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Amylase Activity of a Starch Degrading Bacteria Isolated From Soil Around Tulja-Bhavani Temple of Tuljapur.

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Abstract: *The amylase can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands because it is economical when produced in large quantities. The aim of current study was to isolate amylase producing bacteria from the soil samples collected from Tulja Bhavani Temple of Tuljapur. The isolation was done by serial dilution and plating method. Total 20 bacterial isolate were isolated from the collected soil samples. All isolates were screened for amylolytic activity by starch agar plate method. Among 09 bacterial isolates, only 03 isolates showed the amylolytic activity. Out of there one isolate was selected on the basis of maximum hydrolysis for further study. The 16 s r RNA sequences of selected strain was isolated and sequenced. The isolate was identified as Pseudomonas aeruginosa JCM 5962 (T). On the basis of morphology, biochemical tests and 16 s Rrna sequence done from microbial cell culture of DBT branch NCCS (National Center for Cell Sciences at Pune, Maharashtra) The enzyme production was done by using amylase production media at 37°c in shake flask culture. The enzyme was extracted by centrifugation and then it was purified by ammonium sulphate precipitate 40%. The partially purified fraction was used for the charcterisation studies of amylase.It was 1 fold purified fraction with 90.2 % yield ,fifty fractions (1ml/10min) were collected & the O.D. at 530nm showed major peak, represented by 37-43 fractions.Purified enzyme was seen as single band on SDS-PAGE image, whose apparent MW was 1.5 KDa, Purification fold of sephadex-G - 100 fraction amylase was 1.14 with 50 % yield.*

Keywords: *Amylase production, Enzyme purification, Soil bacteria, Sephadex, SDS-PAGE.*

I. Introduction

Enzymes are proteins which catalyze specific biochemical reactions in a very efficient manner. Enzymes have been used for thousands of years as crude animal and plant preparations or as whole micro-organisms which were allowed to grow on substrate. The industrial production of enzymes dates back to 1894 when fungal 'taka-diastase' was marketed for pharmaceutical use. Among no. of enzymes are produced on large scale amylases achieve largest quantities (300 ton/year of each) and used in commercial operations.

Amylase is an endo-hydrolase. It hydrolyzes starch into components which have 3 or more linear alpha 1, 4 glucan units. It stops hydrolysis when fragments contain an alpha-1, 4-6 linked branch-point residue. The end products of starch hydrolysis are dextrin which are used as adhesives and thickening agents in prepared foods.

Microbial amylases are used for modifying starch in vegetable purees, and in treating vegetables for canning (Bode 1954)

A significant application of the bacterial enzyme is in the continuous process for designing of textile fabrics (Gale, 1941, Wood 1947) another is in preparing modified starch sizing for textiles (Gale, 1941) and starch coatings for paper. Amylases are significant enzymes for their specific use in the industrial starch conversion process, starch liquefaction, paper, food, sugar and pharmaceutical industries. In the food industry amylolytic enzymes have a large scale of applications, such as the production of glucose syrups, high fructose corn syrups, maltose syrup, reduction of viscosity of sugar syrups, reduction of turbidity to produce clarified fruit juice for longer shelf-life, solubilization and saccharification of starch in the brewing industry.

The baking industry uses amylases to delay the staining of bread and other baked product, the paper industry uses amylases for the reduction of starch viscosity to achieve the appropriate coating of paper. Amylases enzyme is used in the textile industry for warp sizing of textile fibers, and used as a digestive aid in the pharmaceutical industry. In preserve work, to isolate microbial strains from soil around Tulja-Bhavani Temple enriched with wheat flour and oil and to screen enzyme amylase producing microbial strains.

Tuljabhavani temple (Tuljapur, Maharashtra) is very ancient temple. People from different corners of Maharashtra and some part of Karnataka and Andhra Pradesh visit this place to worship. They put a lot of cereal flour, edible oil and coconuts etc in campus of temple, since last so many years, an Array of micro flora may be residing on this substratum. Hence we may get novel enzyme producers. We do biochemical assays for selection of microbial strains showing strongest activity, molecular identification and biochemical characterization of selected microbial strains to make enzymes and also production conditions were optimized (temperature, pH) to achieve high enzyme production and better enzyme activity.

This work was undertaken to obtain a new obligate microorganism which had a well-known Amylase production, twenty bacterium strains were isolated and characterized from soil samples that were collected at different regions of Tulja Bhavani Temple, one of which had the highest amylolytic activity was selected for enzyme production.

II. Materials And Methods

1. Soil sample collection:- soil samples were collected from different parts of Tulja-Bhavani Temple, Tuljapur. Soil sample were collected from upper layer and also 3 to 4 cm depth of some different parts of Tulja Bhavani Temple where maximum population of micro-organism was concentrated. 5 gm of soil sample was collected by using clean and dry sterile spatula in a clean polythene bag.
2. Sephadex G-100 was obtained from Emcure pharmaceuticals.
3. Molecular weight calibration kits (SDS-PAGE) gel electrophoresis from Bioera.
4. Buffer ingredients and all other chemicals were AR grade produced from Rajesh Chemicals, Aurangabad.
5. Pure culture:-

For reducing microbial population, 1 gm of soil was dissolved in 10 ml of sterile distilled water to make soil suspension. Serial dilution was made and was plated on nutrient agar by spreading 0.1 ml of the diluted sample for getting isolated single colony. Then the plates were kept for incubation at 37°c for overnight.

After observing growth of bacteria in colony form on plate, then peak up some growth and perform streaking plate method was used to get single colonies of pure culture.

From this pure culture:-

Screening for amylase activity:-

Isolated colonies were picked up from each plate containing pure culture and streaked in straight lines in starch agar plate with starch as the only c-source, after incubation at37°c for 48 hours, individual plates were flooded with freshly prepared Gram's iodine, if in the zone of degradation no blue color forms, (deep blue colored starch iodine complex), which is the basis of the detection and screening of an amylolytic strain. The colonies were showing zone of clearance in starch agar plates, were maintained on to nutrient agar slants and stored at 4°c.

Morphological and Biochemical Characteristics:-

1. Cultural characterization:-
   The isolates were observed under the microscope to obtain the colony morphology i.e. color, shape, size, nature of colony and pigmentation.
2. Microscopic observation (Nature of isolated bacteria)
   The bacterial isolates were gram stained as per the procedure explained by Christian gram and observed under a high power magnifying lens in light microscope.
   Bacterial motility was done by hanging drop method and the motility was examined under microscope 45x magnification showing Brownian movement.
3. Biochemical characterization:-
   Biochemical test such as indole test, methyl red test, simmon's citrate test, urease test, gelatin hydrolysis test, starch hydrolysis test, casein hydrolysis, voges proskayer test were carried out to find the enzymatic activity of isolates organism.

Enzyme production medium:-

The amylase production was carried out in 250 ml conical flasks containing 50 ml medium with the following composition.

5 gram/litre soluble starch, 5 g/l yeast extract, 2.5 g.l $(NH4)2SO4$. 0.2 G/L $MGSO4.7H2O$, 3 g/l KH and 0.25 g/l $Cacl2.2H2O$ incubated and 50°c for 48 hours under shaker incubator and inoculated with two loop of 24 hours old culture of bacteria (Yasser R. Abdul Fattah, 2013), (Ajayi, 2008)

After incubation, the production medium was centrifuged at 6000 rpm for 20 min to separate the cells. The supernatant was collected as it contained the crude enzyme and stored at 4°c till further use. (Varalakshmi et al., 2008) (Oyelete S. B. et al 2011)

Amylase activity for crude sample:-

Amylase activity was estimated by the activity of reducing sugar released during hydrolysis of 1% w/v starch in 0.1 M Phosphate Buffer at pH 6.5, 25°c for 20 min. under heating by dinitrosalicylic method (DNS method) miller 1959.

After 20 min showing yellow orange color changes to dark-orange red color indicated that amount of enzyme are fulfill to release the reducing sugar as glucose from substrate. i.e. one unit of amylase activity was

defined as the amount of enzyme that release 1 mmol of reducing sugar as glucose per min assay. (ConditionVaralakshmi et al, 2008)

Enzyme purification

Enzyme was purified by ammonium sulphate (40%) method. The precipitate obtained by centrifugation was dissolved in 0.1 M phosphate buffer (pH 6.8) and dialysed overnight against 0.05 M phosphate buffer.
Dialysing bag was kept in hot water (60°c) and washed thoroughly to remove adhering material. One end of it was packed with rubber band and through other end sample was passed and packed. The bag was kept in 0.05 M phosphate buffer for 48 hours.
The experiment was carried out under low temperature 4°c to prevent denaturation.
The sample obtained after dialysis subjected to sephadex G-100 column chromatography for further purification.

Enzyme purification by sephadex G-100

Preparation of the gel column and the fractionation procedures was determined as previously mentioned by Ammar (1975). For this purpose sephadex G-100 column has been used sephadex G-100 was used with 0.2 M Phosphate buffer of pH 6.2 and the slurry was allowed to swell for 2 days at room temperature with addition of sodium azide (0.02%) was added to prevent any microbial growth.
One ml of the enzyme preparation sample was applied carefully to the top of the gel and allowed to pass in to the gel by running the column. Buffer was added without disturbing the gel surface and to +ve reservoir fifty fractions (1ml of each) were collected. The concentrated enzyme solution is loaded on sephadexG-100 column. (4.5 cm × 50 cm length) pre-equilibrated with 0.2 M phosphate buffer (pH 6.0) and the elution is carried out by the same buffer containing 0.2 M NaCl (method of El-safey and Ammar 2002) at a flow rate of 1.0 ml/10 min. at room temperature.

The protein content of fractions is determined by measuring optical density at 530 nm. The protein containing fractions are assayed for enzyme activity.

Enzyme purification by gel-electrophoresis:-

Molecular weight of protein is estimated by SDS-PAGE according to Lacmmli (1970) on a vertical slab of polyacrylamide gel at 150V a constant voltage for 5 hrs using alpha-Lactalbumin (14.2 KDa) is used as standard protein molecular weight marker.
The sample was run on an agarose gel. Single band was visualized when observed under the gel doc, which confirmed the purity of sample, as the bands were single, distinct and no traces of contaminants were observed. Results were finally photographed.
.Amylolytic activity and protein contents were carried out for each individual fraction.
The partially purified amylase from sephadex G-100 was collected and then

1. Enzyme activity:-
   Amylase enzyme activity by E1-Safely and Ammar (2002)
2. Protein determination:-
   the protein content of amylase enzyme was determined by the method of Biuret.

Factors affecting activity (Enzyme Kinetics):-

The following factors were studied to obtain the optimal condition for activity of partially purified Amylase. These factors include pH and temperature.
As large quantity of protein solution was required for repeated amylase assays ,40%-60% ammonium sulphate fraction was used for the characterization studies. An effect of temperature was studied from 10 to 80°c,Effect of pH was studied from pH 2.0 to pH 11.9.The optimum temperature of Amylase produced from pseudomonas aeruginousa is found to be 40°c.The optimum pH of amylase produced from Pseudomonas aeruginousa shown at pH 7.0.

III. Result And Discussion

1. Isolation of Amylase Producing Bacteria:-

Total six bacterial isolates were collected and processed by serial dilution and spread plate method for isolation of Amylase producing bacteria. A total 20 different bacterial strains were isolated on the basis of colony morphology. All isolates were primarily screened for amylase production by starch hydrolysis test. Among 20 isolates, 3 isolates hydrolysed the starch on starch agar. The starch showing maximum inhibition zone by enzyme in starch hydrolysis test were selected and further characterized by morphological and biochemical test and also showing to microbial experts.
Screening result of strain pamy 1

Molecular characterization based on 16 s Rrna Sequence:-

The amylase producing strain pamy1 was send to microbial culture collection under National Center for cell Science-DBT branch (Pune, Maharashtra) for to study 16 s rRna sequence analysis and identification of starch sample based on the 16 srRNA sequences, the above strain was confirmed as Pseudomonas aeruginosa.

DNS Assay of crude and purified enzyme:-

In order to get the concentration of enzyme in enzyme sample, a standard graph with known concentration of a standard (maltose) was plotted as shown.

Concentrations of enzyme in crude and purified sample were calculated by reacting the enzyme with 1% starch and comparing the resultant O. D. with standard graph.

Purification of Amylase:-

The isolated sources of Pseudomonas aeruginosa maximum enzyme production was observed at 48 hours. The protease enzyme from Pseudomonas aeruginosa was partially purified by ammonium sulphate fractionation as described previously and also by Sephadex G-100 column chromatography.

The eluent collecting after chromatography are run for SDS-PAGE gel electrophoresis for checking the purity of sample against standard marker Lactalbumin.

The purified enzyme in gel electrophoresis are run straightly. Single band was visualized when observed under gel doc, which confirmed the purity of sample as the bands were single, distinct and no traces of contaminants were observed. Results were finally photographed.

Enzyme optimization:-

Optimization for different parameters such as temperature and pH, on enzyme activity was carried out. Effect of temperature on enzyme activity was determined at different temperature from 10°c to 80°c and effect of pH on enzyme activity was determined at different pH within range 4-9. (K. Jaya Priya, 2011),
(Shembaker et al, 2013)
(Tippsmwat et al, 2006)

Microbial Identification Report
Microbial Culture Collection-NCCS
Sait Trinity, Pashan, Pune- 411021
v.04/2016

PRN (as given by MCC):

A-Apr_!6_070

Parameters Used for Database Search:

EzTaxon-e (http://ezbiocloud.net/eztaxon)
Reference: Kim et al. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 62: 716–721.

Summary of the closest neighbour(s) for your sample:

Sr. No.	Closest Neighbour	Strain	Citation	Accession No.	Pairwise Similarity (%)	Diff/Total nt
1	*Pseudomonas aeruginosa*	JCM 5962(T)	(Schroeter 1972) Migula 1900	BAMA01000316	100.00	0/851

Sequence Text (in FASTA format):

TCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATT
CAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACC
GCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTG
GTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTG
TGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTT
ACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTA
CTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAA
AACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC
ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGAT
AA

End of report

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IV. Conclusion

Various biochemical isolates from soil were studied for amylase activity. amylolytic activity was measured for high enzyme producing strain.Structural staining & biochemical activity results have revealed it is Pseudomonas aeruginuosa by 16srRNA study.

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Table -1 Biochemical Characterisation.

Sr. no.	Test	Observation	Inference
1	Gram staining	Grow on Mackonkey's agar. The slide was examined under the 45x light microscope, blue colored colonies were observed, Oxidase +ve.	+ve.
2	Shape	Rod shaped bacteria	Pseudomonas aeroginosa
3	Motility	Motility was observed under high power lens	+ve Brownian movement
4	MR-VP test	Bacterial respiration occurs shown by to change the medium color to red by adding methyl red indicator	+ve.
5	Citrate utilization test	No color change	-ve
6	Catalase test	No streaming up of oxygen bubbles	-ve
7	Starch hydrolysis	No blue color observed when reacted with iodine	+ve.

Table 2-Purification table for Amylase

Sr. No.	**Step**	**Total volume (ml)**	**Units (µmole/min)**	**Protein (mg)**	**Sp. Activity (units/mg)**	**Purificati on fold**	**% yield**
1	Crude sample	From 100 take only 10 ml	37050	1342	10.32	1	100%
2	Dialysed sample	24	12768	1056	11.7	90.25	100%
3	Sephadex G-100	5	1994.4	16.25	140.16	1.14	50%

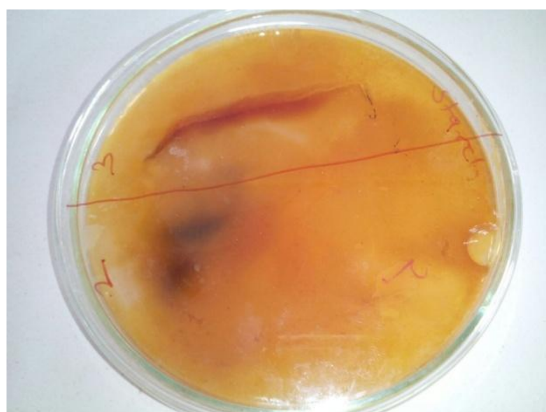

Fig-1. Starch Hydrolyzing Activity of Bacterial Strain

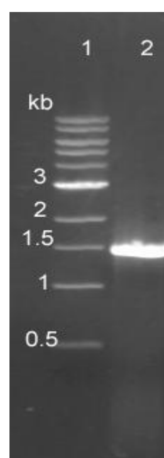

Fig-2. Sds- Gel Electrophoresis Of Purified Amylase

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