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COLD-ACTIVE EXO-INULINASE ENZYME: ISOLATION AND SCREENING OF ENDOPHYTIC MICROBES FROM FRUITS

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ABSTRACT: Microbial inulinases have a great potential for industrial use in the production of fructose from inulin. Optimization of the growth parameters of the microbes is essential to obtain inulinase in sufficient quantity. The present work aims to obtain inulinase from different fruits of endophytic bacteria and optimize its growth parameters. In a total of 22 isolates and among them, each one isolates from each fruit of, Banana, Pineapple and Zapata were isolated by endophytic bacteria were identified as efficient inulinase producers by SEM analysis. The optimum temperature (4 °C) and pH of these organisms were found 5.0 - 7.0 respectively. Inulin was observed to be the suitable carbon source and also a specific substrate Banana powder for these organisms. The work has been done to grow in 4'C and 37 °C, morphological, physiological, and biochemical characters, grow in agar plate with the specific substrate for zone formation, gram staining, and estimation of inulinase enzyme for using different carbon sources to compare with specific activity for protein content. The molecular weight of this inulinase enzyme was estimated at 66 KDa using SDS-PAGE and compare to TLC inulin hydrolysis showed mono and disaccharides as the main end products. The highest enzyme activity was obtained from Banana isolate KARE_B006, which showed exo-inulinase in SDS-PAGE gel electrophoresis.

INTRODUCTION: In the past decades, biocatalysts have emerged as an increasingly attractive domain for science and industry. Nature gives us a lot of biological catalysts, better termed as 'enzymes' in all the living organisms, including bacteria and fungi, to operate most effectively under physiological conditions.

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Enzymes accelerate the rate of reaction millions of times. This unique ability of the enzymes has gained the attention of researchers and is considered a commodity for catalytic conversion technology. Bacteria and fungi are considered abundant sources of various enzymes; hence the study of microbial enzymes and their biocatalytic activity is crucial to the development of microbial technology. Carbohydrates constitute the bulk of the organic matter on the earth.

The enzymes catalyzing the biosynthesis as well as hydrolysis of carbohydrates are very diverse. Among the enzymes that hydrolyze polymers into interesting monomers and oligomers, inulin

hydrolyzing enzymes, inulinases, have attracted much attention in nutraceuticals, pharmaceutical industries. In recent years, inulin hydrolyzing enzymes have also gained enormous applications in food industries for the development of functional foods such as prebiotics, low-calorie sweeteners, and rare sugars.

A substance of inulin, an important material in bioprocesses, gets easily solubilized in warm water, and at high concentrations, does not form a viscous solution. Hence it has many advantages over starch as a raw material. Inulin sources have also received increasing attention as they represent a renewable, inexpensive and abundant raw material for fructose syrup production inulo oligosaccharide (IOS) and fructo oligosaccharides (FOS) production, ethanol fermentation and single-cell protein, single cell oil, citric acid production, *etc*. Inulin has been considered as a possible source for the production of fructose either by acid hydrolysis or by enzymatic degradation. It is a substitute for sugar or fat and contributes to improving gastrointestinal system conditions. Among the possible applications, it is used as an orally delivered drug targeting the colon to delay the absorption of drugs with adverse effects on the stomach.

Fructose obtained from inulin hydrolysis can be further utilized for the production of certain value-added products such as biofuels, biodiesel, citric acid, butyric acid, *etc*. Inulin is also a promising source for inulin-oligosaccharide production, which has enormous health benefits as a dietary fiber. However, the desirability of inulinase with variable properties prompted several workers to explore bacteria for inulinase production. Allais *et al.,* (1986) have isolated thirty-two bacterial strains growing on inulin as a sole carbon source, among which twenty-two were identified as Flavobacterium multivorum. Gill *et al*. (2006) have reported *Streptomyces sp*. GNDU 1 produced extracellular inulinase (0.552 U/ml) after 24 h at pH 7.5, temperature 46 °C, in the presence of 1.0% inulin in the liquid medium. Actinomycetes have also been used for inulinase production (Pandey *et al.,* 1999) Li *et al*., (2011) have screened for and isolated Marini microbium sp. LS-A18 as a source of novel alkaline inulinase with the unique property of high salt tolerance.

This marine isolate was able to produce 14.6 U/ml of inulinase in the media containing 4% inulin, 1% peptone, and 5% NaCl, at pH 7.5 at 37°C, after 96 h. Similarly, Belamri *et al.,* (1994) have isolated thermophilic bacterial *Bacillus stearothermophilus* that produced inulinase at 63.5 °C, pH 6.8 after 7 h. The enzyme was able to hydrolyze inulin into inulotriose as a major product. Laowklom *et al.*, (2012) have reported 1.6 µ/ml of inulinase production by *Streptomyces sp*. CP01 at 28 °C and pH 8, after 24 h. This present study focuses on inulinase from natural strains isolated from different fruits of endophytic bacteria and to optimize its growth parameters. In 25 isolates, and among them, each one isolates from each fruit of, Banana, Pineapple and Sapota were isolated by endophytic bacteria were identified as efficient inulinase producers by SEM analysis.

The optimum temperature (10 °C) and pH of these organisms were found 5.0 - 7.0 respectively. Inulin was observed to be the suitable carbon source and also a specific substrate Banana powder for these organisms. The work has been done grow in 4 °C and 37 °C, morphological, physiological and biochemical characters, grow in agar plate with a specific substrate for zone formation, gram staining, and estimation of inulinase enzyme for using different carbon sources compare to a specific activity for protein content. The molecular weight of this inulinase enzyme was estimated at 66 Kda using SDS-PAGE and compare to TLC inulin hydrolysis showed mono and disaccharides as the main end products. The highest enzyme activity was obtained from Banana isolate KARE_B006, which showed exo-inulinase in SDS-PAGE gel electrophoresis

MATERIALS AND METHODS:

Selection of Fruits: In this market, all the available fruits have been chosen Nenthriran banana, Pineapple, and Sapoda from Simmakkal, Madurai.

Isolation of The Bacteria: The isolation of bacteria was carried out by serially diluting the samples in sterile water and subsequently plating on the Modified agar base medium containing 2.5% Dextran sulphate by using the pour plate method.

Endophytic Microbial Isolation and Screening: The modified medium was used in the production

of exoinulinse contained (gL^{-1} Banana powder 1.2g, Yease extract 3.0g, $NaNO_3$ 3.0g, $CaCl_4$. 0g, Agar 1.5% the pH was adjusted 7.0 and add distilled water up to 100 ml according to Lim *et al.* 1998. The isolated organism was studied by morphological and biochemical also different carbon sources **Table 2**.

Gram Staining: A "smear" of bacteria is made on a microscope slide, fixed, stained, dried, and without using a coverslip, examined with the aid of a microscope. Aseptic technique must be observed when taking samples of culture for making a smear. A smear that is thin enables the study of shape and arrangement of cells to be clearly seen and ensures that the staining procedure is applied uniformly.

Microscopy: A scanning electron microscope (SEM) (Zeiss O 18) and a polarized optical microscope with a digital image analyzer (Olympus SZX12) were used to investigate the distribution and particle size of the TSP fillers and the AgNPs in the nanocomposite films.

Optimization of Culture Condition: Both agar and broth medium was optimized for maximum inuliinase production by addition of Banana powder 2.5%, Sucrose 1.5%, CaCl 0.5%, Yease extract 0.3%, $NaNO_3$ 0.3%, KCl 1.5% and NaCl 0.001%, pH 7.0 incubated for 2 days incubated rotary shaker at 37 °C and 4 °C.

Enzyme Assay: Extracellular enzyme solution (0.1ml) was mixed with 0.1% inulin (1ml) in 0.1ml sodium acetate buffer pH 5.0, and the mixture was incubated at 35 °C for 15 min in dark place. As a result of the reaction mixture, reducing sugar was determined by the 3.5 dinitrosalisilic acid method (Miller, 1959). The one unit of the enzyme expressed in 1µmol of fructose per min.

Protein Determination: The extracellular protein sample was determined by the method of Lowry *et al* (1951), using bovine serum albumin as a standard and by absorbance measurements at 750 nm on a visible spectrophotometer.

Purification of Crude Extracellular Exoinulinase Enzyme Production: The cells were harvested by centrifugation 12,000 rpm at 4 °C for 15 min, and the clear supernatant was purified by salting out with ammonium sulphate (Himedia) at 50-70% saturation, and the mixture was left for 2 h. The precipitate was component centrifugation at 20,000 rpm for 30 min at 4 °C. The pellet was diluted with 0.05m phosphate buffer at pH7.0 and dialyzed against the same buffer for 15-20 h.

Thin Layer Chromatography: Thin layer chromatography was performed on pre-coated silica gel plates from Merk, Germany. The sample was developed using n-butanol, isopropanal, acetic acid, and water (7:5:2:4, volume ratio) as solvent system. Thin-layer chromatography was followed as described by Wei *et al.,* 2009.

Inulin Content (%/mg/g Fresh Matter) of Plants: Initially, inulinase enzyme was found in some plant materials based on a percentage of milligram inulinase enzyme per gram of fresh matter **Table 1**. Primary screening of inulinase-producing microorganisms was screened initially using the enrichment culture technique. Samples in 1 gm quantity, which included chicory, were inoculated in the media containing 1% chicory root powder, 1.5% peptone, 1.5% yeast extracts, and agar 15% incubated at 10-40 °C and pH 5 to 7.0 respectively. Subsequent transfer of growth into fresh media was carried out for further enrichment purposes, after which samples were inoculated on inulin agar plates and were incubated at 37 °C. For isolation of thermophilic microorganisms, plates were incubated at 10-40 °C. The organisms grown on inulin agar plates were analyzed for their colonial characteristics, purified by subsequent transfers, and stored at 10 °C on the inulin agar plate for further studies.

RESULTS AND DISCUSSION: Inulinase enzyme-producing endophytic bacterial isolates were screened in three types of fruits; among them, we screened based on the zone of formation under the different conditions of 37 °C was grown in the agar plate with supplementation of specific carbon sources **Fig. A** and **B**. 22 endophytic bacterial isolates were identified from three different fruits: Banana, Pineapple, and Zapata. Among the 22 isolates, it was screened based on the number of zone formations from Banana one, Sapota three, and Pineapple once isolated subcultured in agar plates and was kept in 37 °C incubators for further study. However, we have checked different growth studies in 37 °C (Normal room temperature) and 4

°C (cold) conditions. Under the aerobic condition, the plate has a zone formation total of five isolates only zone formation for positive responses of extracellular enzyme produced inulinase expression as confirmation (**Fig 5A** and **B**). Our results indicated that the highest activity level of inulinase was recorded in the agar plate under the cold condition for 4 °C was a synthesis of the enzyme **Fig. 5A** & **B** compare to 37 °C (A. Pandey *et al.* 1999). In Further study, the protein was expressed in extracellular for 66kDa in SDS-PAGE analysis, and confirmation due inulinase enzyme produced Banana KARE_B006 isolates **Fig. 8**. The molecular structure of extracellular inulinase enzyme and formation of the enzyme in different fruits **Fig. 1**.

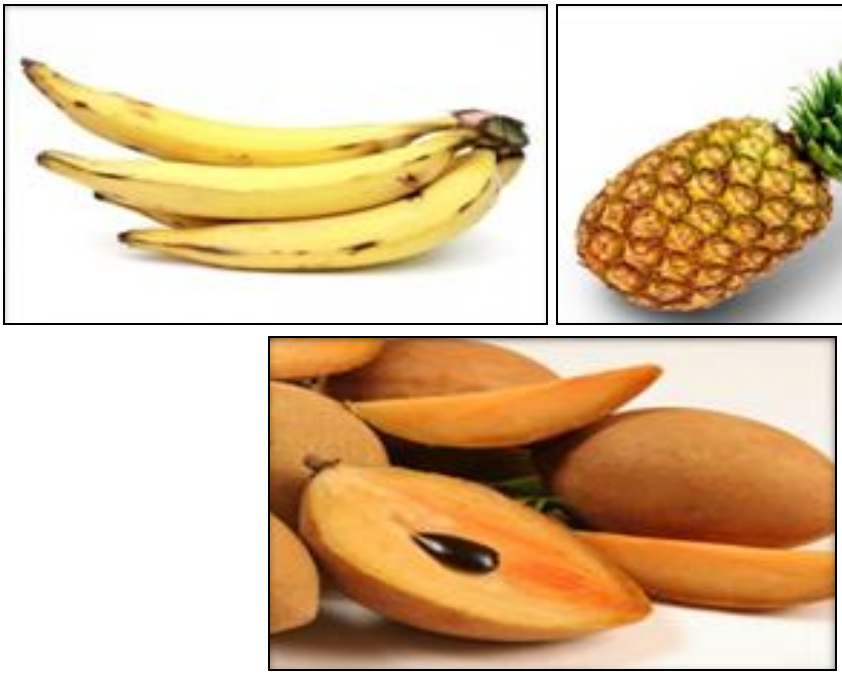


FIG. 1: SHOWN IN FRUITS OF (A) NENTHIRAN BANANA, (B) PINEAPPLE, AND (C) ZAPODA

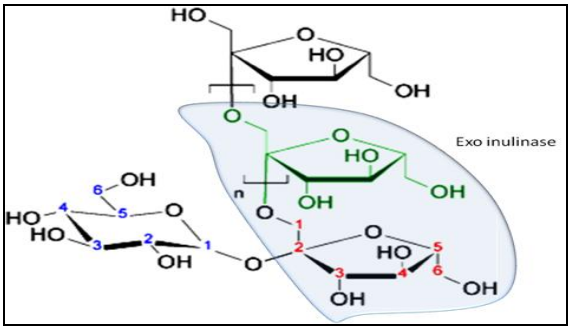


FIG. 2: SHOWN IN MOLECULAR STRUCTURE OF EXOINULINASE ENZYME FORMATION

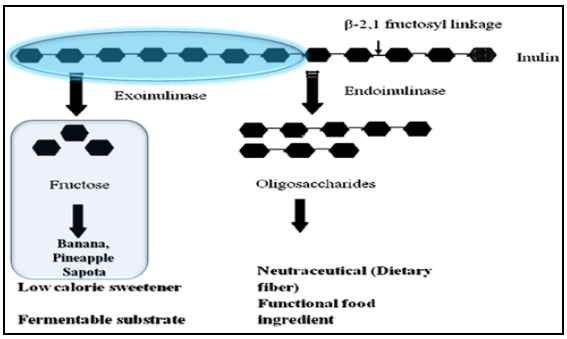


FIG. 3: SHOWN IN EXOINULINASE ENZYME CONVERTED IN TO FRUCTOSE IN FRUITS OF ENDOPHYTIC MICROBIAL METABOLISM

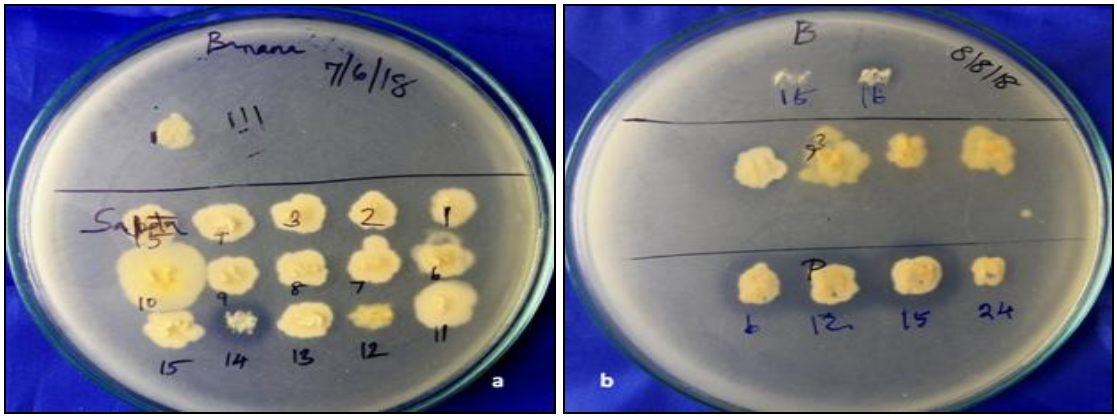


FIG. 4: A & B. SHOWN IN ENDOPHYTIC ISOLATES FROM BANANA, PINEAPPLE AND ZAPOTA FRUITS

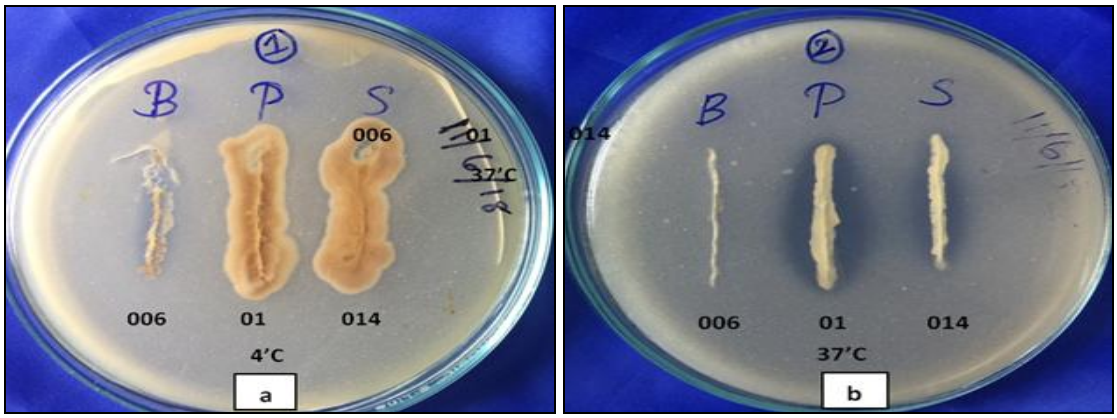


FIG. 5: A & B. SHOWN IN THERMOPHILIC AND COLD-ACTIVE ENZYME-PRODUCING ISOLATES FROM DIFFERENT FRUITS. (B-BANANA, P-PINEAPPLE AND S-ZAPOTA)

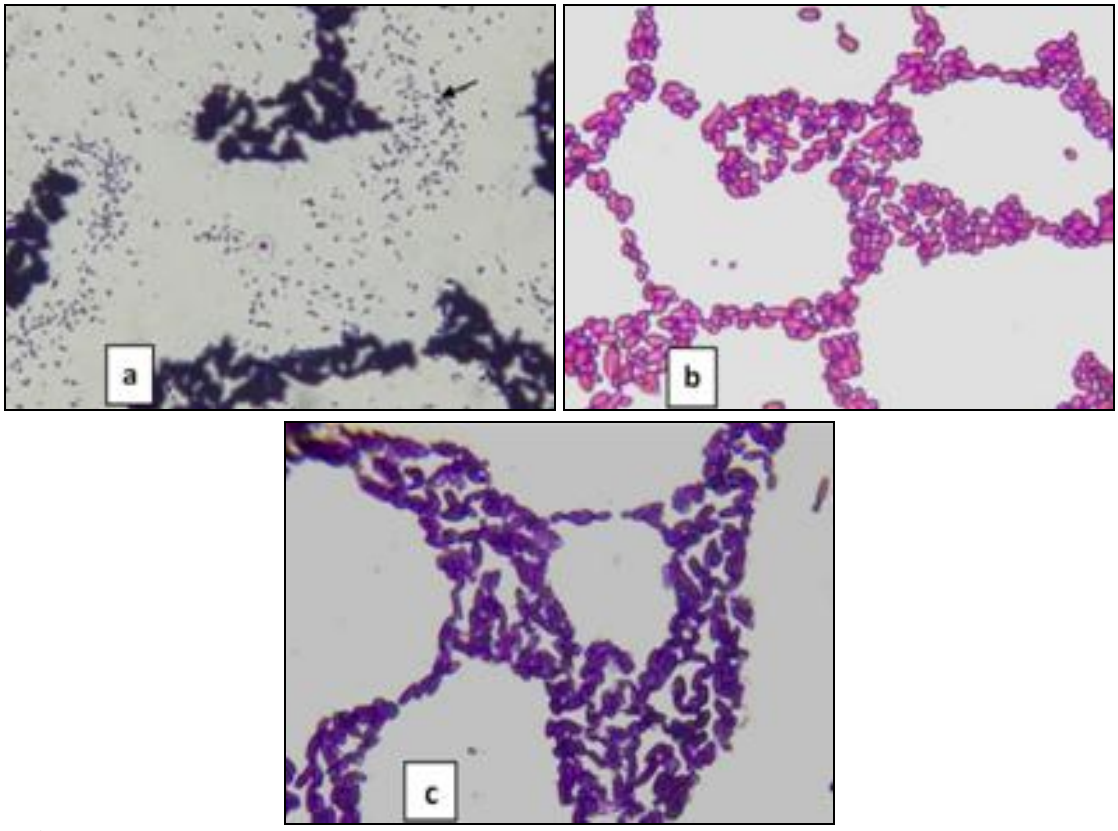


FIG. 6: (A-C). SHOWN GRAM STAINING OF DIFFERENT ISOLATES FROM DIFFERENT FRUITS OF KARE_B006, KARE_P01, KARE_S014

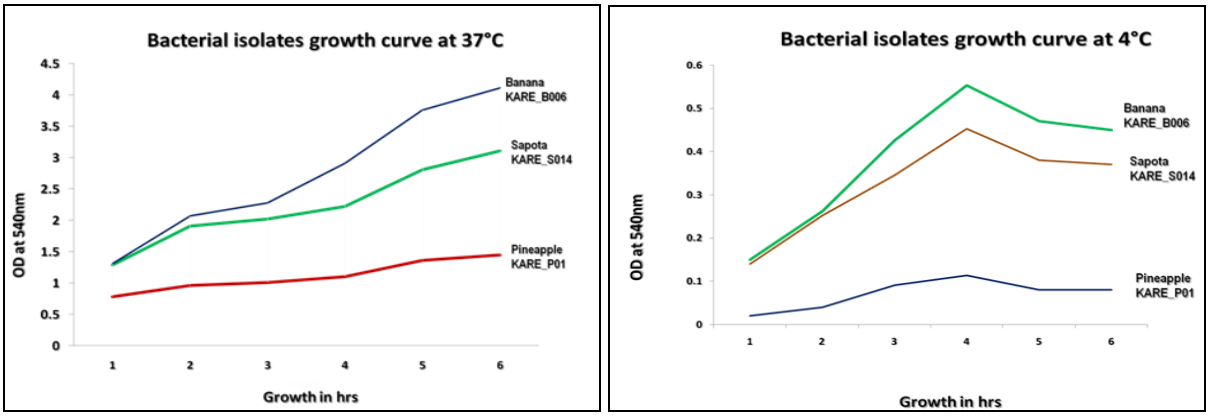


FIG. 7: A & B BACTERIAL GROWTH CURVE FOR THREE DIFFERENT ISOLATES KARE_B006, KARE_P01, KARE_S014 ON TWO DIFFERENT TEMPERATURES (A) 37'C, (B) 4'C.

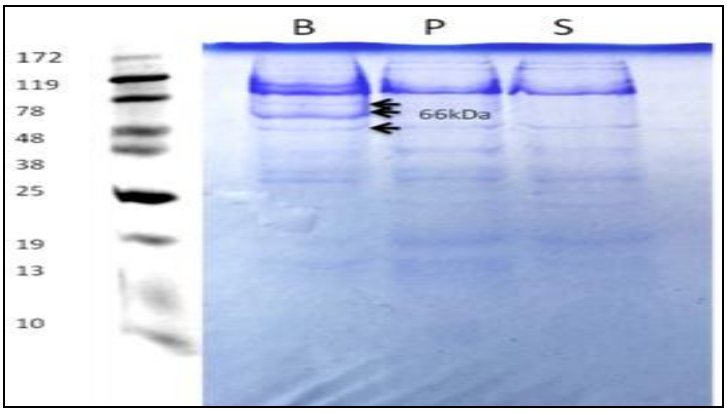


FIG. 8: SHOWN ON SDS-PAGE ANALYSIS OF THREE DIFFERENT STRAINS FROM THREE DIFFERENT FRUITS

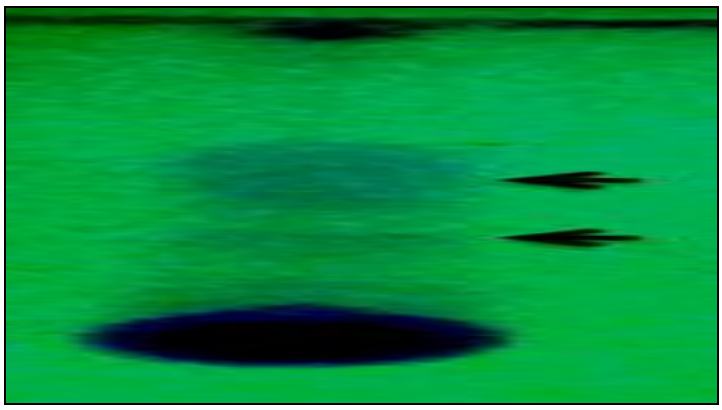
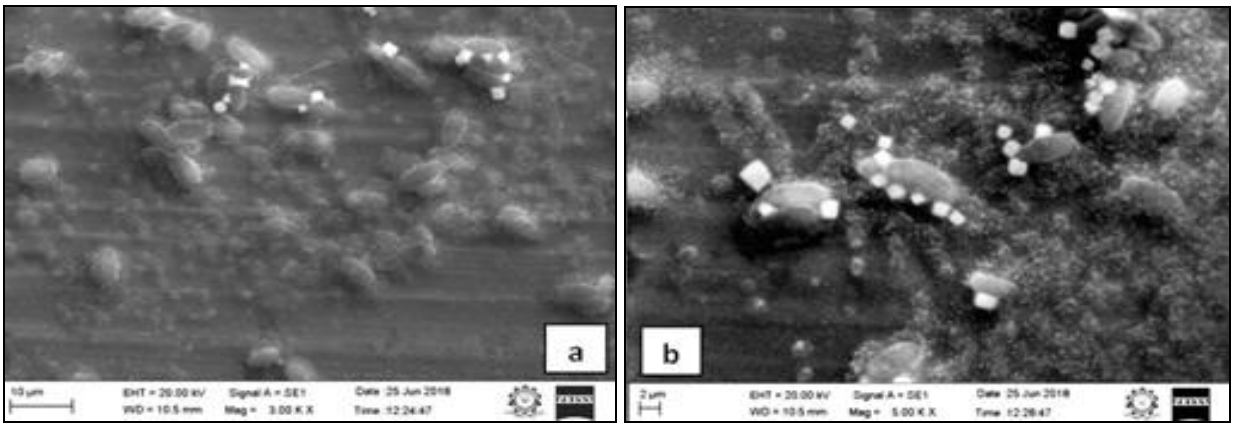


FIG. 9: SHOWN ON TLC ANALYSIS OF BANANA SAMPLES.



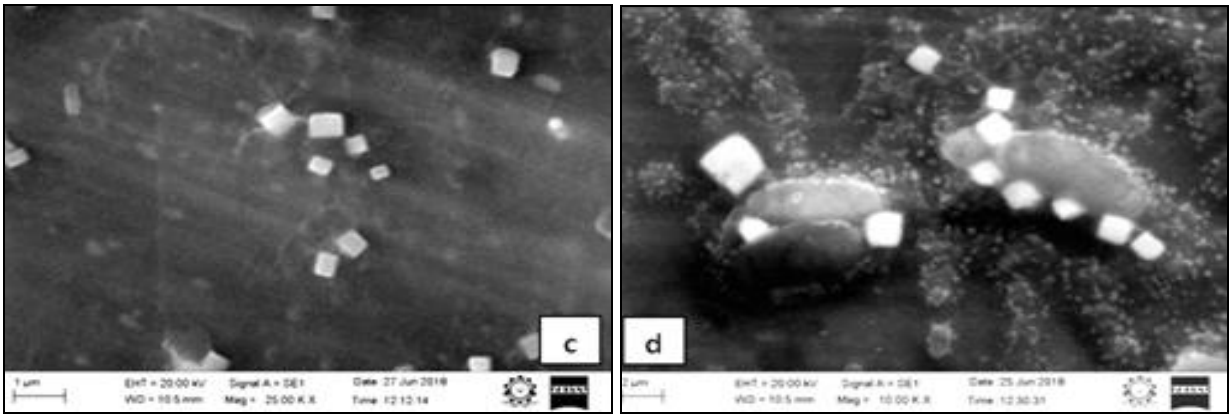


FIG. 11: (A-D) SHOWN MICROSCOPIC EXAMINATION OF SEM ANALYSIS OF KARE_P01 FOR PINEAPPLE ISOLATES

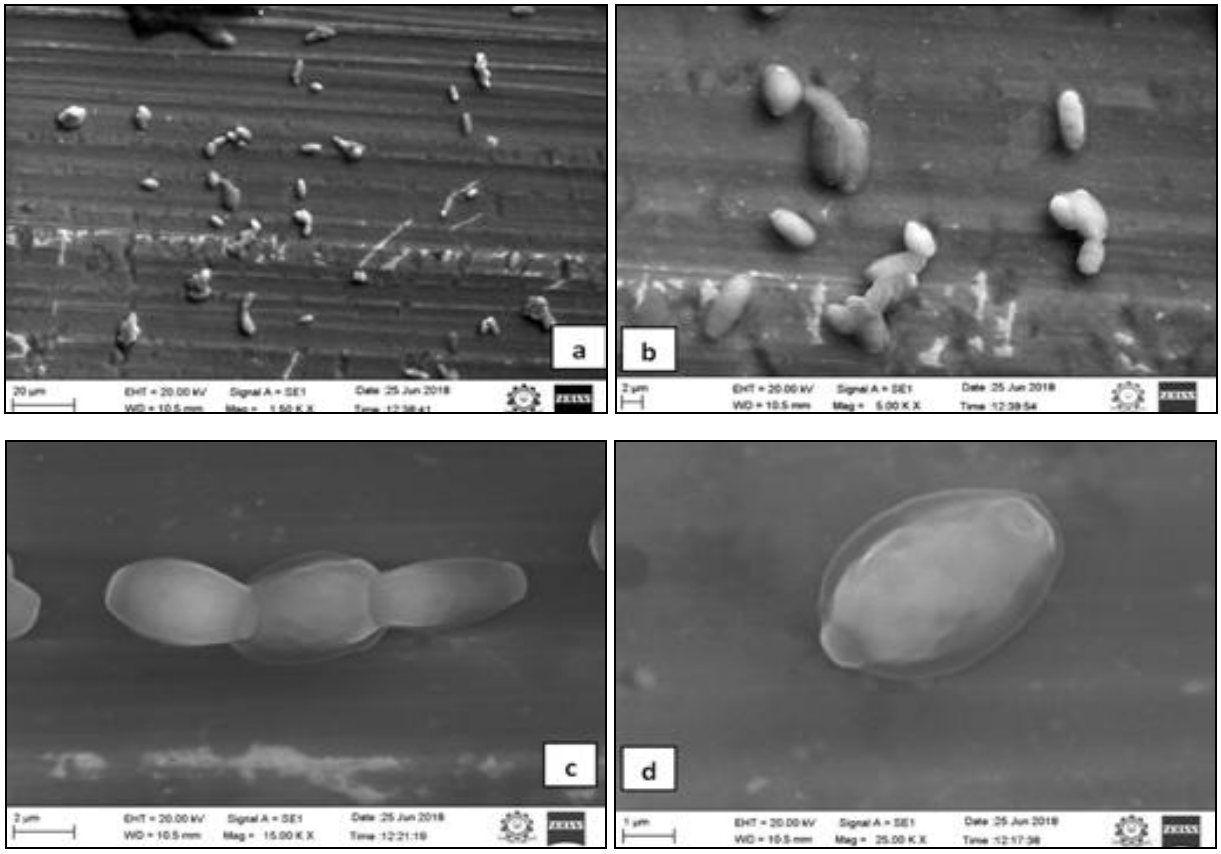


FIG. 12: (A-D) SHOWN MICROSCOPIC EXAMINATION OF SEM ANALYSIS OF KARE_S014 ON CAPSULAR WALL IN AN AROUND THE OLD BACTERIAL CELL

TABLE 1: SHOWS INULIN CONTENT (%/MG/G FRESH MATTER) IN DIFFERENT TYPES OF PLANTS

S. no.	Sources	Sources in the edible part of the plants	Dry matter content (%/mg/g)	Inulin content (%/mg/g)
1	Chicory	Root (tuber)	20-25	15-20
2	Leek	Bulb	15-20	3-10
3	Banana	Fruit	24-26	0.3-0.7
4	Rye	Cereal	80-90	0.5-1.0
5	Barley	Cereal	90-100	0.5-1.5
6	Onion	Bulb	6-12	2-6

Primary Screening of Inulinase Producing Microorganism: During the primary screening, a total of 22 isolates were obtained; among the 22 bacterial isolates, 5 isolates showed a zone of hydrolysis of specific carbon sources and expression around their colonies.

The majority of the isolates were found to be gram-positive rods which included four thermophilic and/or mesophilic cold-active enzyme-producing bacteria isolated from fruits of Banana, Pineapple, and Zapota are grown at 10-50 °C **Fig 4A** & B. Interestingly, bacterial growth study incubating in agar plate and LB broth for two different temperatures was kept in 37 °C and 4 °C. The zone of hydrolysis of bacterial isolates was typically expressed on the agar plates **Fig 3A** & **B**.

The zone formation KARE_B006 in 4 °C alone not in 37 °C and other isolates of KARE_P01, KARE-S014 zone formation at 37 °C on an agar plate **Fig. 5A** & **B**. Further studies on gram staining and SEM analysis were carried out by three isolates of KARE-B006, KARE_P01, KARE_S014, in the case of **Fig 4A** have inulinase enzyme synthesis under 4 °C were similar. Results were found in gram staining and SEM report. Because of inulinase enzyme expressed in cubical crystal adhere to the bacterial cell seen on arrow mark indicate that both gram staining plate. Other than KARE_P01 and KARE_S014 isolate not expressed cubical crystal formation (**Fig. 6** b-c) the similar results obtained by (Reddy *et al.*, 2010).

Microscopic SEM Analysis: Morphology and colonial characteristics of isolates were analyzed in SEM for a different view of (**Fig**. **10, 11, 12** a-d) bacterial cells. However, we have the first time KARE_B006 was seen in the four different views of µm bacterial cells that have surface look like the white cubical structure of inulinase enzyme binding **Fig. 10 AD**. In contrast, KARE_P01 bacterial cells look like budding cells and zone of hydrolysis only in 37 °C and also KARE-S014 was measured. Although the bacterial isolate reKARE-S014 bacterial cells were a capsular wall in matu cells **Fig 11** and **12AD**. Cold active inulinase-producing strains formed colonies on inulin agar plates, which solubilized inulin particles and released reducing sugars in the agar plate **Fig. 5**. It has been reported that in the presence of reducing sugars, (Reddy *et al.*, 2010).

Effect of Carbon Source on Inulinase Production: Production of inulinase is affected by the medium components, especially carbon sources. Inulin containing raw substrates as well as pure sugars was supplemented in 1% concentration as a carbon source in the media containing 1.5% peptone and 1.5% yeast extract.

The maximum inulinase production (10.01≠0.01) from sucrose and high specific activity (1.2 µ/mg) compared to pure sorbitol. Inulin was similar to that of yielded high inulinase activity in Dextrose [(2.50 ≠ 0.003 µ/ml respectively) **Table 3**. Chicory roots are known to store about 15-20% inulin as a reserve carbohydrate (Van loo, 1995).

Asparagus roots are another attractive source of polyfructan. There are many reports on the use of raw inulin-containing substrates for inulinase production has utilized for inulinase production.

TABLE 2: SHOWN IN BIOCHEMICAL STUDIES ON DIFFERENT ISOLATES OF ENDOPHYTIC ORTANISM FROM BANANA, PINEAPPLE AND SAPOTA FOR INULINASE ENZYME PRODUCING KARE_ISOLATES

		ISOLATES- Endophytic organism from fruits																						
		Sapota											**pineapple**						**Nenthram Banana**					
S. no	**Parameters**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
		MORPHOLOGICAL CHARACTERS																						
1 2	Colony color	PL	W	W	W	W	W	W	W	Y	Y	W	PB	W	W	PL	W	W	PL	W	LB	W	W	W
		PHYSIOLOGICAL CHARACTERS																						
3	Grow at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Zone formation	+	-	-	-	+	-	-	-	+	+	+	-	+	+	-	-	-	+	+	+	-	-	+
5	Gram staining	+	-	+	-	-	-	-	+	+	-	+	+	+	+	+		+	+	+	+	+	+	-
		BIOCHEMICAL CHARACTERS																						
6	Catalase	+	-	-	-	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	+	-	+
7	Oxidase	-	-	+	+	-	-	-	-	+	+	-	-	-	+	+	+	-	-	-	-	+	+	-
8	Indole	-	-	+	-	-	+	-	+	+	-	+	-	+	+	+	-	+	+	-	+	+	+	-

	production																							
9	Methyl red test	+	+	+	+	-	+	-	-	+	+	+	+	-	-	-	+	+	+	+	-	-	+	+
10	Voges-Proskauer	-	+	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	-	-
11	Starch hydrolysis	+	-	-	-	-	+	+	+	-	+	-	+	-	-	-	+	+	-	-	+	+	+	-
12	H2S production	+	+	+	+	-	-	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	+	+
13	Gelatin hydrolysis	-	-		+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+
14	Casein hydrolysis	-	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+	+	+	-	+	+	+	-
15	Nitrate reduction	+	+	+	+	-	+	-	+	-	-	+	-	-	-	-	+	+	-	+	-	-	+	+
16	Citrate utilization	+	+	+	+	-	+	-	+	-	-	+	-	-	-	-	+	+	-	+	-	-	+	+
17	Urea hydrolysis	-	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+	+	+	-	+	+	+	-
18	Glycerol hydrolysis	+	+	+	+	-	+	-	-	-	-	+	+	+	-	+	-	+	+	-	-	-	+	+
	Catalase	+	-	-	-	+	+	-	-	-	+	+	+	-	+	+	-	-	+	+	+	+	-	+
	FERMENTATION TESTS																							
19	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	Dextran sulphate	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-
23	sorbitol	+	-	-	+	-	-	+	-	+	+	-	+	-	-	+	+	-	-	+	-	+	+	-

TABLE 3: INULINASE PRODUCTION BY ENDOPHYTIC KARE_B006 ISOLATE ON DIFFERENT SUBSTRATE

S. no	Carbon sources (5%)	Banana dry powder in mg	Inulinase Units/ml	Invertase Units/ml	I/S ratio	Protein mg/ml	Specific activity Units/mg/ml
1	Sucrose	500	10.01± 0.01	50.05± 0.11	0.2	8.8 ± 0.001	1.2
2	Glucose	500	2.8.99± 0.02	14.00± 0.01	0.2	6.0 ± 0.001	0.66
3	Fructose	500	3.1± 0.10	16.21± 0.13	0.19	6.0 ± 0.002	0.53
4	Sorbital	500	1.08± 0.001	5.4± 0.12	0.2	3.0 ± 0.001	0.22
5	Dextrose	500	2.50± 0.003	12.5± 0.003	0.2	8.03 ± 0.02	1.09
6	Inulin	500	17.7± 0.001	88.5± 0.132	0.2	7.71 ± 0.001	2.22

CONCLUSION: A potential inulinase producing endophytic bacterial population is the first time reported KARE-B006, KARE-P01, and KARE-S014 was successfully screened from edible fruits of Banana, Pineapple, and Sapota, as well as utilization of one raw banana powder as inulin sources namely inulin as a substrate for inulinase production by the selected isolate, was performed.

Around a two-fold increase in inulinase, the yield was obtained on both substrates employing optimized conditions for inulinase production. In addition to inulin, the enzyme was also active on sucrose, providing a dual advantage for the production of reducing sugar. Successful exploitation of endophytic microbes synthesis of high cold-active inulinase yields obtained using simple media formulations in cold conditions may prove an attractive alternative for inulinase production at the industrial level.

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CONFLICTS OF INTEREST: Nil

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