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Isolation, Characterization, Identification and Screening of Amylase Producing Bacteria from Soil under System of Rice Intensification (SRI) Technique

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ABSTRACT

Amylases are amongst most widely used enzymes in industries such as food, fermentation, starch processing, textile and paper. In the present investigation, bacteria were isolated from rhizosphere of Rice Agricultural Experimental Farm, Giridh, Jharkhand, screened for the production of amylase and their optimum growth conditions were determined. A total of 36 bacterial colonies were isolated from collected soil samples. Thirty Four isolates are from geneus Bacillus and two are paenibacillus, out of these five bacterial isolates, displayed zones of clearance in starch hydrolysis test. The isolate displaying maximum amylase activity on quantization was selected. Characteristic feature of the strain indicates that it belongs to the genus Bacillus and will be later used for further characterization. Maximum yield of amylase was obtained after 48hrs of incubation. The optimum pH for enzyme activity was found to be at pH 7.0 and the optimum temperature for the activity was found to be at 40^{oC}.

Key words: *Amylase, Starch Hydrolysis, characterization, amylase activity*

INTRODUCTION

Starch degrading amylase enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper[1]. They are degrading enzymes which breakdown the starch into sugars by acting on α- 1,4-glycosidic bonds[2]. Amylases are obtained from various origins like plant, animal, bacterial and fungal. Microorganisms are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production[3,4]. Many microorganisms are able to produce amylases including *Bacillus* spp., *Lactobacillus*, *Escherichia*, *Proteus*, *Strepotmyces* sp., *Pseudomonas* sp. etc. For production of amylase for industrial use, isolation and characterization of new promising strains is a continuous process.

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In the present study, we report the isolation, screening and characterization of amylase producing bacteria from the soil samples collected from Rizosphere of rice from giridh. Production conditions were optimized (temperature, pH, etc.) to achieve high enzyme production and better enzyme activity.

MATERIALS AND METHODS

Sample collected

Soil sample were collected from Agricultural Experimental Farm, Giridh, Jharkhand, India from 3 to 4 cm depth with the help of sterile spatula. Samples were transferred to sterile plastic bags and maintained in aseptic conditions.

Isolation of bacterial cultures

Isolation of bacterial cultures Isolation of soil bacteria was performed by serial dilution and spread plate method as described earlier. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-9}. A volume of 0.1 ml of each dilution was transferred aseptically to starch agar plates. The sample was spread uniformly. The plates were incubated at 37°C for 24 hr. The bacterial isolates were further sub cultured to obtain pure culture. Pure isolates on agar slants were maintained at 4ºC.

Morphological and Biochemical characterization of the isolated strain

Gram staining was performed to know whether the isolate was Gram positive or negative. The isolates were observed under the microscope to obtain the colony morphology i.e. colour, shape, size, nature of colony and Motility. The citrate, catalase, urease, starch MRVP tests for biochemical characterization were performed.

Identification by 16S rRNA PCR

The genotypic identification was done using the 16S rRNA identification method (Hutter et al., 2003) using 519R primer (5'-GAGAGTTTGATCCTGGCTCAG-3') and 27 F primer (5'-CTACGGCTACCTTGTTACGA-3') The 10 ml bacterial DNA extract and controls were amplified with 0.5 mM primers (Table 1), 200 mM of each dNTP (Promega), 10 mM KCl PCR buffer, 2 mM MgCl2 and 1.0 U Taq polymerase (Bioline). Amplification conditions for both PCRs were as follows: 5 min at 94^0C to denature the DNA, followed by 40 cycles of denaturation at 94^0C for 1 min, primer annealing at 55^0C for 1 min and strand extension at 72^0C for 2 min on a Rotor gene thermal cycler. PCR products were separated on a 1.5 % agarose gel and DNA bands were visualized with ethidium bromide. Primers and excess nucleotides were removed from the amplified DNA using a PCR clean-up kit (Qiagen). The amount of DNA in the cleaned-up product was quantified by comparing the intensity of the band to bands of known intensity in a Hyper Ladder marker (Bioline). Spiked samples were included with each PCR to determine whether or not the sample was inhibited. Spiked samples contained 8 ml clinical sample and 2 ml known positive 16S rRNA.

Isolation of Amylase Producing Microorganisms

One gram of the above collected soil sample was weighed and mixed to 9 ml of sterile distilled water. Serial dilution was done up to 10-5 and spread plated into nutrient agar HIMEDIA fortified with 1% starch. Then the plates were kept for incubation at 37°C for overnight.

Screening for Amylase Activity (Starch Iodine Test)

Bacterial cultures were screened for amylolytic activity by starch hydrolysis test on starch agar plate[5]. The pure isolated colonies were streaked on starch agar plates with starch as the only carbon source. After incubation at 37oC for 24-48 hrs, the individual plates were flooded with Gram's iodine (Gram's iodine-250 mg iodine crystals added to 2.5gm

potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is the basis of the detection and screening of an amylolytic strain. The amylase producers displaying maximum diameter of zone of clearance, were further investigated[6]. The pure cultures were sub cultured at regular intervals and starch- nutrient agar slants were maintained at 4 C.

Amylase production medium

A loopfull of bacterial culture was transferred from starch-nutrient agar slants to starch-nutrient broth at pH 7 for activation and incubated in a shaker at 40◦C at 120 rpm for 24 h. Fermentation medium contained soluble starch (10 g/L) peptone (5 g/L), $(NH_4)_2$ SO_4 (2 g/L), KH_2PO_4, (1 g/L), K_2HPO_4 , (2 g/L), MgCl2, (0.01 g/L) at pH 7.The fermentation medium was inoculated with the activated culture (20% v/v) and incubated in shaker at 37^0 C for 24 hrs. At the end of the fermentation period, the culture medium was centrifuged at 10000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Enzyme assay

Amylase activity was assayed as described[7] with some modifications. Briefly, 1.5 ml of 1% starch in 2 ml, 0.1M phosphate buffer (pH 6.5) and 0.5ml of diluted enzyme were incubated for 15 min at room temperature (37^{oC}). The reaction was arrested by adding 1ml of DNS reagent and kept in a boiling water bath for 10 min and diluted with 8ml of distilled water.

The absorbance was measured at 540nm against blank prepared as above without incubation. One unit of α-amylase activity was defined as the amount of enzyme that liberates 1μmole of reducing sugar (maltose equivalents) per minute under the assay conditions[8]. Unless otherwise stated all experiments were carried out in triplicate.

Characterization of α- amylase

Determination of optimum pH

1% Starch was used as a substrate. Substrate solution was prepared in sodium phosphate buffer at pH 6, 6.5, 7, 7.5 and 8 in different test tubes. 0.5 ml each of diluted crude enzyme solution was added into buffer tubes[9]. Then the mixture was incubated at room temperature for 15 min, reactions were terminated by adding 1 ml DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12ml with distilled water and the activity of enzymes was determined by taking the absorbance at 540nm.

Determination of optimum temperature

1.5 ml of substrate was taken into six different test tubes and 2 ml of phosphate buffer pH 7 was added in each test tubes. Tubes were marked with different temperature (at 30, 35, 40, 45, 50, 55°C). 0.5ml of diluted enzyme solution was added in each tube. Then tubes were incubated at specific temperature for 10 minutes. Reactions were terminated by adding 1 ml DNS reagent and the mixture incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12ml with distilled water and the activity of enzymes were determined by taking the absorbance at 540nm.

RESULTS AND DISCUSSION

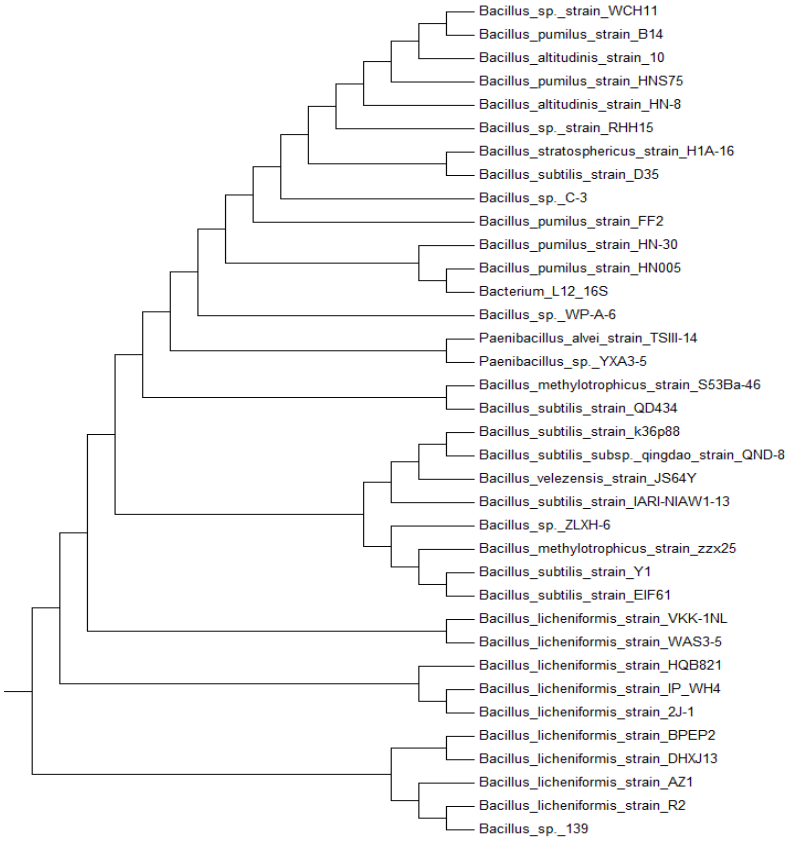
Isolation, biochemical characterization and identification of Isolates:

Thirty-Six bacterial isolate were Characterized and Identified by different biochemical tests and 16s rRNA Sequencing, out of Thirty-six isolate thirty-four are from bacillus and two are paenibacillus strain. Out of these five Bacteria have potential to produce Amylase.**Table 1.**

Table1: Thirty-six isolated strains and their Biochemical Characterization

Sr.no.	bacterial Strains	Gram's reaction	Shape	Catalase test	Citrate utilization	Starch hydrolysis	MR-VP Test	Urease test	Motility
1	Bacillus sp. WHCH11	Gram positive	Bacilli blunt ends	Positive	positive	negative	positive	Negative	Mortile
2	Bacillus Pumulis B14	Gram positive	Bacilli blunt ends	Positive	Negative	Positive	Positive	Negative	Mortile
3	Bacillus altitudinis 10	Gram positive	Rod	Positive	positive	Positive	positive	positive	Mortile
4	Bacillus pumilus HNS75	Gram positive	Bacilli	Positive	Negative	negative	positive	Negative	Mortile
5	Bacillus altitudnis HN-8	Gram positive	Rod	Positive	positive	Positive	positive	positive	Mortile
6	Bacillus sp. RHH15	Gram positive	Rod	negative	positive	Positive	negative	positive	Mortile
7	Bacillus stratosphericus H1A-16	Gram negative	irrigular smooth	Positive	Negative	negative	negative	Negative	Mortile
8	Bacillus subtlis D35	Gram positive	rod	Positive	positive	Positive	positive	Negative	non-motile
9	Bacillus sp. C-3	Gram positive	Rod	negative	positive	Positive	negative	positive	Mortile
10	Bacillus pumilus FF2	Gram positive	Bacilli	Positive	Negative	negative	positive	Negative	Mortile
11	Bacillus pumilus HN-30	Gram positive	Bacilli blunt ends	Positive	Negative	Positive	Positive	Negative	Mortile
12	Bacillus pumilus HN005	Gram positive	Bacilli	Positive	Negative	negative	positive	Negative	Mortile
13	Bacterium L12-16s	Gram positive	Rod	negative	positive	Positive	negative	positive	non-Mortile
14	Bacillus sp. WP-A-6	Gram positive	Rod	negative	positive	Positive	negative	positive	Mortile
15	Paenibacillus alvei TSIII-14	Gram positive	Bacilli	negative	positive	Positive	negative	Negative	non-motile
16	Paenibacillus sp. YXA3-5	Gram positive	Rods	negative	positive	Positive	negative	Negative	non-motile
17	Bacillus methylotrophicus S53Ba	Gram positive	white rods	Positive	positive	Positive	negative	Negative	Mortile
18	Bacillus subtlis QD434	Gram positive	Rod	Positive	positive	Positive	positive	positive	Mortile
19	Bacillus subtlis k36p88	Gram positive	Rod	Positive	positive	negative	positive	positive	Mortile
20	Bacillus subtlis subsp. qingdao	Gram positive	bacilli	Positive	positive	negative	positive	positive	non-Mortile
21	Bacillus velezensis JS64y	Gram positive	Rod	Positive	Negative	Positive	positive	positive	Mortile
22	Bacillus subtlis IARI-NIAW1-13	Gram positive	rod	Positive	positive	Positive	positive	Negative	non-motile
23	Bacillus sp. ZLXH-6	Gram positive	Rod	negative	positive	Positive	negative	positive	Mortile
24	Bacillus methylotropicus zzx25	Gram positive	creamy rods	Positive	positive	Positive	negative	Negative	Non-Mortile
25	Bacillus subtlis Y1	Gram positive	Rod	Positive	positive	negative	positive	positive	Mortile
26	Bacillus subtlis EIF61	Gram positive	bacilli	Positive	Negative	negative	positive	positive	non-Mortile
27	Bacillus licheniformis VKK-1NL	Gram positive	rod	Positive	positive	Positive	positive	Negative	Mortile
28	Bacillus licheniformis WAS3-5	Gram positive	rod	negative	Negative	Positive	positive	Negative	Mortile
29	Bacillus licheniformis HQB821	Gram positive	rod	negative	positive	negative	positive	Negative	Mortile
30	Bacillus licheniformis IP-WH4	Gram positive	rod	Positive	positive	Positive	positive	Negative	Mortile
31	Bacillus licheniformis 2J-1	Gram positive	rod	Positive	positive	Positive	positive	Negative	non-Mortile
32	Bacillus licheniformis BPEP2	Gram positive	rod	negative	positive	Positive	positive	Negative	Mortile
33	Bacillus licheniformis DHXJ13	Gram positive	rod	Positive	Negative	negative	positive	positive	Mortile
34	Bacillus licheniformis AZ1	Gram positive	rod	Positive	positive	Positive	positive	Negative	non-Mortile
35	Bacillus licheniformis R2	Gram positive	rod	Positive	positive	Positive	positive	Negative	Mortile
36	Bacillus sp 139	Gram positive	bacilli	Positive	Negative	negative	positive	positive	Mortile

Table 2: Phyllogenetic tree of 36 bacterial isolates



Selection of α-amylase producing bacteria:

Bacteria isolated from starch rich materials may have better potential to produce enzyme under adverse conditions[10]. Microorganisms that produce amylases could be isolated from places such as soil around mills, cassava farms and processing factories as well as flour markets[11].

During the study, α-amylase producing bacterial strains were isolated from the rizospheric soil of SRI (system of rice intensification). A total of 36 bacterial strains were isolated. Among which 5 strains gave zone of clearance with iodine solution on starch hydrolysis test. These were further selected and quantified. Amongst these, the isolate showing maximum absorbance was further optimized and characterized and found to belong to the genus Bacillus.

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. Most of the starch degrading bacterial strain revealed a pH range between 6.0 and 7.0 for normal growth and enzyme activity[6]. The pH change observed during the growth of microbes also affected product stability in the medium. As shown in table2 the isolate was able to grow in the pH range of 6–8, but pH 7.0 was the optimum for the growth of the cultures. Temperature also plays the significant role in the stability in enzyme activity. 40°C was found to be optimum temperature at which enzyme activity was found to be higher.

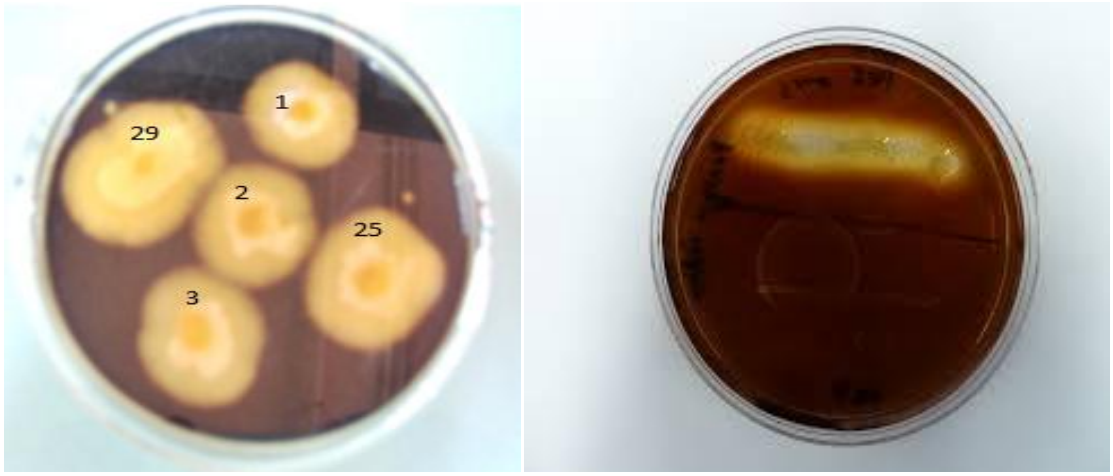


Fig. 1: Five isolates showing positive starch hydrolysis test and *Bacillus licheniformis HQB821*) showing the maximum

Table 3: Quantitation of all the isolates (OD at 540 nm)

Isoates	Absorbance (540 nm)
Bacillus sp. WHCH11	0.326
Bacillus Pumulis B14	0.392
Bacillus altitudinis 10	0.350
Bacillus subtlis Y1	0.462
Bacillus licheniformis HQB821	0.496

Table 4: Effect of varying pH on α- amylase activity of isolate Bacillus licheniformis HQB821

pH	Amylase activity(U/ml)
6	6.18
6.5	8.42
7.0	9.24
7.5	8.11
8.0	7.36

Table 5: Effect of varying temperature on α-amylase activity of Bacillus licheniformis HQB821

Temperature	Amylase activity(U/ml)
30	6.41
35	7.95
40	9.72
45	8.88
50	7.92
55	7.50

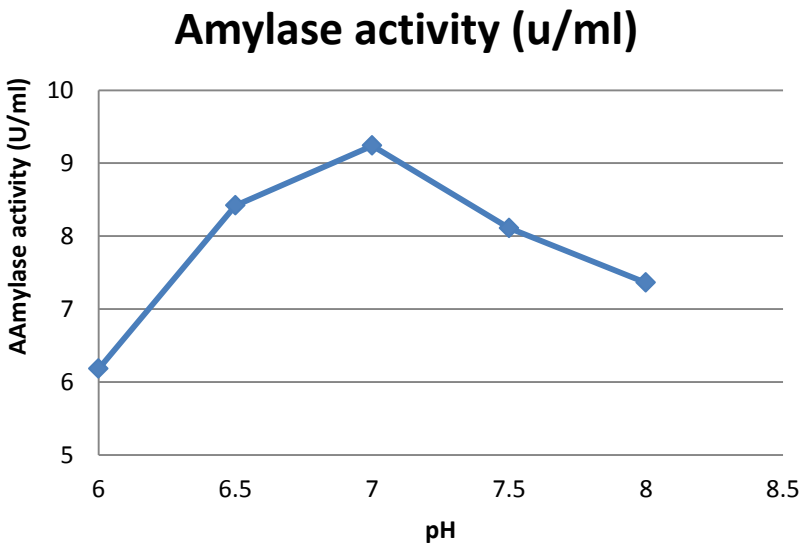


Fig. 2: Effect of different pH on Amylase activity

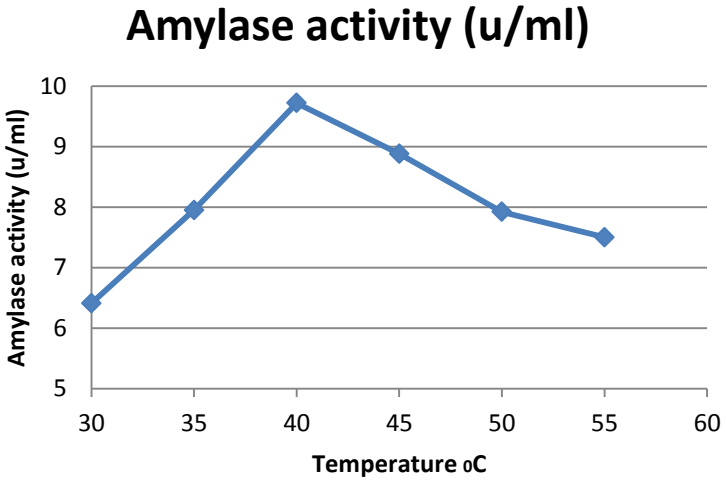


Fig. 3: Effect of different temperature on Amylase activity

CONCLUSION

Strain was isolated from the rizhospheric soil sample of SRI technique which has the capability to produce amylase. The nature of culture conditions, temperature and pH for the optimal production of α-amylase by the isolated bacterial strain has been developed in this study.

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