 4 : Comparison of percent inhibition of fungal α-amylase and bacterial α-amylase with modified production medium (MPM IV-a & MPM IV-b) and production-IV

S.No	Type of medium	Percent inhibition of fungal α-amylase	*Number of inhibitor units/mL	Percent of inhibition of bacterial α-amylase	Number of inhibitor units/mL
1	MPM IV a	70	2.8	-	-
2	MPMIV b	-	-	60	2.4
3	PMIV	32	1.28	26	1.04

*** Initially 1.28 inhibitor units/mL was produced incase of Production medium IV, where as in modified media 2.8units/mL of fungal amylase inhibitor and 2.4 units/mL of bacterial amylase inhibitor**

16 is a marine bacterial isolate was able to produce both fungal and bacterial alpha amylase inhibitors which were confirmed by different primary screening techniques. By optimizing production medium –IV

TABLE 5 : Composition of modified production media and production medium -IV

S.No	Medium	Composition
1	*PMIV	Glucose 2.0 %, soybean flake extract 1.0%, NaCl 0.3% Inoculums level 7.5%, pH 7.0, 100% distilled water.
2	*MPMIV-a	Glucose 2.0 %, Peptone 1.0 %, NaCl 0.3%, pH 7.0, Distilled water 100mL, Inoculums level 10 %
3	*MPMIV-b	Glucose2.0%, Soybean meal 1.0%, NaCl 0.3%, Distilled water 100 mL, Inoculums level 10, pH 7.0

***MPMIV-a—optimized modified production medium-IV for fungal amylase inhibitor; *MPMIV-b—optimized modified production medium-IV for bacterial amylase inhibitor; *PMIV— Initial production medium for both amylase inhibitors**

TABLE 6 : Time course of inhibitor formation in growing culture of SS11/16

S.No	Day/hours	Percent inhibition of bacterial α-amylase±S.D	Number of inhibitor units/mL	Percent inhibition of fungal α-amylase±S.D	Number of inhibitor units/mL
1	1st (24hours)	20±0.1	0.8	15±0.4	0.6
2	*2nd (48hours)	29±0.2	1.16	33±0.1	1.32
3	3rd (72hours)	20±0.1	0.8	26±0.5	1.04
4	4th (96hours)	19±0.25	0.74	24±0.3	0.96
5	5th (120hours)	15±0.23	0.6	19±0.2	0.76

***2nd (48hours) —— Maximum production was observed at 2nd day in both cases of inhibitors.**

TABLE 7 : Effect of preincubation time on degree of inhibition of fungal and bacterial α-amylase-(SS11/16) (cultured broth of SS11/16+bacterial α-amylase) & (Cultured broth of SS11/16+fungal α-amylase)

Sr. No	Time (minutes)	Percent inhibition of fungal α-amylase±S.D	Number of inhibitor units/mL	Percent inhibition of bacterial α-amylase±S.D	Number of inhibitor units/mL
1	5	10±0.2	0.74	17±0.22	0.68
2	10	19±0.3	0.96	22±0.3	0.88
3	*15	27±0.4	1.28	27±0.4	1.08
4	*20	32±0.2	1.16	25±0.45	1.0
5	25	29±0.1	1.16	25±0.1	1.0
6	30	28±0.2	1.12	24±0.2	0.80

***15—fifteen minutes was found to be optimum for maximum inhibition of bacterial amylase; *20-Twenty minutes was found to be optimum for maximum inhibition of fungal amylase**

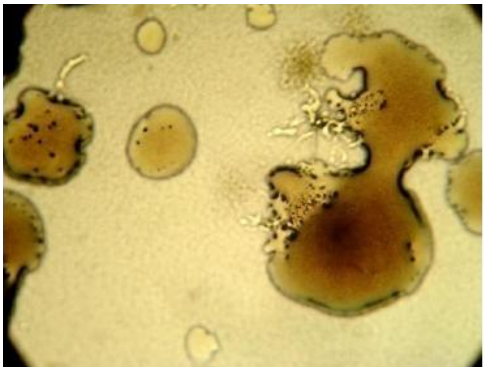

Figure 3 : Identification and characterization of isolate SS11/16: Shape of the colony of SS11/16 is rhizoid, shape of the margin is filamentous and surface texture is smooth and shiny

Figure 4 : Light micrograph of SS11/16 at 400x magnification Rhizoid shaped colony of SS11/16

FULL PAPER

TABLE 8 : Physiological and biochemical properties of isolate SS11/16

S.No	Reaction	Response	Result
1	Gram staining	Blue color rods	Positive
2	Spore staining	Colored	Positive
3	Motility	Spreading of dye throughout the medium.	Positive
4	Growth at 12^{O}C	Growth was not observed	Negative
5	Growth at 25^{O}C	Growth was observed	Positive
6	Growth at 37^{O}C	Growth was observed	Positive
7	Growth at 42^{O}C	Growth was observed	Positive
8	Growth at pH 5.2	Growth was observed	Positive
9	Growth at pH 8.0	Growth was observed	Positive
10	Growth at pH 9.0	Growth was observed	Positive
11	Growth at pH 10.0	Growth was observed	Positive
12	*Growth on NaCl 2%	Growth was observed	Positive
13	Growth on NaCl 5%	Growth was observed	Positive
14	Growth on NaCl 7%	No growth was observed	Negative
15	Growth on NaCl 10%	No growth was observed	Negative
16	Starch hydrolysis	Hydrolyzed zone=10mm Growth zone=24mm	Positive
17	Casein hydrolysis	No hydrolyzed zone was observed	Negative
18	Citrate utilization	Utilized	Positive
29	Gelatin liquefaction	No hydrolyzed zone was observed	Negative
20	H_2S production	No brown color	Negative
21	Methyl red	No color	Negative
22	Voges Proskauer	Red color formation	Positive
23	Nitrate reduction	Brown color	Positive
24	Indole production	No color	Negative
25	Catalase production	Bubbles formation was observed	Positive
26	Oxidase production	Bubbles formation was observed	Positive

Tolerance to sodium chloride: The isolate SS11/16 exhibited growth at 2% and 5% NaCl and no growth was observed at 7% and 10% NaCl. SS11/16 was identified as Paenibacillus lentimorbus and its accession number is MTCC 10472 by IMTEH Chandigarh.

cultural conditions, the yield of inhibitor production was increased by a difference of 38% incase of fungal alpha amylase inhibitor and 34% incase of bacterial alpha amylase inhibitor production. SS11/16 is a Gram positive *Paenibacillus*, exhibits motility and spores get stained with Malachite green. The bacterial colony is in rhizoid shape and the edge is filamentous, the surface of the colony is very smooth and shiny indicates that it is virulent and grows aerobically at 37^{O}C on nutrient agar with characteristic smooth colonies, which indicates the pathogenicity of organism. No growth was observed at 12^{O}C and growth was observed at 25^{O}C, 37^{O}C and 42^{O}C. Growth was observed at different pH 5.2, 8.0, 9.0 and 10.0. This was the first report on Bacillus sps producing amylase inhibitors..

CONCLUSION

So far many α-amylase inhibitors have been reported, belonging to the genus *Streptomyces* and one from the fungi- *Cladosporium herbarum*. Now a fungal and bacterial α-amylase inhibitor has been isolated from isolate SS11/16.

SS11/16 was identified as *Paenibacillus lentimorbus* and its accession number is MTCC 10472 by IMTEH Chandigarh.

Even though the isolate SS11/16 was isolated from sea sediment, it grows well in the presence of distilled water, indicating that the isolate might have been washed away from river water in to the sea.

Fungal and bacterial amylase inhibitors can be used for weed control in crop cultivation.

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