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Original Research Article

Amylase degrading bacteria from soil and their RAPD profiling

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A B S T R A C T

Amylases are known to be produced by a variety of bacteria and fungi and their applications at industrial level have stimulated interest to explore their amylolytic activity in several microbes to be used as bio-resources.In this study amylase degrading bacterial colony characterization visual inspection was carried out in which circular shape, pinkish and creamish color and smooth margin was found. Besides these elevation and opacity was raised and was transparent. All the bacteria were found gram positive. Motility test was positive for both JP IMViC test was done for biochemical characterization in which MR, VP, Indole and Citrate test, gelatinase, phosphates and urease test was negative for both JP. Catalase test and amylase test was positive for both of strain.Highest bacterial growth was recorded at 35 ^{0}C and pH 7.0 with 1% salt concentration and bacteria optimum growth for was recorded in 72h.Genetic diversity analysis was performed by RAPD based markers. Total 18 markers used for the genetic diversity analysis. Both the bacterial showed 80% dissimilarities to each other that in the basis of genetic analysis and biochemical test also indicated the same.

Keywords

Amylase, Morphological, Biochemical, RAPD profiling

Introduction

Microorganism are the most important source of enzyme production, as we know many of enzyme, each with a specific role are required in nature to break down compounds during the biodegradation process (Pandey *et al*., 2000; Schmidt *et al.*, 2002). In an interesting way the first enzyme produced industrially was an amylase from a fungal source in 1894, used as a pharmaceutical help for the treatment of Digestive disorders (Pandey *et al.*, 2000).

Amylases establish a group of industrial enzymes, which only covers approximately 30% of the enzyme. They have vital sources of many commercial microbiological processes including renewable Energy, Pharmaceuticals, Saccharification or Liquefication of starch, Detergent industries, Textiles (commercially for preparation of sizing agents), Fibers, Paper industries, Foodstuffs, Baking, Clarification of haze formed in Beer or Fruit juices and for

pretreatment of animal feed to improve digestibility also, manufacture of corn and Chocolate, Syrups, widely used in starch processing, (Leveque *et al.,* 2000; Vijayalakshmi *et al.,* 2012).

Amylases could be extracted from different sources such as plant, animal and microbes including bacteria and fungi (Kathiresan and Manivannan, 2006), These Bacteria are screened from natural resources including soil, Biogas plant, Kitchen waste and domestic waste water for its ability to grow on cheap substrates, producing enzymes at high stable rate and no toxic substances (Bahadure R.B.*et al.,* 2010; Kunal M *et al.,* 2011).Amylases are among the most important enzymes used in microbiology, particularly in process involving starch hydrolysis. Though amylases originate from different sources (plants,soil, animals and microorganisms), the microbial amylases are extensively produced and used in industry, due to their productivity and thermo stability (Burhan*etal.,* 2003).

As the evolved NILs have shown higher survival than the RPs, the amylase gene itself may confer higher survival by improving digestibility, or some other closely linked genes flanking the amylase locus may be responsible for better viability of the NILs. Against this background, the RAPD profile among the DPs, RPs, and NILs were analyses with the objective of identification of DNA markers linked to and bordering the amylase locus paving the way for further characterization of chromosome segments conferring higher survival in the evolved lines (Ashwatha*et al.,* 2009).

The present work mainly deals with the characterization of amylase degrading strains and to see the RAPD profiling on the growth of amylase degradingbacteria and are focused on following objectives 1) Screening and identification of amylase producing strain from soil 2) Morphological and biochemical characterization of identified strain 3) Estimating of temperature, pH and salt tolerance capability and RAPD profiling of bacterial strain

Materials and Methods

Material

Isolation of Amylase Producing Microorganisms

Soil samples was isolated from (sugarcane field) Aditya Biotech Agricon Research and Development Centre Nandanvan Road, Chandanidih, Raipur (C.G.) PIN - 492001, were Morphologically, Biochemically, Physiologically characterized and DNA isolated for RAPD profiling. 1.0g of the freshly collected soil was mixed with 9.0 mL of sterile distilled water in sterile test tube, serial dilutions were followed. 0.5mL of 10-5 dilution was pipetted into a sterile petri dish and overlaid with 20ml of nutrient agar. This was incubated at 37^0C for 24 hours.

Methods

Media Preparation

Amylase is an exoenzyme that hydrolysis starch, a polysaccharide into maltose, a disaccharide and some monosaccharide such as glucose. Starch agar is a differential medium that tests the ability of an organism to produce certain exoenzyme, including a-amylase and oligo-1, 6-glucosidase that hydrolyze starch. The following composition of starch agar medium:

Screening for Amylase Activity (Starch Iodine Test) Isolated colonies were picked up from each plate containing pure culture and streaked in straight lines in starch agar

plates with starch as the only carbon source. After incubation at 37ºC for 24-48 hrs., 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 colonies which were showing zone of clearance in starch agar plates were maintained on to nutrient agar slants.

Morphological and Biochemical Characterization

The Gram staining was performed to differentiate microorganism either gram positive or gram negative, Motility test is performed to determine whether the bacteria is motile or not. Indole production test, Methyl red test, Voges- proskauer test, Citrate utilization test, Catalase test, Gelatin hydrolysis, urease, phosphates test and Amylase production test were carried out.

Fermentation Test

Fermentation of various carbohydrates like Sucrose, Lactose, and Maltose etc. by the bacteria was tested by inoculating the broth with individual sugars as a sole carbon source and phenol red as an indicator. Phenol red is a pH indicator which is red in color at alkaline pH while in acidic pH it is yellow. If the bacteria ferment the sugar in the test after incubation the color of broth will change from red to yellow. The change color of broth from red to yellow indicates that the bacterium has ability to ferment the sugar in test.

Temperature

Temperature is an important role for the production of amylase. The effect of temperature on amylase production was studied by the incubating the culture media at various temperatures25, 30 35, 40, 45, and 50°C along with arbitrary control at 37°C. The enzyme assay was carried out after 24 hours of incubation.

pH

The effect of pH for amylase production was determined by culturing the bacterium in the production media with different pH. The experiment was carried out individually at various pH 5, 6, 7, 8 and 9. The enzyme assay was carried out after 24 hours of incubation.

Salt

Salinity test was used for the identification of high salinity tolerance and low salinity tolerance bacteria which grow in high saline medium and low saline medium. The media used for this test was NAM medium containing 1% to 6% sodium chloride.

Growth Curve

The amylase production by the selected experimental microorganisms was determined by optimizing the media by adding different bacteria in the production media. The experiment was carried out individually at various incubation periods such as 24, 48, 72 and 96h.

PCR amplification

RAPD PCR was performed on bacterial DNA samples using different primers (from suing universal primer) according to the method described by Williams *et al*. 1990. The thermal cycle profile was as follow: 5 min initial denaturation at 95ºC for 1 cycle, 1 min deneturation at 94ºC for 40 cycles , 1 min anneling at 35ºC for 40 cycle, 2 min

extraction at 72ºC for 40 cycle, followed by a final extension at 72ºC for 10 min 1 cycle. PCR product was analyzed in Gel Electrophoresis (Tarson M1-01) 2% agarose gel stained with ethidium bromide. Gels were photographed by Gel Documentation system (Bio Rad- Gel doc EZ imager). The phylogeny tree of bacterial isolates was made according to statistical program analysis (NTSYSpc 2.02)

Results and Discussion

Colony Characterization

For colony characterization colonies were pinkish and creamish, circular, smooth, bacilli, raised and translucent having shiny. Mishra S. and Behera N. (2008) and Rwarinda Angelo U. and Rangabhashiyam S. (2013) also found that the colonies were creamish, bacilli and edges translucent. OsfarSjofjan and Tri Ardyati (2011) colonies were pink in color.

Screening of Amylase Producing Bacteria

The bacteria isolated from soil were screened for amylase production on starch agar medium. From the soil samples 7 bacterial strains were isolated. But later during screening it was found that only 2 strains showed amylase activity. The four potential isolates were identified by standard morphological and biochemical characterization (Fig. 1 and 2).

Morphological and Biochemical Characterization

For morphological character study two strains were isolated from soil (sugarcane field) Aditya Biotech Agricon Research and Development Centre Nandanvan Road, Raipur District (C.G.). The strain identified as JP was gram negative and JT was gram positive .The bacteria give positive result in motility test; this was in accordance with the Sjofjan O. and Ardyati T. (2011) colonies were pink in color and gram negative. Study by Mishra S. and Behera N. (2008) and Samanta A. (2013) who found in their studies that the amylase degrading bacteria was Gram positive.

All the biochemical tests were carried out to screen the bacterial cultures which were then identified as amylase degrading bacteria. Amylase strains not reduced gelatinase and also gave positive result for catalase test describing that the amylase strains have catalase enzyme to catalyze the hydrolysis of hydrogen peroxide (Table1). Sjofjan O. and Ardyati T. (2011) also found that the amylase does not produce gelatinase enzymes as medium containing gelatin. In the cellulose test only JT have shown very good positive degradation of cellulose. In fermentation test lactose, sucrose and maltose are gives negative result. Vaseekaran *et al.,* (2010) found that amylase producing bacteria gives negative result on urease test. Kunal *et al., (*2012) found their study that amylase degrading bacteria was gives positive result to Gelatin hydrolysis, H_2Sproduction and in fermentation test Lactose was not produced acid.

Physiological Test

Temperature

Temperatures revealed that the all the two bacteria yielded maximum amylase production at 35^{o}C. Mishra S. and Behera N. (2008), reported that the thermal stability (Fig.1).

Effect of pH

All the four isolates were allowed to grow in media of different pH ranging from 4.0 to 9.0. Maximum enzyme activity was

observed in medium of pH 7.0. Alariya*et al.*, (2013)reported same findings (Fig.2).

Salts

Salinity test was used for the identification of high salinity tolerance and low salinity tolerance bacteria which grow in high saline medium and low saline medium. The media used for this test was NAM medium containing 1% to 6% sodium chloride in the present study, Sodium chloride was found to be the most suitable inorganic nitrogen source for amylase degrading bacteria (Fig.3).

Growth Curve of Starch Degrading Bacteria

Growth Curve: Effect of incubation period on amylase production showed that 72 hours was the optimum duration for maximum amylase enzyme activity. Above this period the amylase enzyme activity started to decrease. This is because, the cells may reach the decline phase and displayed low amylase synthesis (Table 2 and Fig.4).Bacillus sp. shows that the amylase production was detected from 48-72 hours and reached maximum activity at 48 hours byVijayalakshmi*et al.*, (2012); Alariya*et al.*, (2013).

Genetic Diversity Analysis

Genetic diversity analysis was performed by RAPD based markers. Total 18 markers used for the genetic diversity analysis. The range of alleles amplified was 6 to 10 for all the primers. Total 108 alleles amplified. The dendogram generated by using NTSYS-pc ver. 2.02software UPGMA matrix. Both the bacterial showed 20% similarities to each other that mean they are 80% dissimilarities to each other. Hanafy*et al.*, (2007**)** found 7% dissimilarities between species during RAPD profiling (Figure.5).

Table.1 Biochemical characterization of the bacterial strain

SN	Biochemical Characterization	JP	JT
1	Indole	_	_
2	MR	_	_
3	VP	_	_
4	Citrate	_	_
5	Catalase	+	+
6	Gelatin hydrolysis test	_	_
7	Amylase	+	+
8	Cellulose	_	+
9	Phosphates	_	_
10	Urease	_	_
11	Lactose fermentation	_	_
12	Sucrose fermentation	_	_
13	Maltose fermentation	_	_

Fig.1 Growth of amylase degrading bacteria on different temperature

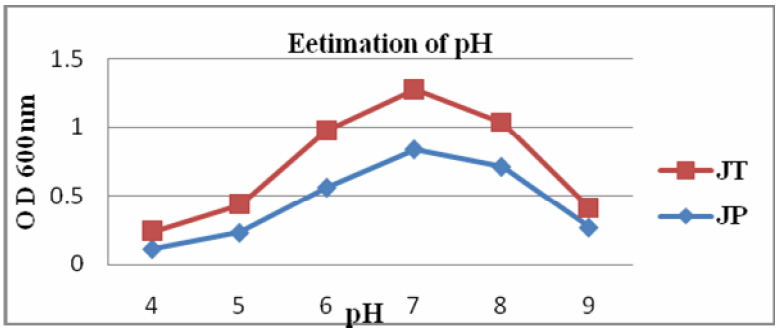


Fig.2 Growth of amylase degrading bacteria on different saline medium

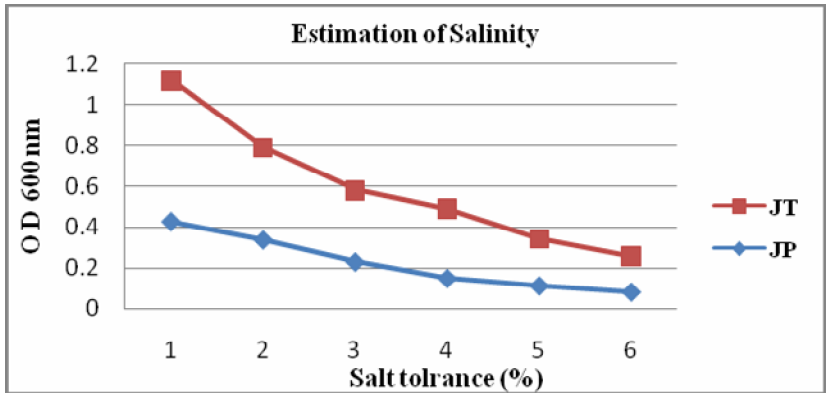


Fig.2 Growth curve of amylase degrading bacteria on different time

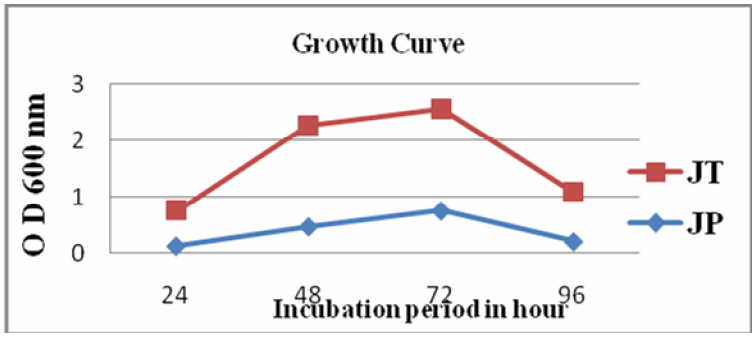


Table.2 Growth Curve of Starch Degrading Bacteria

GROWTH CURVE					
S.NO.	STRAIN NAME	24 H	48 H	72 H	96 H
1	JP	0.1222	0.4709	**0.7548**	0.2014
2	JT	0.6292	1.7858	**1.8040**	0.8872

Fig.3 Dendogram showing the genetic relationship among amylase degrading bacteria

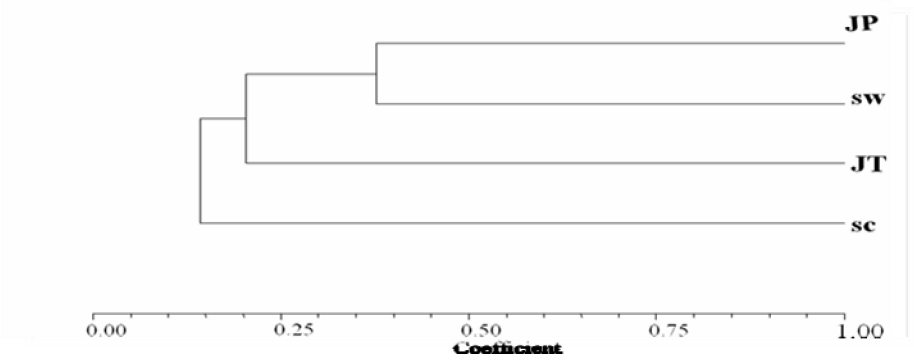
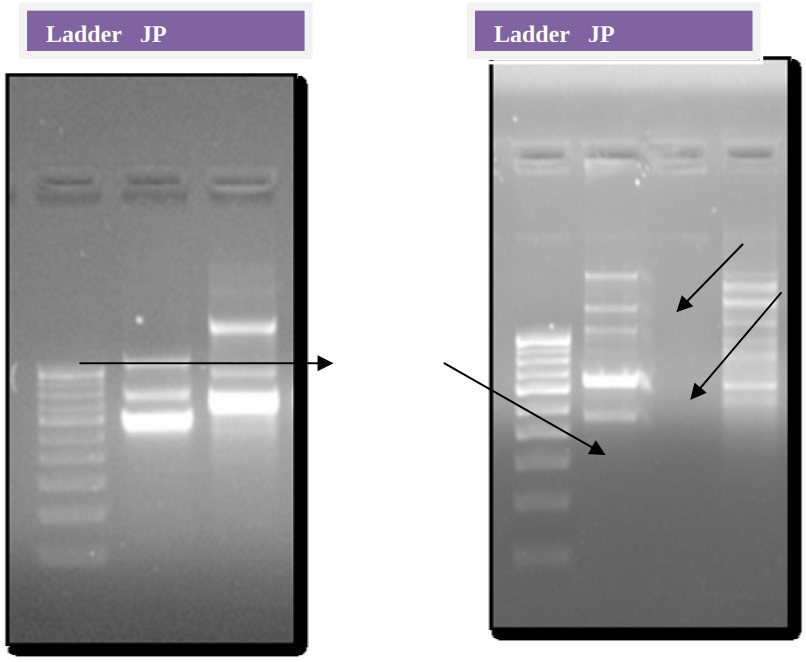


Fig.4 RAPD analysis of amylase degrading bacteria.



Rbac 05 Rbac 08

The enzyme has found many applications in commercial processes, including thinning and liquefaction of starch in alcohol, brewing and sugar industries (Richele, et al., 1998). Amylases are known to be produced by a variety of bacteria and fungi and their applications at industrial level have stimulated interest to explore their amylolytic activity in several microbes to be used as bioresources.The purview got further increasing with the discovery of new strains of microorganisms and development of more efficient production. (Vijayalakshmi*et al.*, 2012)

In this study amylase degrading bacteria and RAPD profiling was studied. For

characterization of bacteria colony, morphological, biochemical and physiological characterization were carried out. In colony characterization visual inspection was carried out in which circular shape, pinkish and creamish color and smooth margin was found. Besides these elevation and opacity was raised and was transparent.

Morphological test namely Gram's staining and motility test was carried out. The bacterium JP was found to be Gram negative and JT was gram positive. Motility test was positive for both JP and JT. IMViC test was done for biochemical characterization in which MR, VP, Indole and Citrate test, gelatinase, phosphates and urease test was negative for both JP and JT. Catalase test and amylase test was positive for both of strain. Only JT strain showed cellulase positive. In fermentation test three different carbon sources, lactose, sucrose and maltose were used but no one strain found positive for these fermentation tests.

Highest bacterial growth was recorded at 35°C and pH 7.0 with 1% salt concentration and bacteria optimum growth for was recorded in 72h. Genetic diversity analysis was performed by RAPD based markers. Total 18 markers used for the genetic diversity analysis. Both the bacterial showed 80% dissimilarities to each other that in the basis of genetic analysis and biochemical test also indicated the same.

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