ISSN No:-2456-2165

Isolation, Identification and Characterization of Amylase Producing Microorganism from Soil

Amrutha Philip[1], Smitha Mathews[2], Dona Thomas[3] and Shradha Sanil[4]
[1]Department of Zoology, Assumption College, Changanacherry, Kottayam, Kerala, India

Abstract:- The enzyme Amylase catalyses the hydrolysis of starch into oligosaccharides and sugars. Bacteria isolated from the soil are the most common sources of amylase. In this study, soil samples (n=30) collected from different sites of Changanacherry were taken for screening amylase-producing bacteria. The samples were serially diluted and representative bacterial flora was isolated by plating on the nutrient agar media. The amylase production of these isolates was assessed using starch agar. Thirteen isolates were selected for further investigations. The growth conditions of these isolates were optimized and they were analysed for antagonistic activity. Two strains were selected for assessing antibacterial resistance patterns by the well diffusion method and to check the antibiotic sensitivity against 12 antibiotics using the disk diffusion method. The microbiological and biochemical characteristics of these strains were also determined. They were tested for extracellular amylase activity at elevated temperatures using the DNS assay method. The strain showing maximum amylase activity (2.395 units/ml) was identified as Alcaligenes faecalis using 16S rDNA-based method. The selected thermophilic strains can be employed to degrade starchy waste materials in monoculture or in a consortium, as well as for the industrial production of amylase. The study highlights that there is an increased scope for identifying industrially important bacteria from soil.

Keywords: *Soil, Bacterial strain, Antagonism, Extracellular amylase, Isolates.*

I. INTRODUCTION

With the increase in population during recent years, the problem of waste management has become an area of great concern. The eco-friendly management of the waste produced has become the major problem faced by environmentalists. Most of the waste produced is either dumped in a landfill or subjected to incineration. The organic waste produced can be degraded using bacteria which help in the bioremediation of the organic waste. Bioremediation is a process where biological organisms are used to remove or neutralize an environmental pollutant by metabolic process. The process of bioremediation is catalysed by microbial enzymes. The microorganisms, using these enzymes, degrade the waste and use this energy for their metabolic activities and growth. Amylase is a hydrolytic enzyme that degrades starch into simple sugars and is mainly produced by bacteria that use starch as their primary source of energy. Of the many uses of bacterial amylase, it also can be used to degrade organic waste such as kitchen and food waste. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics [[1.]]. Soil acts as a major source of amylolytic microorganisms. The above study was aimed at isolating extracellular amylase producing organisms from soil that can be further employed for waste degradation as a monoculture or in a consortium.

II. MATERIALS AND METHODS

A. Collection of samples

The samples were collected aseptically from Changanacherry (9º26′47.4”N76º32′24.8”E). They were carried to the lab in sterile polythene bags for further analysis and stored at 5̊C.

B. Screening and Isolation of Amylolytic Organisms

The samples were serially diluted upto the dilution of 10⁻6 and 0.1mL of last two dilutions were plated in nutrient agar plates fortified with starch. These plates were incubated at 37ºC for 24 hours. The plates were then flooded with Lugol'sIodine . The colonies that have a clear zone around it against the dark background are selected. These colonies are then purified using quadrant streaking. The pure cultures were subcultured in Nutrient Agar slants; incubated at 28ºC to achieve vigorous growth and then preserved in 20% glycerol vials at -20ºC [[3.]].

C. Determination of moisture content and pH of the soil

The petri plates were weighed and their weights noted down. 40g of soil was weighed and placed in the petri dish and were reweighed. The soil was placed in the hot air oven and dried overnight at 106°C. The samples were taken out from the oven and allowed to cool. The plates were weighed again with the dry soil. The moisture content was calculated using the following equation % moisture content

$$= \frac{\text{weight of moist soil (M)-weight of dry soil (D)}}{\text{Weight of dry soil (D)}} \times 100$$

To test the pH of the soil samples. 5g of each soil sample was taken into tube number 1 and transferred to tube number 2. 2mL of pH reagent (pH-1) was added to it and shaken for 5-10 minutes. One drop of decolouriser (D-1) was added and mixed well. The sample solution was filtered into bottle number 3 and 2 drops of pH reagent (pH-2) were added and shaken for 1-2 minutes until the colour developed. The colour was then compared with the standard pH chart.

D. Determination of physico chemical properties of soil

The analysis of nitrogen, phosphorus and potassium content was done.For nitrogen analysis 5g of each soil sample was taken in tube number 1 and transferred to tube number 2. 2mL of nitrogen reagent was added to it and shaken for 5-10 minutes. One drop of decolourizer (D-1) is added and mixed well. The sample solution was filtered to bottle 3 and two drops of nitrogen reagent (N2) was added and shaken for 1-2 minutes until colour developed. The colour developed was compared with the standard nitrogen chart. The same was repeated for phosphorus and potassium using their respective reagents.

E. Test for Antagonistic Activity

The antagonistic activity of the isolated strains was tested using cross streak. In this method, the strain to be tested is streaked along the middle of the agar plate. The indicator strain is streaked by a single streak perpendicular to the test strain and incubated at 37˚C for 24 hours. All isolates were tested against each other for antagonistic activity. The strains that showed antagonism were separated.

F. Optimization of growth conditions - pH and Temperature

The growth conditions of all the isolates were determined by growing them in different pH in different temperatures. Nutrient broth of different pH ranging from 2, 5.5, 7 and 9 were used. The isolate was inoculated into nutrient broth of all pH. The cultures from each pH were incubated in five different temperatures ranging from 8ºC, 21ºC, 32ºC, 37ºC and 60ºC for 24 hours. The same procedure was followed for all the ten isolates.

G. Antibiotic Susceptibility using Disc Diffusion Test

The disc diffusion method for antibiotic susceptibility was done [[5.]]. The strains to be tested are plated on Muller-Hinton Agar plate using the lawn culture method. The antibiotic discs of different antibiotics are placed at different corners of the agar plates. These plates are incubated at 35ºC for 24 hrs. The plates are studied for inhibition zones and the zones are measured in nearest millimetre to study the susceptibility of the organisms. The susceptibility to the following antibiotics were checked - Gentamicin, Tetracycline, Penicillin, Norfloxacin, Ceftriaxone, Ciprofloxacin, Co-trimoxazole, mezlocillin, Amikacin, Carbenicillin, Tobramycin. The zones were observed after 24 hours of incubation.

The MAR Index for each isolate was calculated using the formula:

$$\text{MAR Index} = \frac{\text{Antibiotic Resistance Shown}}{\text{Total number of antibiotics used}}$$

H. Test For Antimicrobial Activity Against Pathogenic Organisms

Well Diffusion Method was employed for the analysis of antimicrobial activity [[6.]]. The agar plates were inoculated with the pathogenic strains using a sterile swab on the entire agar surface. Holes of diameter 6-8mm were punched on the surface of the agar using a sterile cork borer. 10µl of the isolated sample was inoculated in the well using a micropipette. The samples were incubated at 37ºC for 24 hours and the result was obtained. Antimicrobial activity of the isolates were tested against six pathogenic organisms namely *Pseudomonas, Klebsiella, Escherichia coli, Staphylococcus aureus, Vibrio cholera* and *Vibrio parahaemolyticus.*

I. Microbiological and Biochemical Identification of the Isolated Bacterial Strains

The bacterial colonies were checked for colony morphology and gram staining properties. Various biochemical tests were also carried out to identify the characteristics of the isolated strains. The biochemical tests include Carbohydrate Fermentation Test for Glucose, Lactose and Sucrose, Indole test, MRVP Test, Citrate Test, Urease Test, Catalase Test, Oxidase Test and TSIA Test.

J. Enzyme Assay-DNSA Assay Method

The enzyme production media was prepared and autoclaved. The selected isolate was inoculated into fermentation media and incubated in a shaker incubator at 120 rpm, 37°C for 3-4 days. After the incubation, the culture centrifuged at 12,000 rpm for 15 min to get cell free culture filtrate. This is used as an enzyme to determine amylase activity. The amount of reducing sugar, produced by the hydrolysis of starch by amylase, can be estimated by DNS (3,5-dinitrosalicylic acid) method, thus can measure the activity of amylase. Two test tubes were taken, one as test and other as control. The enzyme assay is performed in tubes containing 0.5 ml phosphate buffer (100 mM. pH 7) and 0.5 ml substrate (1% starch). 0.5ml. of the enzyme is added to the reaction tube labelled test and incubated for 1 hour at 30⁰ C. The reaction is terminated at the end of incubation by adding 1 ml. of DNSA reagent to both test and control. To the tube labelled control 0.5 ml. of the enzyme is added. Then the assay tubes were kept in a boiling water bath for 10 minutes and 1 ml of distilled water was added to each tube. The absorbance of the red coloured complex developed is recorded at 540 nm using UV-Visible spectrophotometer. A standard curve is prepared using glucose standard solution (0.5 mg/mL). Enzyme unit (U) is defined as the amount of enzyme required to catalyse 1µmol of substrate per minute under the assay conditions [[7.]].

$$\text{Enzymatic activity u/ml/min} = \frac{\text{mg of glucose liberated} \times \text{total assay volume} \times \text{df}}{\text{ml of enzyme used} \times \text{time of incubation (min)}}$$

where:
df = dilution factor
ml. enzyme = ml. of enzyme added
time of incubation: 30 min

K. Identification

The strain which was found to have maximum amylase activity was identified using molecular analysis. Genomic DNA was isolated from the samples provided using Sigma Aldrich DNA extraction Kit. The quality was evaluated on 0.8% Agarose Gel. The 16s gene fragment was amplified

ISSN No:-2456-2165

by polymerase chain reaction (PCR) from the above isolated genomic DNA. The size and quality were evaluated on 1.5% Agarose Gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing of PCR amplicon was performed. The sequences were then used for similarity searches using the Basic Local Alignment Search Tool for identifying the sample.

III. RESULTS AND DISCUSSION

A. Isolation and characterization of Physico-Chemical properties of Amylolytic Bacteria

A total of 8 soil samples were taken. These samples were serially diluted (till 10^{-6} dilution) and plated on starch agar plates. After an incubation of about 24- 48 hours, the amylolytic (starch degrading) bacterial isolates were identified using the appearance of a clear zone around the streaked lines(Figure: 1).

Fig. 1: Growth of amylolytic bacteria on Starch Agar Media and presence of zone of clearance

The best isolate with highest amylase activity and different colony morphology were selected (13 isolates viz.ST M_1, ST M_2, ST M_3, ST M_4, ST Ms, ST Ms_3, ST D_1, ST K_1, ST WRS_1, ST WRS_2, ST P, ST Vc & ST Mo). The moisture content and pH of the soils from which the samples where isolated was tested. The sample from which STM_3 was isolated had a moisture content of 9.001% and pH-6. A pH of 6 and moisture content of 9.46% was exhibited by the soil from which $STMS_3$ was isolated. The other samples have a varying pH from 4 to 9 and a moisture content of 4%.The physicochemical properties like the Nitrogen, Phosphorus, and Potassium content of the isolated soil samples were analysed. The soil from which the isolate ST M_3 was obtained, the concentration of Nitrogen was 151-200kg/acre, that of Phosphorus was 8-10 kg/acre and Potassium had a concentration >150kg/acre. The soil from which the isolate ST MS_3 was obtained, the concentration of Nitrogen was 151-200kg/acre, that of Phosphorus was >15kg/acre and Potassium had a concentration of 50-80kg/acre. Soil pH and mineral Nitrogen availability appears to have a cooperative effect on bacterial abundance with soil pH being the key influencing factor [[8.]].

B. Antagonistic Activity of the Selected Strains

The first step in screening of isolates was testing for Antagonistic activity. Each isolate was tested (streaked on nutrient agar plates) with all of the other isolates to determine antagonistic activity (Table 1). Out of the 13 isolates tested for the antagonistic activity ST M_4, ST WRS_1, ST WRS_2 showed maximum antagonistic activity against the various strains tested. These three isolates were separated and the remaining 10 isolates were subjected to further studies.

ISSN No:-2456-2165

Sl.no	Test Strains	Target Strain												
		ST M_1	ST M_2	ST M_3	ST M_4	ST MS	ST M_{S3}	ST D_1	ST K_1	ST WRS_1	ST WRS_2	ST P	ST Vc	ST Mo
1	ST M_1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	ST M_2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	ST M_3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	ST M_4	-	-	-	-	-	-	-	+	-	-	-	-	-
5	ST Ms	-	-	-	-	-	-	-	-	-	-	-	-	-
6	ST Ms_3	-	-	-	-	-	-	-	-	-	-	-	-	-
7	ST D_1	-	-	-	-	-	-	-	-	-	-	-	-	-
8	ST K_1	-	-	-	-	-	-	-	-	-	-	-	-	-
9	ST WRS_1	-	-	-	+	-	-	-	-	-	+	-	-	-
10	ST WRS_2	-	-	-	+	-	-	-	-	+	-	-	-	-
11	ST P	-	-	-	-	-	-	-	-	-	-	-	-	-
12	ST Vc	-	-	-	-	-	-	-	-	-	-	-	-	-
13	ST Mo	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1: The Antagonistic property of the isolated strains

C. Optimization of Growth Conditions

The remaining isolates viz; ST M_1, ST M_2, ST M_3, ST Ms, ST Ms_3, ST D_1, ST K_1, ST P, ST Vc, ST Mo were then optimised under different growth conditions- pH and temperature. All isolates are transferred to a media with different pH and temperatures mentioned in the procedure. Out of 10 isolates 2 of them were able to grow at high temperature (60°C). It is desirable that α-amylase should be active at high temperature of gelatinization and liquefaction to economise the process [[9.]]. Therefore, there has been a need for thermophilic and thermostable alpha amylase. Thus, the strains ST Ms_3 and ST M_3 were selected for further studies.

D. Antibiotic Susceptibility using Disk Diffusion Test

The antibiotic susceptibility test of the isolates ST Ms_3 and ST M_3 to various antibiotics viz; Gentamicin, Penicillin, Streptomycin, Tetracycline, Norfloxacin, Ceftriaxone, Ciprofloxacin, Co-trimoxazole, Mezlocillin, Amikacin, Carbenicillin and Tobramycin were tested using agar disc diffusion method. The strain ST M_3 and ST Ms_3 showed resistant to mezlocillin, amikacin, carbenicillin, tobramycin. In addition, ST M_3 was found to be resistant to co-trimoxazole and ST Ms_3 to ceftriaxone.ST M_3 was sensitive to Gentamicin, Penicillin, Streptomycin, Tetracycline, Norfloxacin, Ceftriaxone, and Ciprofloxacin. ST Ms_3 was found to be sensitive to Gentamicin, Penicillin, Streptomycin, Tetracycline, Norfloxacin, Co-trimoxazole and Ciprofloxacin (Table: 2 & Figure 2). The acquisition of an antibiotic resistance genotype may actually increase the fitness of certain bacteria in the absence of antibiotic

selective pressure, possibly allowing rapid emergence and dissemination of antibiotic resistance on a worldwide scale [[10.]]; [[11.]].

SR.NO	ANTIBIOTIC	SAMPLE	
		ST M_3	ST Ms_3
1.	CO-TROMOXAZOLE	RESISTANT	SENSITIVE
2.	MEZLOCILLIN	RESISTANT	RESISTANT
3.	AMIKACIN	RESISTANT	RESISTANT
4.	CARBENICILLIN	RESISTANT	RESISTANT
5.	TOBRAMYCIN	RESISTANT	RESISTANT
6.	CEFTRIAXONE	SENSITIVE	RESISTANT
7.	CIPROFLOXACIN	SENSITIVE	SENSITIVE
8.	GENTAMICIN	SENSITIVE	SENSITIVE
9.	PENICILLINE	SENSITIVE	RESISTANT
10.	STREPTOMYCIN	SENSITIVE	SENSITIVE
11.	TETRACYCLINE	SENSITIVE	SENSITIVE
12.	NORFLOXACIN	SENSITIVE	SENSITIVE

Table 2: Susceptibility of amylolytic bacteria to various antibiotics

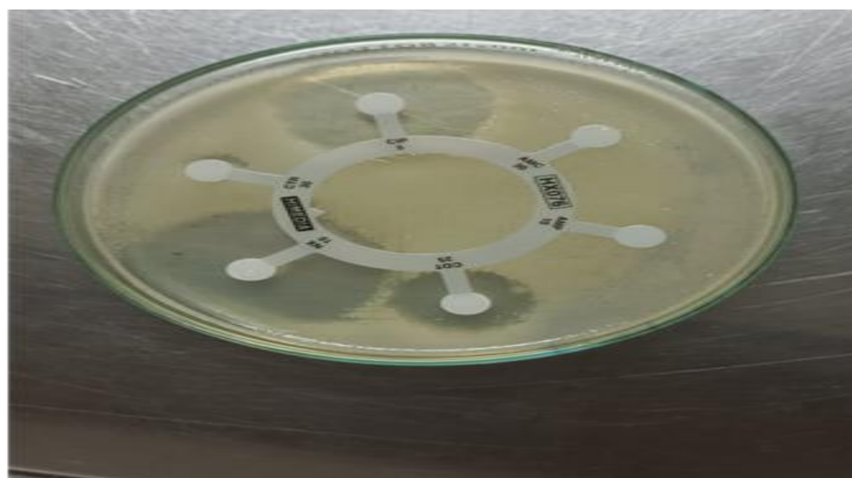

Fig. 2: Antibiotic susceptibility of amylolytic bacteria

The MAR Index of STM3 was found to be 0.41 and that of STMs3 was found to be 0.5. The MAR index is an effective, valid and cost-effective method that is used in source tracking of antibiotic resistant organisms [[12.]].Bacteria having MAR index ≥ 0.2 originate from a high-risk source of contamination where several antibiotics are used.[[13.]][[13.]][[15.]].

E. Antimicrobial Activity using Well Diffusion test

The antimicrobial activity of the two isolates to various pathogenic strains such as Pseudomonas, Klebsiella, Escherichia coli, Staphylococcus aureus, Vibrio cholera and Vibrio parahaemolyticus revealed the antimicrobial property of sample ST M_3 towards Pseudomonas and ST M_3 & ST Ms_3 towards S.aureus. The results are expressed as table in Table: 3.

SL. NO	PATHOGENIC STRAIN	ST M_3	$STMs_3$
1.	*Pseudomonas*	-	+
2.	*Klebsiella*	-	-
3.	*Escherichia. coli*	-	-
4.	*Staphylococcus aureus*	+	+
5.	*Vibrio cholera*	-	-
6.	*Vibrio parahaemolyticus*	-	-

Table 3: Test for Antimicrobial Activity Against Pathogenic Organisms

F. Biochemical Characteristics of Characteristics

Colony morphology of the selected strains indicated that ST M_3 produced off-white, irregular, boil like, shrinked colonies while ST Ms_3 produced whitish, feathery, thin, flared, irregular edges. The Gram staining proved ST M_3 to be a Gram-negative coccus while ST Ms_3 to be Gram-negative rod-shaped bacteThe biochemical analyses of the isolates were carried out and the results are summarized Table: 4.

Sl. no	sample	Colour of colony	Nature of colony	Gram nature	shape
1.	ST M_3	Off -white	Irregular, Boil like, Shrinked	Gram negative	Cocci
2.	ST Ms_3	Whitish	Feathery, thin flared, irregular edges	Gram negative	Rod

Table 4: Colony morphology and staining property

The strain ST M_3 was tested positive for Methyl red, citrate and oxidase test. It was also noted that the organism produced an acid slant and acid butt indicating that it had the capacity to ferment glucose. On The other hand, the strain ST Ms_3 showed an alkaline slant and alkaline butt indicating the absence of carbohydrate fermentation. The strain was also tested positive for Voges Prausker test, citrate test, catalase production and oxidase production. ria. The results are summarised in Table: 5.

SL. No	BIOCHEMICAL TEST	RESULTS	
		ST M_3	ST Ms_3
1.	Carbohydrates Fermentation		
a)	Glucose	-	-
b)	Fructose	-	-
c)	Sucrose	-	-
2.	Indole Production	-	-
3.	MRVP Test		
a)	Methyl Red (MR)	+	-
b)	Voges Proskauer (VP)	-	+
4.	Citrate	+	+

5.	Urease	-	-
6.	Catalase	-	+
7.	Oxidase	+	+
8.	TSIA Test	A/A	K/K H2S production

Table 5: Biochemical characteristics of the amylolytic bacteria

A/A : Acid slant Acid Butt **K/K :** Alkaline slant Alkaline Butt

G. Ezyme Assay

The quantitative assay of the enzyme production of each strain was carried out using the DNSA method. The absorbance of the colored compound was measured using a colorimeter. ST M_3 had an enzyme activity of 2.1726 Units/mL and enzyme activity of ST Ms_3 was 2.395 Units/mL. The quantitative assay helped in the identification of more efficient amylase producing arterial strain, which can be used for industrial purposes.

SAMPLE	ENZYME ACTIVITY (Units/mL)
ST M_3	2.176
ST Ms_3	2.395

Table 6: Enzyme activity of the isolates

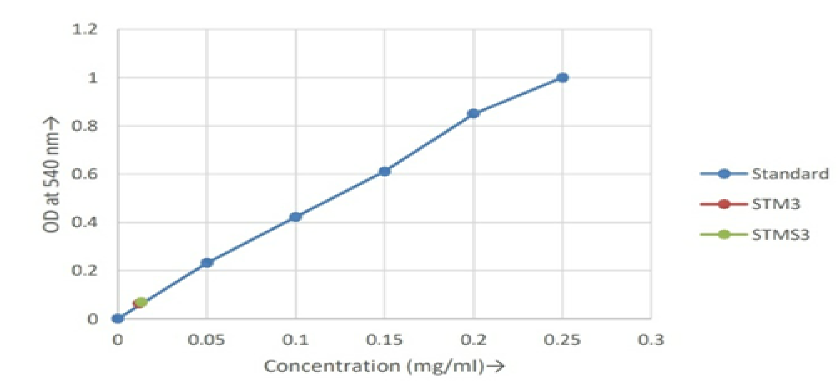

Fig. 3: Absorbance of isolates at 600nm

H. Molecular Identification

The strain showing maximum amylase activity was identified using molecular methods. Based on the BLAST analysis done for the sequencing data, the sample (STMs3) showed 99.77% identity with Alcaligenes faecalis (NCBI Accession No: CO048039.1) with query coverage of 100%. The following is the BLAST sequence of the bacterial isolate (861bp).

ACCGCGTTAGCTGCGCTACTAAGGCCTAACGGACCCCAACAGCTAGTTGACATC
GTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTG
TCTGAGCGTCAGTATTATCCCAGGGGGCTGCCTTCGCCATCGGTATTCCTCCACA
TATCTACGCATTTCACTGCTACACGTGGAATTCTACCCCCCTCTGACATACTCTAG
CTCGGCAGTTAAAAATGCAGTTCCAAGGTTGAGCCCTGGGATTTCACATCTTTCT
TTCCGAACCGCCTACACACGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACC
CTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTGCAGATA
CCGTCACCAGTATCCCGTATTAGGGGATACCTTTTCTTCTCTGCCAAAAGTACTTT
ACAACCCGAAGGCCTTCATCATACACGCGGGATGGCTGGATCAGGGTTTCCCCCA
TTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC
CCAGTGTGGCTGGTCGTCCTCTCAAACCAGCTACGGATCGTTGCCTTGGTGAGCC
TTTACCCCACCAACTAGCTAATCCGATATCGGCCGCTCCAATAGTGAGAGGTCTT
GCGATCCCCCCCTTTCCCCCGTAGGGCGTATGCGGTATTAGCCACTCTTTCGAGT
AGTTATCCCCCGCTACTGGGCACGTTCCGATATATTACTCACCCGTCCGCCACTCGCCGCCAAGAGAGCAAGCTCTC
TCGCGCTGCCGTTCGACTTGCATGTGTAAAGCA TCCCGCTAGCGTTCAATCTGAGCCAGGATCAAAC

Fig. 4: Blast result of $STMs_3$

IV. CONCLUSIONS

The present study was conducted to isolate and identify the potential microorganisms that produce extracellular enzymes. Out of the 30 soil samples screened, 13 isolates producing extracellular amylase were recognised. Based on the physicochemical properties (pH, moisture, nitrogen, phosphorus and potassium content), antagonistic activity, and antimicrobial activity, tolerance towards different pH (2.2-9) and temperature (37°C-60°C) two strains were selected. They were identified using morphological and biochemical and molecular characteristics. The strain ST Ms_3 showed maximum enzyme production.

They can be used for the degradation of waste on a large scale. Since the disposal of waste is a large area of concern due to the growing population, there is a need to invent economically feasible ways for waste disposal. The use of naturally occurring microorganisms from soil is one of the ways in which this can be achieved. Soil has an abundance of microorganisms and can be screened for extracellular enzyme producing bacteria. The isolated strains can further be utilised to degrade waste. They can be either used as monocultures or in consortium to make the process more efficient. This provides an easy and economical way to handle the biodegradable wastes such as kitchen waste and litter.

ACKNOWLEDGMENTS

We would like to express our gratitude to Research Promoting Council of Assumption College Autonomous, Changanacherry, Kottayam, Kerala, India for financially supporting the project and the Department of Zoology, Assumption College, Changanacherry for providing access to the Microbiology lab for the completion of the research. We are also grateful to Dr. Sudha K, Department of Biotechnology, St. Peter's College, Kolencherry for providing the necessary guidance throughout the research. We also thank Ms. Sabina Sabu for her assistance in completing the work.

REFERENCES

[1.] de Souza PM, de Oliveira Magalhães P. Application of microbial α-amylase in industry - A review. Braz J Microbiol. 2010 Oct;41(4):850-61. doi: 10.1590/S1517-83822010000400004. Epub 2010 Dec 1. PMID: 24031565; PMCID: PMC3769773.

[2.] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3769773/

[3.] Kenneth E. Sanderson, Daniel R. Zeigler,[10] Storing, shipping, and maintaining records on

bacterial strains, Methods in Enzymology, Academic Press, Volume 204,1991, Pages 248-264.

[4.] https://doi.org/10.1016/0076-6879(91)04012-D

[5.] L. Barth Reller, Melvin Weinstein, James H. Jorgensen, Mary Jane Ferraro, Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices, *Clinical Infectious Diseases*, Volume 49, Issue 11, 1 December 2009, Pages 1749–1755, https://doi.org/10.1086/647952.

[6.] Gonelimali, F . D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M., &Hatab, S. R. (2018). Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. Frontiers in microbiology, 9, 1639.https://www.frontiersin.org/articles/10.3389/fmicb.2018.01639/full

[7.] Raul, D., Biswas, T., Mukhopadhyay, S., Kumar Das, S., & Gupta, S. (2014). Production and partial purification of alpha amylase from Bacillus subtilis (MTCC 121) using solid state fermentation. *Biochemistry Research International*, *2014*. https://doi.org/10.1155/2014/568141

[8.] Wang Q, Wang C, Yu W, Turak A, Chen D, Huang Y, Ao J, Jiang Y, Huang Z. Effects of Nitrogen and Phosphorus Inputs on Soil Bacterial Abundance, Diversity, and Community Composition in Chinese Fir Plantations. Front Microbiol. 2018 Jul 19;9:1543. doi: 10.3389/fmicb.2018.01543. PMID: 30072961; PMCID: PMC6060263.https://doi.org/10.3389%2Ffmicb.2018.01543

[9.] Imran Pancha, Deepti Jain, Anupama Shrivastav, Sanjiv K. Mishra, Bhumi Shethia, Sandhya Mishra, Mohandas V.P., BhavanathJha,Athermoactive α-amylase from a Bacillus sp. isolated from CSMCRI salt farm, International Journal of Biological Macromolecules, Volume 47, Issue 2, 2010, Pages 288-291,.https://doi.org/10.1016/j.ijbiomac.2010.04.006

[10.] Luo N. Pereira S. Sahin O. Lin J. Huang S. Michel L. Zhang Q. (2005) Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. Proc Natl Acad Sci USA102: 541–546. https://doi.org/10.1073/pnas.0408966102

[11.] Enne V.I. Bennett P.M. Livermore D.M. Hall L.M. (2004) Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. J Antimicrob Chemother53: 958–963. https://doi.org/10.1093/jac/dkh217

[12.] Chitanand MP, Kadam TA, Gyananath G, Totewad ND, Balhal DK. Multiple antibiotic resistance indexing of coliforms to identify high risk contamination sites in aquatic environment. Indian J Microbiol. 2010 Jun; 50(2):216–20. pmid:23100831.https://doi.org/10.1007/s12088-010-0042-9

[13.] Mthembu, M.S. (2008) The Usefulness of Multiple Antibiotic Resistance (MAR) Indexing Technique in Differentiating Fecal Coliform Bacteria from Different Sources. Doctoral Dissertation, University of Zululand, Empangeni.

[14.] Sandhu, R., Dahiya, S. and Sayal, P. (2016) Evaluation of Multiple Antibiotic Resistance (MAR) Index and Doxycycline Susceptibility of Acinetobacter Species among Inpatients. Indian Journal of Microbial Research, 3, 299-304.

[15.] Ejiofor, S.O., Edeh, A.D., Ezeudu, C.E., Gugu, T.H. and Oli, A.N. (2016) Multi-Drug Resistant Acute Otitis Media amongst Children Attending Out-Patient Clinic in Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka, South-East Nigeria. Advances in Microbiology, 6, 495-501.https://doi.org/10.4236/aim.2016.67049