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Prevalence, Screening and Characterization of Starch Degrading Bacteria from Sago Industry Wastes

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Abstract: ***The sago processing industries generate high concentrations of organic pollutants. These industries required about 20,000 to 40,000 litres of water per ton of sago processing. They let out the water with hightoxic and organic matter causing serious threats to the environment and aquatic life. Potential starch degrading microorganisms are required to degrade the organic contaminants in the sago industry wastes before being generated. Sago wastes were collected from sago industries using sterilecans and polythene bags. The bacterial strains were obtained by adopting serial dilution methods and they were identified up to the generic level by their morphology and biochemical characteristics. The potential strains were screened by starch hydrolysis test and they were identified to the species level by 16s rDNA sequencing. The aligned sequences were evolutionary genetics analysis format using MEGA v.2.1 software and construct phylogenetic trees using the neighbour-joining method and SDS-PAGE zymogram analysis starch degrading bacteria producing amylase enzyme.There are 103bacterial strains were isolated and identified. They were belonging to the genera of Bacillus (72%), Micrococcus (8%), Enterobacteriaceae (6%), Corneybacterium (5%), Aeromonas (4%), Alcalinges (3%) and Pesudomonas (2%). Among the isolates, there are 5 strains namely S5, S7, S16, S20 and S28 were selected as potential starch degraders. The database of the strains wassubmitted to the NCBI gen bank and got the accession numbers (S5-KY213660, KY213661, KY213662, KY230509 and KY230510).The molecular mass of the amylase was estimated to be ~45 kDa by SDS-PAGE and zymogram analysis. The strains S5, S7, S16, S20 and S28 showed more efficiency for the degradation of starch. These strains can be used to degrade starch from the waste substrates fabricating with amylase and glucose as the products.***

Key words- Sago effluent, waste water sediment, sago sludge, bagasse, tapioca peel.

I. INTRODUCTION

Cassava (Manihotesculenta spp. esculenta) a plant by perennial crop belonging to the family Euphorbiaceae, is known as tapioca in Asian countries. This root crop has a high starch content of up to 90% (dry weight). It grows well on infertile land with minimal input of fertilizers and especially in a tropical climate cultivated by small scale farmers in Africa, Latin America and Asia. It is a world sixth most important food crop and a basic food for more than 700 million people in several countries [1]. Cassava is cultivated in about 88 countries, but five countries account for about 67% of the production [2]. India has third rank among the Asian countries in the production (about 5.0±5.5 million ton fresh roots), the average yields on a per hectare basis are highest in India [3]. The total cultivation in Salem districts in 34,000 hectares, besides about 8-10 large-scale starch factories and 150-200 small scale sago and starch production units are operating in Salem districts, Tamil Nadu, which contributes nearly 50% of the total production of the state generating about 40-60 tonnes of solid waste per annum [4].

Cassava wastes and the residues are generated during the separation of flour and starch from cassava, which involves several steps: peeling and washing, grating, pressing, disintegration, sifting, drying, milling and screening. In general, there are four categories of waste/residue streams: peels from initial processing, fibrous by-products from crushing and sieving, starch residues after starch settling and wastewater effluents [5]. The solid waste/residue from cassava starch and flour processing is termed as bagasse, pulp or thippi[3, 6]while the processing; wastewater is called manipueira or starch liquid wastes[7]. The liquid waste water however is produced in more than 3 times of cassava roots mass [8].

The effluent generated from the root washing tank and sedimentation tanks forms the major source of pollution. Accordingly, sampling and characterisation of wastewater from these two unit operations and also of the combined waste were carried out from different sago units [9]. Sago effluent from the various processes is treated using a series of open lagoon treatment system before being discharged into the river [10]. It is a rich carbohydrate residue generated in large amounts during the production of cassava flour, the major nutrients present on cassava waste are sugars and mineral salts [7, 3]reported that processing of 250 to 300 tons of

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cassava root results in approximately 1.16 tons of cassava peels, 280 tons of cassava pulp and 2655 m3 of starchy wastewater. Cassava bagasse approximately 600 to 650 tons per day of cassava bagasse is disposed from sago industry in India [11]. The generated wastewater has a very high chemical oxygen demand, biochemical oxygen demand and total solid, high amount of starch and fibers which causes environmental problems due to its high organic load [7, 12, 13, 14and 15].

It causes high pollution owing to its organic content and more bio degradability it is a fibrous residue, contains about 50% starch on a dry weight basis [16, 17].

Wastes are usually stored in an open field; they spoil rapidly, not only causing environmental problems, including landfill without any treatment, contamination of water bodies [3, 18]. Hence, the objective of this study was the isolation of bacterial strains from the wastes and identification at genus level using colony morphology and biochemical characteristics. These strains were screened for the starch degradation by starch hydrolysis methods and the potentials strains were confirmed by molecular aspect. This research will provide a basis for using starch degradation, for the conversion of glucose from starch. In future, these strains and the consortium can be used for glucose production.

II. MATERIALS AND METHODS

A. Study Area And Sample Collection

Sago effluent, waste water sediment, sago sludge, bagasse and tapioca peels were collected from sago mills, located in Salem district of Tamil Nadu, India. Varalakshmi sago factory (Latitude: 11.8255537, Longitude: 78.6278915) and Annamalai sago factory (Latitude: 11.8255537, Longitude: 78.6278915) which produces more than 250 metric ton of starch per year. The sludge and the effluents from the processing units were collected in sterile plastic cans and tapioca peels, bagasse was collected in sterile polythene bags and transported to the laboratory in an ice box.

B. Isolation Of Bacteria

Isolation of bacteria from the soil sample obtained from waste dumping land of sago mill was carried out by the serial dilution method. After the incubation of the plates at 37^{o}C for 24h, the bacterial colonies were selected on the basis of colony morphology and the colours. The isolated colonies were purified by repeated streaking and preserved in agar slants at 4^{o}C [19, 20].

C. Bacterial Identification

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations. The parameters investigated included colonial morphology, gram staining, end ospore formation, oxidase test, catalase test and starch hydrolysis. To identify the strains, further tests were made as given in Bergey's Manual of Determinative Bacteriology[21].

D. Screening Of Starch Degrading Bacteria By Plate Assay Method

The isolates were screened for starch degradation by starch hydrolysis test on starch agar plate. All the isolates were inoculated in starch agar plate and incubated at 37°C for 48 hrs. After incubation, the plates were flooded with 0.1% gram's iodine solution to form a dark red colour background in the media. Positive isolates that hydrolyze starch indicates a clear zone formation around the colony [22].

E. Genomic Dna Extraction Of Bacteria

The genomic DNA was extracted from the isolated bacterial colony using a protocol modified from the method described by [23]. The 12 hrs cultures of bacterial isolate were taken in the micro centrifuge tube. The tube was centrifuged at 10,000 rpm for 10 min. The pellet was collected and 90 µl of 10% SDS was added.

The tubes were incubated at 37ºC for 1.5 hrs. After incubation, 150µl of 5M Nacl were added prior to the addition of 100µl of 10% Cetyltrimethyl ammonium bromide. The sample was mixed thoroughly and incubated at 65ºC in water bath for 30 min, after incubation, phenol chloroform is oamyl alcohol in the ratio of 25:24:1 (vol/vol) were added. The tube was centrifuged at 13,000 rpm for 15 min and the aqueous layer was separated into a fresh tube. Then it was precipitated with 70% ethanol and centrifuged at 7000 rpm for 5 min [24]. Pellets were suspended in 30µl of TE buffer. The DNA sample was separated according to their molecular weight under electrophoresis system. Finally, the DNA band was visualized under the Gel Documentation system. The DNA concentration was determined by measuring the absorbance at the ration 260/280 nm and the DNA suspension was stored until it was used for PCR and further analysis.

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F. Pcr Amplification Of 16s Rdna Gene

The selected bacterium was identified on the basis of its 16S rDNA sequence. DNA from the bacterial cells was isolated using QIAamp DNA Purification Kit (Qiagen, Japan) and electro phoresed in agarose gel. Fragment of 16S rDNA gene was amplified by PCR upto 30 cycles (using the following profile: initial denaturation, 95°C for 2min; final denaturation, 94°C for 30s; annealing, 52°C for 30s; extension,72°C for 90s; final extension,72°C for 10min). Amplified PCR product was purified using Qiagen Mini elute gel extraction kit (Qiagen, Japan). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27 F AGAGTTTGATCCTGGCTCAG and 1492 R GGTTACCTTGTTACGACTT primers using BDTv3.1 Cycle sequencing kit on (ABI3730xl) Genetic Analyzer[25]. A single discrete PCR amplicon band of 1500bp was observed when resolved on 1.2% agarose gel.

G. Phylogenetic Analysis

The reference sequences required for comparison were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank). The aligned sequences were then manually checked for gaps in each row and saved in molecular evolutionary genetics analysis (MEGA) format using MEGA v.2.1 software. Pair wise evolutionary distances were computed using the Kimura 2-parameter model [26, 27]. To obtain confidence values, the original dataset was resembled 1,000 times using the bootstrap analysis method. The bootstrapped dataset was used directly for constructing the phylogenetic tree with the MEGA program or for calculating multiple distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using the neighbour-joining method [28].

H. Enzyme Activity Assay

Amylolytic activities were measured by the method of [29]: 50 µL of soluble starch in 25 mm Glycine-NaOH buffer, pH 9.0 was mixed with 50 µL of enzyme solution. After incubation at 60°C for 10 min, the enzymatic reaction was terminated by the addition of 50 µL 1 M HCl. To the reaction mixture, 50 µL iodine solution containing 2% KI and 0.2% I2 and 800 µL 25 mm Glycine-NaOH buffer, pH 9.0 was added. Absorption was measured at 600 nm and the activity was calculated using soluble starch as the standard. Hydrolysis of 1 mg mL^{-1} soluble starch in 1 min was defined as 1 U mL^{-1} of enzyme activity. Specific activity is given as U mg^{-1} protein.

I. Protein Determination, Sds–Page And Zymogram Analysis

Protein concentration was determined by the method of using bovine serum albumin as a standard [30].Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) under non reducing conditions was carried out according to [31]using 10% acrylamide gels. Electrophoresis was run at 150 V for 60 min at room temperature.

After electrophoresis, a part of the gel was silver stained. To identify amylase band (clear zone on a blue background), the other part of gel was incubated for 45 min at 70°C in 1% soluble starch containing 25 mm Glycine-NaOH buffer, pH 9.0 and then stained with iodine solution. Non-denaturing gel electrophoresis (native-PAGE) was performed similar to the SDS-PAGE procedure but lacking sodium dodecyl sulfate. Zymogram staining was performed by incubating the gels for 45 min at 70°C in 1% soluble starch in 25 mm Glycine-NaOH buffer, pH 9.0.

III. RESULTS AND DISCUSSION

A. Bacterial Population From Samples

There are 103 heterotrophic bacterial strains were isolated and identified up to genus level, based on the morphology and biochemical characteristics. Among 103 strains, 81 strains were gram positive and 19 strains were gram negative. *Bacillus* species found to be predominant and they were about 72% followed by 8% of Micrococcus, 6% of members of Enterobacteriaceae, 5% of Corynebacterium, 4% of Aeromonas, 3% of Alcaligenes and 2% of Pseudomonas. All the isolates were subculture and maintained to obtain a pure culture on nutrient agar and used for further studies (Table 1; Fig. 1). Similar reports bacteria isolated from starch rich materials may have better potential to produce enzyme under adverse conditions [22]. Microorganisms that produce amylases could be isolated from places such as soil around mills, cassava farms and processing factories as well as flour markets [32]. During the study, amylase producing bacterial strains was isolated from sago waste contaminated with decaying materials including flour mill waste water.

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B. Screening Of Starch Hydrolysis Strain (Plate Assay)

All the strains were processed for starch degradation by plate assay. About 60% strains showed positive starch degradation with clear zone formation (25-45mm). Out of 62 strains, the best 5 strains namely S5, S7, S16, S20 and S28 were selected based on the clear zone and the ability of starch degradation. The entire positives isolated were belonging to the genera of *Bacillus* sp. They were selected as potential starch degraders and retained for further study of amylase activity (Table2). Our results are good agreement with the study carried out by [33].

C. Molecular Identification

The 16s rDNA sequence was amplified using the primers 27 F and 1492 R from chromosomal DNA of the strain used as a template, the yield amplification of 1500bp viewed in agarose gel electrophoresis. By performing a BLAST search within the Genbank data base, the most similar sequence was identified, later based on the selected sequence of close neighbours, a phylogenetic tree was constructed (Fig. 2 and 3). At the close of the analysis *Bacillus* strains S5, S7, S16, S20 and S28 (Genbank accession number KY213660, KY213661, KY213662, KY230509 and KY230510) were identified as being identical i.e. with 95% identity. Thus, with respect to the phylogenetic tree of the 16S rDNA, strain is considered to be representative of the *Bacillus* spp. Similarly reports molecular sequence search similarities were conducted using BLAST [34, 35]. Phylogenetic analysis with reference sequence data of bacteria under study were aligned with reference sequence homology from the NCBI database using multiple sequence alignment of MEGA 5.0 program [36].

D. Determination Structure Of The Amylase Enzyme

Since the *Bacillus*sp amylases are routinely extracellular enzymes, it was possible to partially purify the enzyme from the supernatant of the isolate; we applied a strong anion exchange column (Resource-Q column, Pharmacia) for such purpose. To determine determination structure of amylase, SDS-PAGE and Native PAGE were employed, together with their zymogram to observe the amylase bands. SDS-PAGE result under non reducing conditions showed one protein bands with estimated molecular masses of ~45 kDa, respectively. All of the protein bands displaying amylolytic activities were detected from their zymogram (Fig. 4). However, the zymogram of native-PAGE result identified only a single amylolytic protein with a molecular mass of ~45 kDa (Fig. 4). Our findings suggested that the quaternary structure of the amylase of the strain*Bacillus*spit was a heter odimeric complex consisting of single band approximate subunits ~45 kDa. Apparently, molecules under denaturing conditions such as in SDS-PAGE were partially separated into its subunits. The illustrates that the raw starch degrading amylase of *Bacillus* sp, ALSHL3 has molecular weight approximately 72 kDa. Amylase from genus Bacillus usually appeared as a monomer with a molecular weight in the range of 68.0 to 76.0 kDa[37]. Taken together, it can be considered that *Bacillus* sp.

IV. CONCLUSION

The sago processing industries generate large amounts of wastewater with extremely high concentrations of organic pollutants. This study screened the potential heterotrophic starch degrading bacteria isolated from the sago factory wastes. A total of 130 bacterial strains were isolated. In its, 81strains were gram positive and 19 were gram negative bacteria. Among the strains only five strains were selected based on starch degradation (clear zone formation) ability and strains to conform the molecular level and sequences to submit NCBI genbank to construct the phylogenetic tree. By doing so, a stable amylase with higher enzyme activity can be identified which may have wide industrial applications and high amylase producing potential connecting with solid waste treatment.

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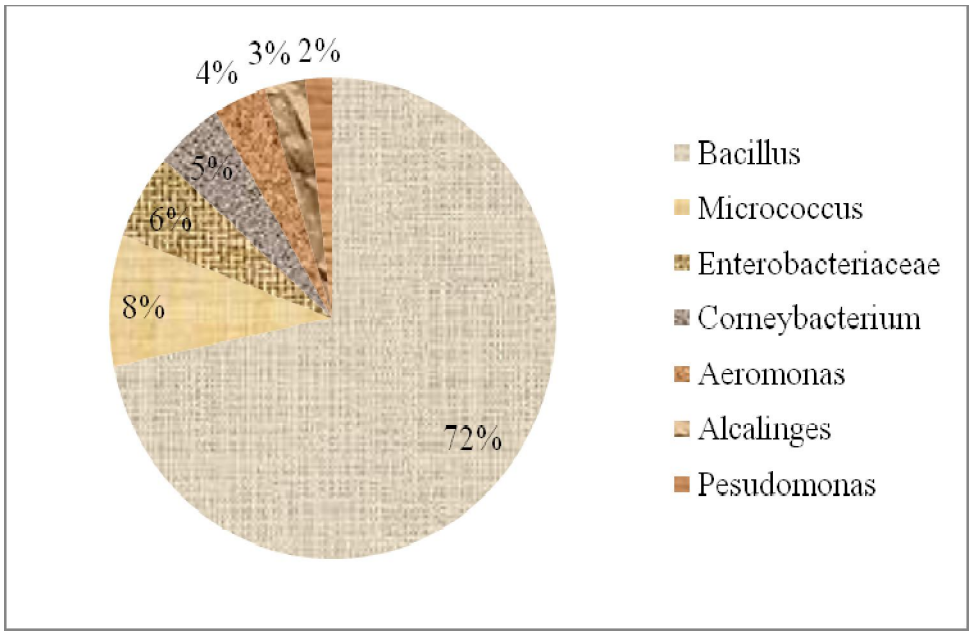
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Table 1: Bacterial diversity in sago mill wastes

Sampling area	Nature of the samples	Bacterial genera	No of strains
Varalakshmi sago factory	1. Effluent	*Enterobacterium*	1
		Aeromonas	3
		Bacillus	8
	2. Waste water sediment	*Bacillus*	17
		Aeromonas	1
		Micro coccus	3
	3. Sago sludge-I	*Bacillus*	16
		Pseudomonas	2
		Micrococcus	2
		Corneybacterium	2
		Enterobacteria	2
		Alkaligens	1
Annamalai sago factory	4. Bagasse	*Bacillus*	10
		Lactobacillus	1
		Enterobactericae	1
		Alcaligenes	1
	5. Tapioca peelings	*Bacillus*	13
		Enterobactericiae	1
		Corneybacterium	1
		Micrococcus	1
	6. Sludge-II	*Bacillus*	10
		Enterobacteria	1
		Micrococcus	3
		Alcalinges	1
		Corneybacterium,	1
		Total	103

Fig. 1.Microbial diversity of isolated bacterial strains from sago waste.



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Table 2: Starch hydrolysis of bacterial strains (Plate assay)

S. No	Phenotypic name	Gen Bank accession number	Starch hydrolysis (mm)
1.	S5	KY213660	25
2.	S7	KY213661	27
3.	S16	KY213662	25
4.	S20	KY230509	24
5.	S28	KY230510	25

Fig. 2.Molecular identification of effective starch degrading bacterial strains.

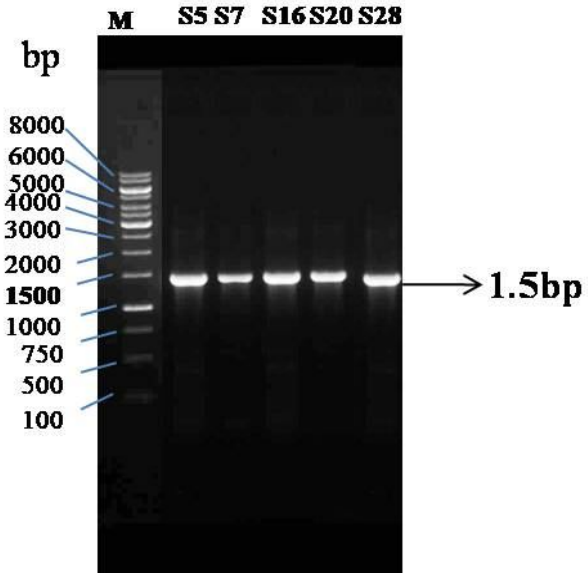
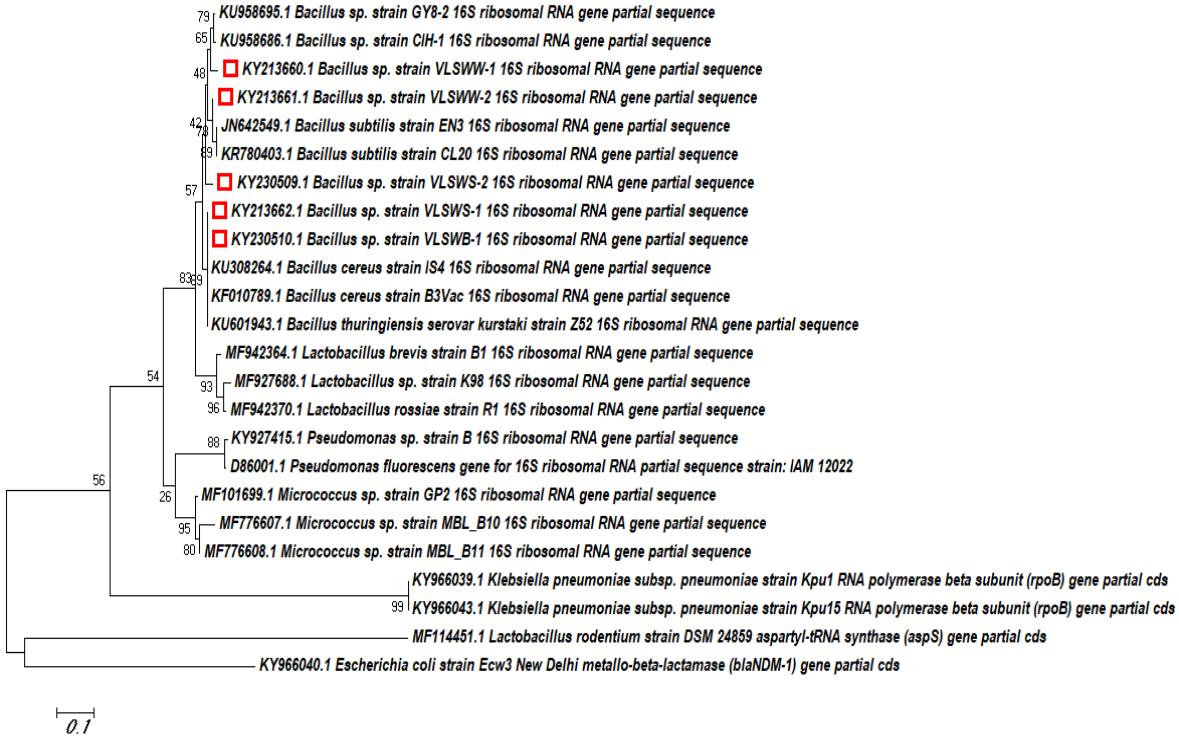


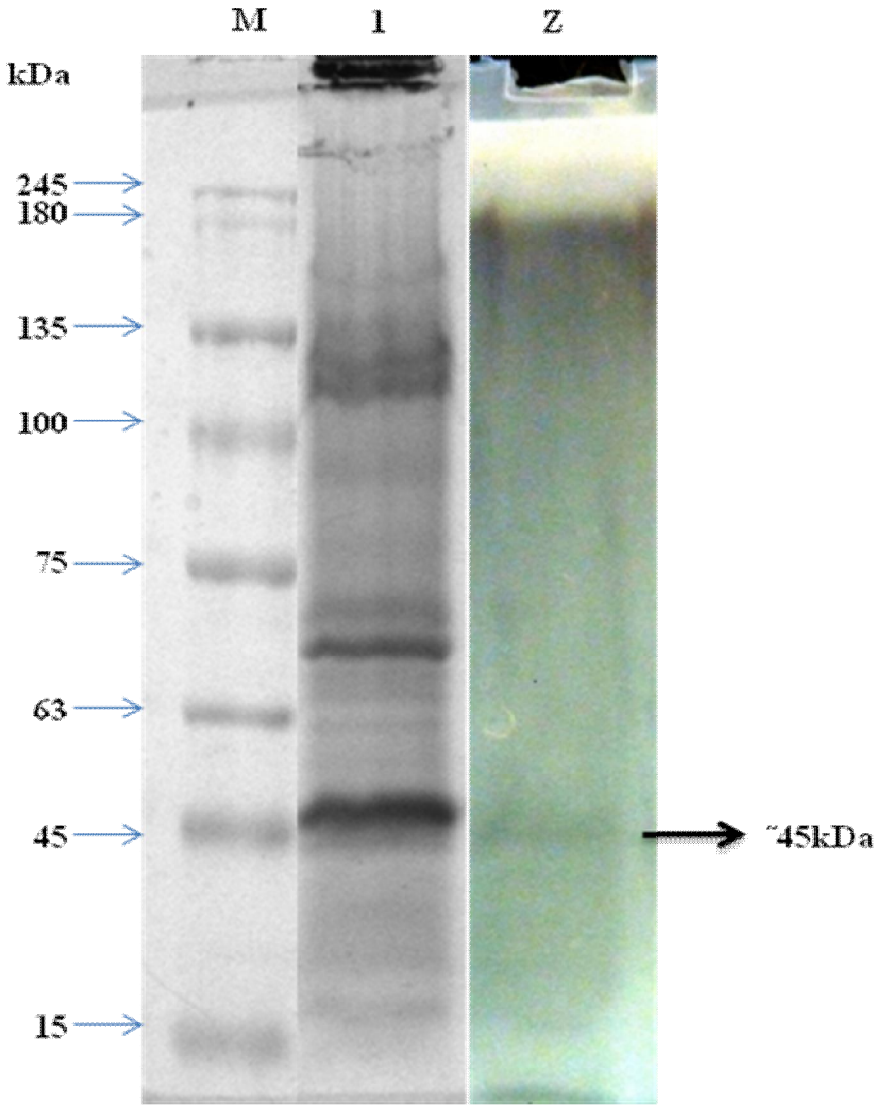
Fig. 3.Phylogenetic analysis of selected starch hydrolysis *bacillus* strains.



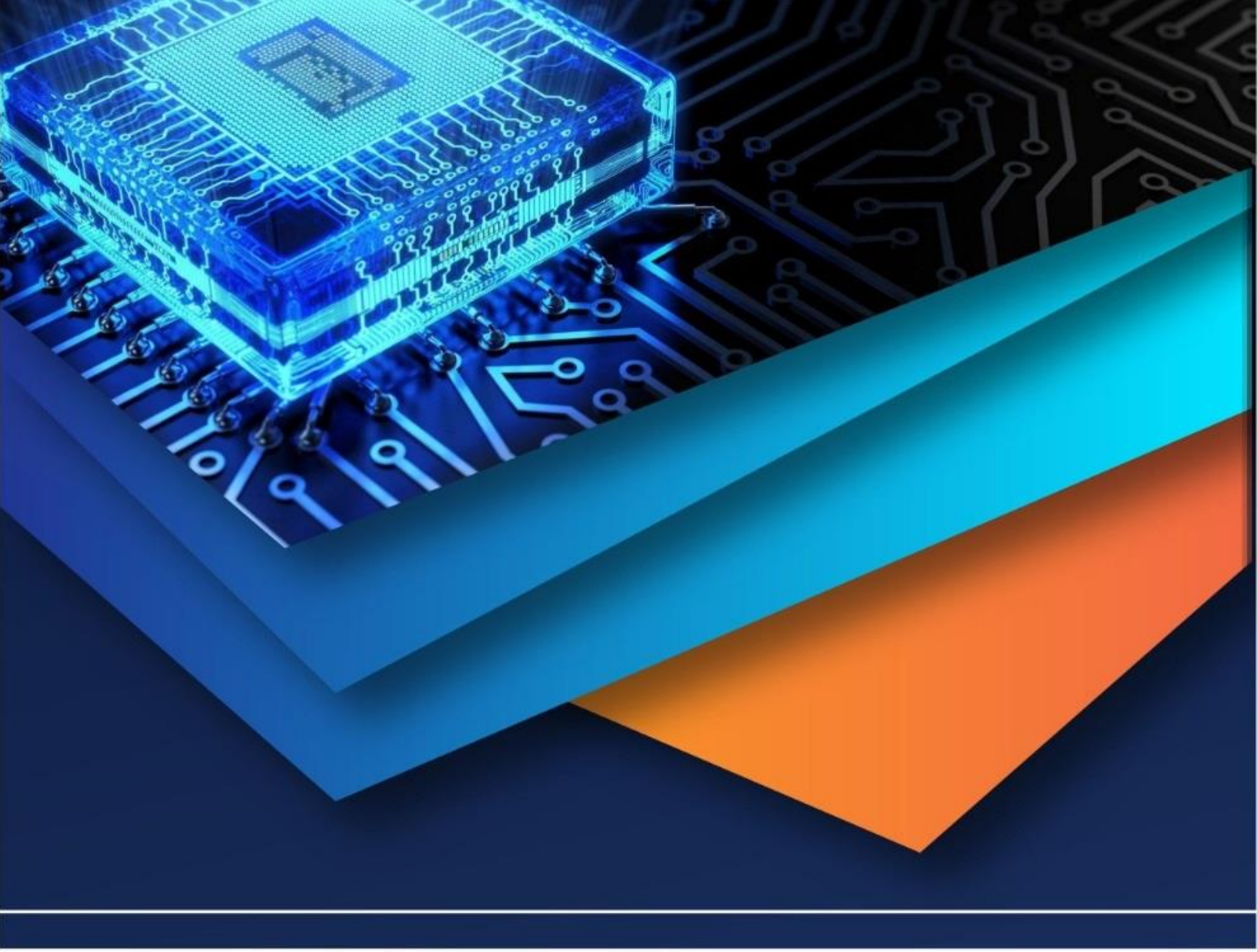
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Fig. 4.Stacrch degrading bacteria production of amylae enzyme confored SDS PAGE and zymogram analysis. (M- Protin marker,1-Crude samole, Z-Cymogram analysis)



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