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RESEARCH ARTICLE

ISOLATION, SCREENING AND CHARACTERIZATION OF AMYLASE PRODUCING BACTERIA FROM SOIL

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Abstract
The enzymes from microbial sources are more stable and obtained cheaply. Amylases are amongst most widely used enzymes in industries such as food, fermentation, starch processing, textile and paper. In the present investigation we report the isolation, screening and characterization of amylase producing bacteria from the soil samples collected from local area of Bhopal. Production conditions were optimized (temperature, pH etc.) to achieve high enzyme production and better enzyme activity.

Keywords: Enzyme, Isolation, Amylases, Characterization

INTRODUCTION

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. [1]Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylase scatalyze hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyze from the non-reducing end, successively resulting in short end products.

Amylases originate from different sources (plants, soil, animals and microorganisms), the microbial amylases are extensively produced and used in biotechnology with applications ranging from food, fermentation, textile to paper industries, due to their productivity and thermo stability.[2]

Bacteria of the *Bacillus* genus are among the most widespread microorganisms in nature, they can be found in soil, water and air. *Bacillus subtilis* constitutes a rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore. The *Bacillus subtilis* genome is totally sequenced, leading to generation of a great amount of basic knowledge in this bacterium.[3]Developments of molecular and genetic methodologies are well established in *B. subtilis*. *Bacillus subtilis* grow efficiently with low-cost carbon and nitrogen sources, because its enzymes are very efficient breaking down a great variety of proteins, carbohydrates and lipids from animal and vegetable origin.

The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. A highly thermostable and alkaline a-amylases is available from the

mesophile *Bacillus* sp.[4]The thermophilic bacterium *Bacillusstearo thermophylus* offers an alternative for commercial production of thermostable a-amylases.

MATERIALS AND METHODS

Soil sample collection

For bacterial isolation, 10 g of soil was collected from kitchen garbage containing area from residential area of Bhopal. Composite samples of 10g Soil were carried to the laboratory in an ice-box and isolation of Amylase producing bacteria was completed within 48h of sample collection. [5]

Isolation of Amylase producing bacteria:

Media preparation & Bacteria culture

[A] Nutrient agar media: It's a general purpose media which allows the growth of almost all bacteria, and it may be used to culture, subculture or storage of pure isolated bacterial colonies. The composition of media is;

1. Beef extract 3 gm,
2. Peptone 5 gm,
3. Sodium chloride 5 gm, and
4. Agar 15 gm
5. The entire component dissolves in 1000 ml of distilled water.

Serial dilution:

The serial dilution was prepared for isolation of micro-organism (bacteria) through spread on respective culture media plate. These plates are incubated at 37^{o}C for 3 days.

The procedure is as follows:

1. Take 9 test tubes with 9 ml sterile distil water and mark it as 10-1, 10-2, 10-3… up to 10-9.

2. Dissolve 1 gm soil sample in 10 ml of sterile distil water in another tube with vigorous shaking.

3. Now under aseptic condition take 1 ml suspension and mix it with tube 1 and shake it vigorously.

4. Again transfer 1 ml suspension from tube 1 to next tube 2 and so on up to 9th tube.

5. In such a way we may get a series of dilutions, having dilutions of as 10-1, 10- 2, 10-3,… up to 10-9, which could be used for culture microbes by spreading various dilutions on media.

6. Incubate the plates at 37oC for 48 hours, then observe a microbial growth.

Sub culturing:

The separate colonies of master plates were sub cultured on separate NAM plates and again incubated for 3 days at 37^0C, these procedure was repeat three times to the get the pure cultures of the bacteria of interest.

Preservation

The purified isolates were then transferred to the slants of nutrient agar media. The one -dram vial containing purified isolate, nutrient broth medium and sterilized glycerol was kept in the polyethylene bags, properly tied and preserved as stock culture.

Identification of Bacteria

Colony morphology and Microscopic study:

The morphological traits evaluated comprised colony morphology, mucous production and change in pH of the medium during growth and growth rate. Mucous morphology analysis was based on type, elasticity and appearance, while colony morphology parameters were diameter, form, transparency and color.

Gram's staining procedure:

Grams staining reaction was performed to evaluate type of strain.

Requirement: Crystal violet, Gram's iodine, Ethanol (95%) and Counter stain.

Endospore Staining:

Endospore Staining was performed to evaluate type of strain.

Requirement: Malachite green stain, safranin stain, filter paper pieces, distilled water, and sprit lamp.

Biochemical characterization of the test isolates

The 3 isolated colonies of bacterial spp. were biochemically characterized for gram reaction, catalase test, gelatin hydrolysis, starch hydrolysis, IMViC test and TSI test as per the standard methods (Holding and Collee, 1971).

IMViC TEST

Indole production test requirement

- Tryptone broth or peptone broth
- Kovec's reagent.

The Tryptone broth was inoculated with culture. Now 5 ml of 24 hours old culture was taken and 0.2 ml of Kovec's reagent was added. A cherry red red color in the alcohol layer indicates the positive indole test.

Methyl red test

MR-VP broth: Composition; Peptone 5 gm, Dipotassium hydrogen phosphate 5 gm, Glucose 10% solution 50 ml all the component dissolve in 1000 ml of distilled water

Methyl red indicator solution: Composition; Methyl red 0.1 gm, Ethanol 300 ml dissolves in 200 ml distilled water.

The tubes of MR-VP broth was inoculated with the test culture and incubated at 37^{o} C for 24 hour then after incubation 5-6 drops of methyl red solution was added. A bright red colour indicating a pH of 4.2 or less indicates positive test and yellow or orange.

Voges-proskauer test

MR-VP broth: Composition; Peptone 5 gm, Dipotassium hydrogen phosphate 5 gm, Glucose 10% solution 50 ml all components were dissolved in 1000 ml distilled water.

40% potassium hydroxide solution: The test culture was inoculated in the MR-VP broth and incubated at 37^{o}C for 48 hrs. After 48 hrs of incubation 1ml. of 40% potassium hydroxide and 3ml of a 5% solution of alpha-naphthol in an absolute ethanol was added. A positive reaction is indicated by the development of pink colour in 2.5 min becoming crimson in 30min. The tube can be shacked at intervals to ensure maximum aeration.

Simmons citrate test: Composition:

Sodium chloride 5 gm, Magnesium sulphate 0.2 gm, Ammonium dihydrogen phosphate 1 gm, Potassium dihydrogen phosphate 1gm, Sodium citrate 5 gm, Agar 20 gm, Bromothymole blue 40 ml, pH 6.8 make up 1000 ml with distilled water.

Simmons citrate medium (a modification of koser's media with agar and an indicator) was dispense in test tube, then sterilized at 121^{o}C for 15 min and allowed to set as slope inoculate, saline suspension of the organism to be tested is inoculated on the agar slant. Then incubate for 24hrs at 37^{o}C. A positive test shows a blue color on the streak of growth retention of original green color shows no growth on the line of streak indicates a negative test.

Catalase test:

A nutrient agar slant was inoculated with the test culture or on any other medium lacking blood and incubated at 37oC for 24 hrs following incubation 1 ml of 3 % H2O2 trickled was down the slant. Now examined immediately and after 5 min for the evolution of bubbles, which indicates the positive test.

Motility Test:

This test is done to help differentiate species of bacteria that are motile from non-motile.

Gelatin hydrolysis:

The test was perform to determine capability of microorganisms to produce gelatinase enzyme and use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme. The actively grown cultures were inoculated in nutrient gelatin medium and grown for 48h. on subjecting the growing culture to low temperature treatment at 4◦c for 30min, the cultures which produce gelatinase remains liquefied while others due to presence of gelatin becomes solid.

Composition: Peptone 5gm, Beef extract 3gm, Gelatin 12gm mix with 1000ml distilled water.

Starch hydrolysis:

The test was performing so as to determine capability of microorganisms to use starch as carbon source. Starch agar media were inoculated with test isolates, incubated and analyzed. In the presence of starch, the production of extracellular enzymes occurs indicating the potential of the organism to use starch. Iodine test was to determine capability of microorganisms to use starch. Drop of iodine solution (0.1N) were spread on 24h old cultures grown on starch agar media plate. Formation of blue color indicated non-utilization of starch.

Composition: Peptone 5gm, starch 0.5%, Beef extract 3gm, agar 17gm mix with 1000ml distilled water.

Triple sugar iron test:

The test was performed to determine the capability of isolates to use various carbohydrate sources e.g. sucrose, glucose, and lactose etc. s media for growth. Triple sugar iron agar media composition is as follows;

Composition: Beef extract 3gm, yeast extract 3gm, peptone 15gm, Nacl 5gm, lactose 10gm, sucrose 10gm, ferrous sulphate 0.2gm, sodium thiosulphate 0.3gm, phenol red 0.24gm, agar 15gm, distilled water 1000ml, pH 7.0

Color on the butt and the slant was observed. On this basis capability of organism to use

Sucrose Fermentation

This test is done to help differentiate species of the family *Enterobacteriaceae.* This tests the bacteria's ability to ferment sucrose and production of acid end-product

Media and Reagents Used: Sucrose broth contains beef extract, gelatin peptone, and sucrose. Phenol red indicator is added to indicate an acid end-product.

For this test a loop full of bacterial culture is inoculated in the medium in sterile condition and incubates at 37^0C for 48 hrs. After the incubation the cultures were taken for observation. A positive result is yellow after indicator is added (indicating lactose fermentation) A negative result will have no color change or will be reddish.

Lactose Fermentation

These tests for the bacteria's ability to ferment lactose.

Media and Reagents Used: Lactose broth contains beef extract, gelatin peptone, and lactose. A phenol red indicator is added to indicate acid production from fermentation.

For this test a loop full of bacterial culture is inoculated in the medium in sterile condition and incubates at 37^0C for 48 hrs. After the incubation the cultures were taken for observation. A positive result is yellow after indicator is added (indicating lactose fermentation) A negative result will have no color change or will be reddish.

Glucose fermentation and Gas production:

This test is done to help differentiate species of the family *Enterobacteriaceae*. This tests for the bacteria's ability to ferment glucose and produce gas and/or an acid end-product.

Media and Reagents Used: Glucose broth contains beef extract, gelatin peptone, and glucose. A phenol red indicator is added to indicate an acid end-product. A Durham tube is added to indicate gas production. For this test a loop full of bacterial culture is inoculated in the medium in sterile condition and incubates at 37^0C for 48 hrs. After the incubation the cultures were taken for observation. A positive result for gas is a bubble in the Durham tube and completely negative result has no color change or reddish color and no bubble.

Designing of fermentation media to production of Amylase and charging of bacteria:

For the production of Amylase, the fermentation media of 100ml was prepared and inoculated with Amylase producing *spp.* and incubate for five days with regularly shaking for the mass cultivation of bacteria. Compositions of fermentation media is as follows:

Composition:

1. Peptone : 6gm
2. Magnesium sulphate: 0.5gm
3. Starch: 1gm
4. Distilled water : 1000ml
5. pH : 7

Screening of bacterial isolates for Amylase production:

To determine the production of Amylase in prepared fermentation medium the culture were streaked on Starch agar plate and incubate for 24 hrs at 37^0C then observe the results.

Physiological Test

Temperature and pH

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. 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.

Cell harvesting and separation of fermentum:

After the 5 days incubation of fermentation media the cells were harvested by centrifugation process. The bacterial culture was taken in centrifugal tubes and centrifuge at 5000rpm for 30mins.after this the extract suspension were collected separately and apply triple filtration technique to avoid any impurity in the suspension.

Enzyme Activity:

The activity of Amylase was determined or estimated through colorimeter and take the absorbance at 620 nm which determine its activity in this hydrolysis of starch is determined by amylase the absorbance was decreased which shows starch hydrolysis by amylase.

Screening of Amylolytic Activity:

Required Material:

1. 1% starch solution
2. Iodine solution
3. Test tubes
4. Micropipettess

For the revealing of amylase in extracted samples starch degradation test were performed; the process of this method is as follows;

- 6 test tubes were taken in which 5 test tubes were poured by 1% starch solution in different concentration from 100% to 6.25%.
- One test tube was taken as negative control filled with distilled water.
- Test tubes were shifted for incubation for 24 hrs in dark condition.
- After incubation period in each test tubes 1ml starch solution was added to verify the activity of amylase.
- The reaction is taking on for observation.

RESULT AND DISCUSSION

Sample collection

Soil Sample was successfully collected from three different area of kitchen garbage containing area from residential area of Bhopal (M.P.) for the isolation of Amylase producing bacteria, under supervision and support of Scan Research Laboratories, Bhopal. For bacterial isolation, 10 g of soil was collected. Soil sample were collected from upper layer of the land where maximum population of microorganism was concentrated. Soil sample was collected by using clean and dry sterile spatula in clean polythene

Table 1: Different Soil samples collected from different regions for Amylolytic Study of Microbial Load.

SN	Collected Sample	Location	Codes
1.	*Soil 1*	Subhash nagar, Bhopal	S-1
2.	*Soli 2*	Indrapuri, Bhopal	S-2
3.	*Soil 3*	Ashoka Garden, Bhopal	S-3

Isolation of bacteria from soil

Serial dilution & Spread plate Method:

In 10-fold serial dilution method, three dilutions were selected for spread plate method from all three samples and apply on nutrient agar plate, i.e. 10^3A, 10^3B, 10^4A after the growth of micro-organism the cells were selected for plate count method and for streaking.

Bacterial Count

The results of bacterial count performed for the microbiological quality analysis of Total bacterial load are described in the table 3.1. for dilution plate count dilution no. 10^4 was chosen and taken for count. The total numbers of bacteria per ml or per gm of sample were successfully calculated using Standard plat count. The results are described in the table 3.2. The results from the table clearly describe the total microbial load in each soil sample. As the Soils are the foundation of all terrestrial ecosystems and are home to a vast diversity of bacteria, archaea, fungi, insects, annelids, and other invertebrates as well as plants and algae. Soil microbes, bacteria, archaea, and fungi play diverse and often critical roles in these ecosystem services.

This microbial standard plate count may contain both harmful and beneficial microorganisms.

Table2: Results of the bacterial count performed for the microbial load present in the soil samples.

S.N	Code	SPC	DPC (at 10^{-4} dilution)
1	S-1	1183	323×10^5
2	S-2	2109	544×10^5
3	S-3	937	328×10^5

***SPC= standard plate count, DPC= dilution plate count.**

Streak plate method:

After the incubation, from the all spread plate of selected dilution we found different type of bacterial colonies. Pick each one different colony and streak them on nutrient agar plate separately. The process was performed again and again until we found a single and pure form of strain. On the basis of colonial morphology, shape and size following strains were selected for study.

Table 3: Results of the bacterial colony select for the present Study.

S.No.	Sample Code	Strain Code	Appearance On Nutrient Agar Medium
1	S-1	10^3A	Large muous colony
2	S-2	10^3 B	Small white colony
3	S-3	10^4A	Small yellow colony

Sub culture:

The mix colonies appeared on three NAM plates after incubation were further sub cultured for pure culture preparation. 3 colonies were sub cultured on another NAM plates. This procedure repeated three times.

Identification of Bacteria

Microscopic study:

***Gram's staining*[10]**

In microscopic study Strain 10^3A shows Pink rod shaped colonies (Gram negative bacilli),10^3B shows Purple rod shaped bacteria arranged in chain form (gram positive bacilli) where as 10^4A shows Purple round shaped arranged in chain form (Gram positive cocci)

Endospore Staining

In Endospore staining test results, only 10^3B colonies are shows positive test which is green spores arranged on rod shape structures of gram positive bacterial cell wall.

Biochemical characterization of the test isolates:

The 3 isolated colonies of bacterial spp. were biochemically characterized for gram reaction, catalase test, gelatin hydrolysis, starch hydrolysis, IMViC test and TSI test as per the standard methods.

For the studies on total bacterial enumeration and screening of amylase producing bacteria in the soil sample chosen in present study, basically we were used the biochemical identifications tests after isolation method. The strains were successfully isolated from the selected samples using their respective selective media and identified on the basis of their colony morphology, microscopic characters, and biochemical behavior on specific media and metabolic tests in a partial manner determined in Burgey's manual of determinative bacteriology flow chart. The results of the tests for bacterial identification are

depicted in the table no. 4. On the basis of growth on selective & differential media, the strains were used for the partial identification tests as indicated in Bergey's manual.

Table 4: Results of microbiological & biochemical tests for confirmation of bacterial identification.

S. No.	Tests applied		Results of bacterial strains		
			10^3 A	**10^3 B**	**10^4 A**
1.	Grams staining		Negative Rod	Positive Rod	Positive cocci
2.	Endospore Staining		Negative	Positive	Negative
3.	Catalase		Positive	Positive	Positive
4.	Motility test		Positive	Positive	Negative
5.	Indole production		Positive	Positive	Negative
6.	Methyl red Reduction		Positive	Negative	Positive
7.	Vogus Proskaur		Negative	Positive	Negative
8.	Cirtrate Utilization		Negative	Positive	Negative
9.	TSI		Positive	Positive	Negative
10.	H_2S		Negative	Positive	Negative
11.	Starch hydrolysis		Negative	Positive	Negative
12.	Gelatin hydrolysis		Negative	Positive	Positive
13.	Carbohydrate fermentation	Sucrose	Acid & Gas	Acid	Acid
		Lactose	Acid &Gas	Acid	Acid
		Glucose	Acid	Acid	Acid

Catalase test

Oxygen is sometime toxic. Small amount of superoxide free radicals are found during the normal respiration of Bacterial spp. that use oxygen as the final electron acceptor. Obligate anaerobes form some oxygen free redicals that are toxic to the cell. Hence, if bacteria want to grow in oxygen environment, enzymes like catalase and superoxidase dismutase must be present for neutralization of the toxic form of oxygen (oxygen redical).

In present study, between three selected colonies for the isolation of Bacterial amylase only 10^3B culture cells gives positive result for catalase test. The result of catalase test is depicted in table no. 4.

Gelatin hydrolysis:

The test was perform to determine capability of microorganisms to produce gelatinase enzyme and use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme. The actively grown cultures were inoculated in nutrient gelatin medium and grown for 48h. on subjecting the growing culture isolates to low temperature treatment at 4◦c for 30min, the isolate 10^3B and 10^4A both are produce gelatinase remains liquefied while others due to presence of gelatin becomes solid.

Starch hydrolysis:

This test was performed for the detection of amylase producing microorganism that hydrolyzed starch in to simpler form. Only 10^3Bstrains are the microorganism that shows starch hydrolysis positive.

IMVIC TEST:

(a)Indole production test requirement

In this test, the all three bacterial spp. under consideration is grown in peptone Water Broth. It contains tryptophan, which under the action of enzyme tryptophanase is converted to an Indole molecule; pyruvate and carbon dioxide. The indole is then extracted from the broth by means of xylene. To test the broth for indole production, Kovac's reagent is added. A positive result is indicated by a Pink/Red layer forming on top of the liquid are shown in 10^3A and 10^3B strains.

(b) Methyl Red test and Voges–Proskauer test

These tests both use the same broth for bacterial growth. The broth is called MRVP broth. After growth of bacterial *ssp.*, the broth is separated into different tubes according to bacterial strains different tubes, one for the Methyl Red (MR) test and one for the Voges-Proskauer (VP) test that means for each bacterial strain, two broth tubes are required one for MR test and one for VP test. The pH indicator for MR test Methyl Red is added to one tube and a red color appears at pH lower than 4.2, and indicated positive test. The VP test uses alpha-naphthol and 40% potassium hydroxide to indicate a positive or negative test. Strain 10^3A and 10^4A shows positive result for MR test and strain 10^3B shows positive test for VP.

(c) Simmons citrate test:

This test uses Simmon's citrate agar to determine the ability of Bacterialstrainsto use citrate as its sole carbon source. The citrate agar is green before inoculation, and turns blue as a positive test indicator. For this test only 10^3B strain shows positive result.

TSI test:

The test was performed to determine the capability of isolates to use various carbohydrate sources eg. Sucrose, glucose, lactose, etc as media for growth. Color on the butt and the slant was observed. On this basis capability of organisms to use carbohydrates, three possible observations could occur.

In the present study the screening of bacterial amylase producing spp., the isolation, identification and mass production were performed. The soil samples were collected from Kitchen garbage fields and serial dilutions were done.

Out of 3 isolates, we were chosen 10^3B strain as it shows the positive result for Starch Hydrolysis test and on the basis of identification and biochemical characterization, the strains expressions are similar to the bacterial spp. ***Bacillus subtelis***. (According to Bergey's Manual).

Mass cultivation of bacteria in Starch fermentation media for production of Amylase:

After all identification and biochemical characterization of pure strain of *Bacillus Subtelis* it was inoculated in fermentation media of Amylase called Starch fermentation media (SFM) and incubate for seven days with regular shaking at 30 to 37^0C for the production of medium [7]

Screening of bacterial isolates for Amylase production:

In the screening test of fermentation medium for Amylase production bacterial culture which are streaked on starch agar plate were treated with Iodine solution. Appearance of clear zone around the bacterial growth shows positive test for amylase.[8]

Physiological Test:

Effect of Temperature and pH

Temperatures revealed that the all the two bacteria yielded maximum amylase production at 35^{o}C. Thermal stability at atmospheric temperature are increase the growth in cellular mass. 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.

Cell Harvesting to extract the Amylase fermentum:

After the fermentation process of seven days the media were successfully centrifuged at 5000rpm for 30 mins. at room temperature. Then the suspensions were collected separately in sterile condition and apply triple filtration method to avoid any contamination.

Amylolytic Activity:

On the basis of observation; test tube containing starch concentration of 25%, 12.5% and 6.25 % were degrade completely at the given incubation period where as 50% and 100% degrade slowly. The results are as following

Table 5: results of Amylolytic Activity

S. NO.	Concentration of Starch	Observation
1	100 %	Light bluish color
2	50%	Light bluish color
3	25%	Colorless
4	12.5%	Colorless
5	6.25%	Colorless
6	Control(D/W)	Brownish due to iodine

Optical Density of testing samples:

Optical densities of testing five samples were taken with the help of colorimeter at 540nm.finding readings are shown in table below:

Table 6: Optical Density of testing samples

S.NO.	Concentration of Starch	Observation	OD at 620 nm
1	100 %	Light bluish color	0.82
2	50%	Light bluish color	0.78
3	25%	colorless	0.47
4	12.5%	colorless	0.44
5	6.25%	colorless	0.23
6	Control(D/W)	Brownish due to iodine	0.34

On the basis of finding results we found that at lower starch concentration 25%, 12.5% and 6.25 % degrade completely from extracted amylase by *Bacillussubtilis* bacteria whereas at higher concentration that is 100% and 50% of starch take time to degrade completely from extracted amylase.[9]

Conclusion

It was concluded that the soilis a potential source for amylase producing microorganisms. Isolate produce amylases at culture conditions and different factors greatly regulates the growth and production of amylases. The optimum temperature and pH for the activity of the amylase obtained from this strain were 35^{o}C and 7.0.The isolates evaluated the higher concentration that is 100% and 50%Amylolyticactivity was obtained from *Bacillus subtilis.*

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