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Isolation of Thermotolerant Inulinase-producing Bacteria from Compost using Enrichment and Baiting Techniques

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Despite the growing demand for thermostable inulinases, only a limited number of microorganisms have been identified as producers of inulinase. While fungi and yeasts are known to produce large amounts of inulinase, bacterial inulinases exhibit greater thermostability, making them ideal for diverse industrial applications. This investigation aims to isolate thermotolerant bacteria from compost using two isolation methods - enrichment and baiting. The two techniques are compared in terms of the population of bacteria isolated and the mean enzyme activity across different temperatures. Both methods yielded a comparable number of isolates, but their performance differed in terms of enzyme activity and optimum temperature. The enrichment technique exhibited an optimal temperature of 46.3°C with an enzyme activity of 2.96 U/mL, whereas the baiting method showed 44.4°C and 1.56 U/mL, respectively. These findings suggest that enrichment is a more effective strategy than baiting for isolating thermotolerant bacterial producers of inulinase. The investigation resulted in the isolation of *Bacillus sonorensis* and a novel thermophilic strain, *Aneurinibacillus thermoaerophilus*, both capable of producing the inulinase enzyme.

Keywords: Baiting technique, Compost, Enrichment technique, Inulinase, Thermotolerant bacteria

Introduction

Inulinases are enzymes that catalyse the hydrolysis of inulin, a polysaccharide primarily composed of fructose units. Inulin is found in various plants such as chicory, Jerusalem artichoke, and garlic. These enzymes convert inulin into fructose and fructooligosaccharides (FOS), both of which hold significant industrial and health applications.[1,2] Fructooligosaccharides enhance the growth of gut bacteria, thus improving digestive health as a prebiotic and fructose is used as a low-glycaemic sweetener in food industries.[2] However, there are two challenges associated with inulinase for its industrial application: (i) Limited solubility of inulin at room temperature.[3] (ii) Higher risk of microbial contamination at room temperatures, hence necessitating industrial processes to be carried out at elevated temperatures (60°C). However, many inulinases lose activity at high temperatures, highlighting the need for thermostable inulinases.[4]

Among microorganisms, fungal strains are good producers of inulinase whereas bacterial inulinase exhibits good thermostability.[5] *Aspergillus* spp and *Penicillium* spp are the most common fungal inulinase producers.[5] Chen *et al.* (2009) isolated *Aspergillus ficuum* JNSP5-06, from soil samples which produced 25 U/mL inulinase in 5 days at 30°C.[7] *Mucor circinelloides* isolated from *Asparagus racemosus* produced 7.1U/mL of inulinase at 30°C.[8] Among bacteria, those belonging to the genus *Bacillus* show growth at high temperatures ranging from 60°C to 70°C. *Bacillus* spp. isolated from the Bulgarian hot spring exhibited optimum inulinase activity at 65°C and the enzyme retained 100% activity after heat treatment at 50°C for 1 hr.[9] Gao *et al.* (2009) isolated *Bacillus smithii* from soil with optimum inulinase activity at 70°C which was stable for 9 hrs at this temperature.[10] Drent *et al.* (1999) used dahlia tubers for enrichment at high temperatures and isolated *Clostridium thermoautotrophicum* which had an optimum growth temperature at 58°C with no loss of enzyme activity at 60°C for 3hrs.[11]

In the process of selecting an isolation source, it is important to consider the natural conditions that support microbial life development.[12] Despite demand, to the best of our knowledge, no studies seem to have been done on the isolation of thermotolerant inulinase producers from compost in

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the Indian subcontinent. Compost, with its varying temperature and nutrient-rich environment, offers a promising source of thermotolerant microorganisms capable of producing thermostable inulinases.[13] The composting process undergoes a thermophilic phase with temperatures reaching 60 – 80°C. Generally, fungi do not thrive at temperatures above 65°C, hence bacteria are the predominant microorganisms that are functionally active during thermophilic degradation of compost.[14–17]

Identifying and selecting microorganisms with desired traits for various applications is crucial in biotechnology. The microbiological process involves isolating microorganisms from different environments, culturing them under specific desired conditions, and testing them for particular metabolic properties.[12] Among others, two traditional methods employed for isolating microorganisms are - enrichment and baiting techniques. These methods enhance the likelihood of recovering strains with desirable enzymatic properties and can significantly influence the diversity and specificity of the isolated bacterial populations.[18]

Baiting technique is a method used to isolate specific microorganisms from a mixed culture or environmental sample. It involves using a specific substrate or "bait" that is attractive to the target microorganism, allowing it to grow and dominate the culture. Previous literature studies have demonstrated that specific substrates, such as inulin-rich plant materials, can be utilized as baits to selectively enrich the population of inulinase-producing microorganisms.[19,20] Baiting can also enhance the recovery of fastidious organisms that may not thrive in standard culture conditions, as demonstrated by the successful isolation of diverse bacteria associated with marine sponges using co-cultivation methods.[21] Furthermore, the baiting technique can be tailored to target specific metabolic pathways or ecological niches, allowing for a more focused approach to isolation.[22]

Enrichment technique involves creating an environment that favours the growth of specific microorganisms by selective substrate, medium composition, culturing conditions and frequent transfer, making it easier to isolate and study them.[23,24] This method has successfully isolated microbial species from complex environments, including soil and compost.[25] Allias *et al.* (1987) have isolated thirty-two bacterial strains producing inulinase from soil using an enrichment technique where they used inulin as the sole source of carbon and energy.[26] Enrichment strategies for isolating nitrile-hydrolysing bacteria from soil by using different nitriles and amides as the sole source of nitrogen have also been developed.[27]

It appears however, that there is a lack of sufficient studies to compare the baiting and enrichment techniques. Both the techniques have been used independently for isolation of microorganisms.[19,20,26,27] Nevertheless, there have been attempts to compare these two techniques but under a different context, for instance Ballhausen *et al.* (2015) have compared short term ‘liquid hyphal Baiting method with long term baiting (transfer-enrichment) method and have concluded that long-term transfer enrichment technique is better than the short-term baiting method to isolate mycophagus soil bacteria.[18] The present study has attempted to compare the two traditional methods of isolation – baiting and enrichment technique, for isolating thermotolerant inulinase-producing bacteria from compost by examining their impact on the population of bacteria isolated and enzyme activity across different temperatures.

The objectives of the present study include:

1. To isolate thermotolerant, inulinase-producing bacteria from compost using both enrichment and baiting techniques.
2. To examine the effect of temperature on inulinase activity and enumerate the population of isolated organisms in each technique.
3. To identify an optimal temperature, if it exists, for maximum enzyme activity.

Materials and Methods

Compost-Sample Collection for Isolation of Thermotolerant Inulinase-Producing Bacteria

Compost was collected in clean containers from three different two-week-old windrows at the Karnataka Compost Development Corporation (KCDC) Madiwala, Bengaluru (Geo location: 12.894187704019025, 77.65015929382432). The samples were mixed and stored at room temperature and used within a week. At the time of collection, the temperature of the compost was 53.5°C. Before isolation, the compost was preheated to 53°C overnight in an incubator.

Isolation Techniques

Enrichment Technique

The methodology described by Allias *et al.* (1987) was followed with minor modifications.[26] Five

different trays containing 100 g compost each were pre-treated by spraying with 10% onion solution. The trays were incubated in a moist chamber at 40°C, 45°C, 50°C, 55°C and 60°C for one week. After pre-treatment, "enrichment transfer" was done for all these temperature ranges by suspending 0.5 g of compost in 30 mL of Inulin broth media containing 0.5% ammonium sulphate, 0.5% yeast extract, and 2% inulin and incubated at various temperatures as mentioned above. On the second day, 1.5 mL of culture from each flask was transferred into 30 mL of fresh media and incubated for another 24 hours at the corresponding temperatures. On the third day, 0.3 mL of culture was inoculated into 30 mL of fresh media and incubated for 24 hours. After 24 hours, 1 mL of the culture was serially diluted and pour-plated onto solid inulin media containing 2% agar, subsequently the plates were incubated at the respective temperatures for 24 hours. The resulting individual colonies were sub-cultured and maintained as pure cultures.

Baiting Technique

Onion being a good source of inulin was used as bait to isolate inulinase producers. The procedure followed by Umesh Kumar *et al.* (1989) was adapted with slight modifications.[28] Large pieces of onion were cut and buried in 250 gm of compost. The compost trays were incubated in a moist chamber at the same temperature ranges recommended for the enrichment technique. After 48 hours, the onion pieces were transferred to sterile Petri plates and incubated in a moist chamber for an additional 48 hours to enrich inulin-hydrolyzing bacteria. The bacteria-enriched onion pieces were then ground in 1mL of sterilized 2% peptone, serially diluted, and pour-plated onto inulin agar media. The plates were incubated at the corresponding temperatures for 24 hours. The resulting bacterial colonies were subcultured and maintained as pure cultures.

Colony characteristics like shape, size, elevation, margin, colour, surface and opacity were observed, Gram staining was performed and the number of viable cells was enumerated by calculating the total Colony forming Units per millilitre (CFU /mL) as per published literature .[29,30]

Biochemical Tests

The following biochemical tests were done for the purpose of identifying the bacterial isolates obtained during the course of the study- methyl Red, Voges-Proskauer, Citrate Utilization, Triple Sugar Iron, Starch Hydrolysis, Nitrate reduction, Gelatin Liquefication, Casein hydrolysis [46].

Molecular Identification

Genomic DNA was extracted from the bacterial culture and evaluated on 1% agarose gel, which showed a single high-molecular-weight band. Molecular identification was carried out by amplifying a ~1500 bp fragment of the 16S rRNA gene using primers 16SrRNA-F/R. The PCR product was visualised, purified, and sequenced bidirectionally with the BDT v3.1 Cycle Sequencing Kit on an ABI 3730×l Genetic Analyzer. Forward and reverse reads were assembled to obtain a consensus sequence with Aligner software. The sequence was analysed through BLAST against the NCBI GenBank "nr" database, and the ten closest hits were aligned using ClustalW. and phylogenetic tree were constructed in MEGA 11 to determine the taxonomic identity.[44,45]

Statistical Analysis

In both these techniques (Baiting and Enrichment), the population of thermotolerant bacteria was enumerated. Significant differences in the population count between the two techniques were analysed using one-way ANOVA with temperature (40°C, 45°C, 50°C, 55°C and 60°C) as the main factor with three replications. The response curves were fitted.

Inulinase Assay

The pure cultures were inoculated into 100 mL inulin broth medium and incubated for 24 hours. One mL of the cultured media was centrifuged at 8000 rpm for 10 minutes. The supernatant was collected and used as crude enzyme extract. Inulinase activity was determined by the DNS method.[31] The methodology given by Gavrailov & Ivanova (2016) was followed for the enzyme assay.[32] Fructose (10 µmoles/mL) was used for the standard graph. The reaction mixture contained 100 µl of crude enzyme extract and 100 µl of 1% inulin (from chicory, Himedia), and the volume was made up to 1mL using phosphate buffer (0.2M, pH 7.0). The test tubes were incubated at 50°C for 10 minutes in a water bath. The reaction was stopped by adding 2 mL of DNS reagent. The readings were taken in a Shimadzu UV-1780 spectrophotometer at 540nm. One unit of inulinase activity was defined as the amount of enzyme that releases one µmol of reducing sugar (fructose) per minute from inulin under the assay conditions.

Statistical Analysis

The enzyme activity of the organisms isolated through the enrichment and baiting technique at four different temperatures was calculated. Since there were two isolation techniques each carried out at four temperatures- 40°C, 45°C, 50°C, and 55°C, it made the experiment factorial with 2 × 4 = 8 treatment combinations. In the course of the experiment, three microorganisms were selected under each treatment combination and the readings were taken in triplicates. Thus, the number of replications became nine for each treatment combination. A two-way ANOVA with interaction was carried out to determine if isolation methods and temperature have any impact on enzyme activity. It was followed by post-ANOVA tests and response curves.

Results and Discussion

The current study was conducted to compare baiting and enrichment method for isolating thermotolerant bacteria from compost. The experiments designed included to estimate and compare the population of thermotolerant bacteria followed by their inulinase activity between the two isolation techniques. Thirty-four bacterial isolates were obtained from compost samples incubated at temperatures ranging from 40 to 60°C, using both enrichment and baiting techniques (Table 1).

The mean number of organisms isolated by both the techniques is as mentioned in Table 2. Based on the colony morphology, the number of different types of bacteria isolated was more or less identical by both the techniques (Tables 3 and 4).

The dominant colonies recorded at a lower temperature range using both techniques were off-white, opaque, circular in shape having either a smooth and glistening or dull surface. Most of these colonies were non-spore forming Gram-positive short rods. Additionally, a few pinpoint colonies which were Gram-negative rods were also observed along with cocci- shaped medium-sized slightly raised colonies. However, the baiting technique also revealed the presence of yellow, opaque colonies which were Gram-positive cocci. Nevertheless, at higher temperature range most of the colonies were opaque, creamy white, circular, having either a smooth or wrinkled surface. Upon Gram staining, all these colonies appeared as spore-forming Gram-positive long or short rods. These results are in agreement with the studies made by Chandna *et al.* (2013) who reported similar type of colony morphological characteristics for the compost-based bacterial isolates.[33] Comparing the colony morphological studies at low and high temperature ranges, there appears to be a gradual shift in the bacterial population from mesophiles to thermophiles. Chinakwe *et al.* (2019) have reported that an increase in temperature beyond 45ºC in compost piles causes population shift of mesophilic bacteria to thermophilic bacteria.[34] This is also consistent with the compost temperature of 53.5ºC recorded at the time of sample collection when the composting process was in its thermophilic phase.

Population of Thermotolerant Bacteria

Upon increasing the temperature from 40 to 60°C, with an increase of 5°C at each level, there was a significant exponential reduction in the population of thermotolerant bacteria isolated by both techniques. (Table 5). Fitted response curves are given in Fig 1. Since the R^2 is 91% for both techniques, the model is a good fit in explaining the effect

Table 1 — Number of types of bacteria isolated based on colony morphology using baiting and enrichment technique

Temperature (°C)	Number of bacteria types	
	Baiting technique	Enrichment technique
40	5	5
45	5	4
50	3	3
55	3	4
60	2	0
Total	18	16

Note: The table 1 presents the number of morphologically distinct bacterial types isolated at different incubation temperatures using baiting and enrichment techniques; Values represent counts obtained from triplicate experiments; Classification was based on colony morphology

Table 2 — Mean number of thermotolerant bacteria (CFU/mL) isolated by baiting and enrichment technique at different temperatures

Temperature (°C)	Number of bacteria isolated (CFU/mL)	
	Baiting Technique	Enrichment Technique
40	45.0×10^6	966.7×10^6
45	4.7×10^6	448.0×10^6
50	2.3×10^6	122.0×10^6
55	1.1×10^6	62.3×10^6
60	0.6×10^6	—

Note: Values in table 2 represent the mean colony-forming units per milliliter (CFU/mL) of thermotolerant bacteria isolated using baiting and enrichment techniques at different temperatures; Each value is the mean of three independent experiments (n = 3); A dash (–) indicates no bacterial growth observed at that temperature

Table 3 — Colony morphology of thermotolerant bacteria isolated by baiting technique

	Isolate	Size	Shape	Margin	Elevation	Colour	Surface	Opacity	Grams character
40°C	BT401	Medium	Circular	Even	Convex	Brown	Smooth and Glistening	Opaque	Gram- positive short Rods; single: No spores
	BT402	Small	Circular	Even	Convex	Off White	Smooth and Glistening	Opaque	Gram-positive Cocci in pairs
	BT403	Medium	Circular	Even	Convex	Brown	Smooth and Glistening	Opaque	Gram-positive short Rods; single: No spores
	BT404	Pin Point	Circular	Even	Flat	Off White	Glistening	Translucent	Gram-positive short Rods; single: No spores
	BT405	Small	Circular	Even	Convex	light yellow	Smooth	Opaque	Gram-positive Cocci in groups
	BT406	Large	Irregular	Wavy	Flat	Dull White	Dull	Translucent	SUBMERGED
45°C	BT451	Pinpoint	Circular	Even	Flat	Off white	Dull	Opaque	Gram-positive rod
	BT452	Medium	Circular	Even	Flat	Off white	Dull	Opaque	Gram-positive rods in chains
	BT453	Large	Irregular	Wavy	Flat	Off white	Wrinkle	Opaque	Gram-positive short rod
	BT454	Large	Irregular	Wavy	Flat	Off white	Dull	Opaque	Gram-positive long rod
	BT455	Medium	Irregular	Wavy	Flat	Off white	Dull	Opaque	Gram-positive long rod
50°C	BT501	Medium	Irregular	Wavy	Umbonate	Creamy white	Wrinkle	Opaque	Gram-positive rods in long chains with sheath
	BT502	Small	Circular	Even	Flat	Creamy white	Glistening	Opaque	Gram-positive Rods; single; Spore formers
	BT503	Pin Point	Circular	Even	Flat	Creamy white	Smooth	Opaque	SUBMERGED COLONIES
55°C	BT551	Medium	Irregular	Filamentous	Flat	Off white	Rough	Opaque	Gram-positive thin rods
	BT552	Pin point	Punctiform	Even	Flat	Cream	Smooth	Opaque	Gram-positive short rods
	BT553	Small	Circular	Even	Raised	Cream	Rough	Opaque	Gram-positive thick rods
60°C	BT601	Pin point	Circular	Wavy	Flat	Creamy white	Dull	Opaque	NO GROWTH ON SUBCULTURE
	BT602	Small	Circular	Even	Umbonate	Creamy White	Dull	Translucent	

Note: The table 3 summarizes colony morphology and Gram characteristics of thermotolerant bacterial isolates obtained using the baiting technique at different incubation temperatures; Observations are based on standard microbiological methods; "Pin point" refers to colonies <1 mm in diameter; "Submerged" indicates colonies growing below the agar surface; "No growth on subculture" denotes inability of the isolate to grow on subsequent transfers

of temperature on the population of thermotolerant bacteria isolated by baiting and enrichment techniques.

In the enrichment technique, the rate of decrease in the number of thermotolerant bacteria was faster compared to the baiting technique. Assuming that similar types of bacteria are isolated in both the techniques, at 40°C which is close to mesophilic temperature, the number of bacteria isolated from enrichment technique appears to be approximately 21 times more than baiting technique. Further in the thermophilic range (45 to 60°C) a similar trend is observed, however, the difference is greatly reduced with increase in temperature (90 times more at 45°C, 61 times more at 50°C and 62 times more at 55°C in enrichment technique). No growth was seen at 60°C (Table 2).

Enzyme Activity

ANOVA table (Table 6) indicates that there is a significant difference between the isolation methods and temperature levels with regard to enzyme activity. Also, the interaction between isolation method and temperature is significant.

The Enrichment technique produces significantly more inulinase activity than the baiting technique by 0.41 U/mL, which is an increase of 38% (Table 7). Standard error of this difference is 0.18. Hence, the enrichment technique shows more inulinase activity than the baiting method by 0.41 ± 0.18 U/mL.

The average enzyme activity varies significantly across different temperatures. Response curves were fitted to determine the optimal temperatures for enzyme activity in baiting and enrichment techniques.

Table 4 — Colony morphology of thermotolerant bacteria isolated by enrichment technique

	Isolate	Size	Shape	Margin	Elevation	Colour	Surface	Opacity	Grams character
40°C	ET401	Medium	Circular	Even	Convex	White	Smooth and Glistening	Opaque	Gram-negative Cocci
	ET402	Pin Point	Circular	Even	Flat	Creamy white	Smooth	Opaque	Gram-negative short rod
	ET403	Medium	Irregular	Wavy	Flat	Creamy white	Smooth and Glistening	Opaque	Gram-positive short rod
	ET404	Medium	Circular	Even	Flat	Dull white	Smooth	Translucent	Gram-positive very short rods
	ET405	Medium	Circular	Wavy	Umbonate	Dull white	Smooth	Opaque	Gram-positive short rods
45°C	ET451	Pin point	Circular	Even	Flat	Off white	Dull	Opaque	Gram-positive long rods; No Spore
	ET452	Large	Irregular	Wavy	Umbonate	Off white	Wrinkled	Opaque	Gram-positive short rod; Spore former
	ET453	Medium	Circular	Even	Raised	Off white	Dull	Opaque	Gram-positive short rod; Spore former
	ET454	Small	Circular	Even	Flat	Pale white	Dull	Opaque	Gram-positive long rods; No Spore
50°C	ET501	Pin Point	Circular	Even	Flat	Creamy White	Smooth	Opaque	Gram-positive short rods
	ET502	Medium	Circular	Even	Umbonate	Creamy white	Wrinkle	Opaque	Gram-positive rods
	ET503	Large	Circular	Wavy	Flat	Creamy White	Wrinkle	Opaque	Gram-positive rod; spore former
55°C	ET551	Pinpoint	Circular	Even	Flat	White	Smooth	Transparent	Gram-positive short rods; No spore
	ET552	Very small	Circular	Even	Flat	Creamy white	Smooth	Opaque	Gram-positive long rods; No Spore
	ET553	Small	Irregular	Even	Flat	Creamy white	Smooth	Opaque	Gram-positive rods; Spore formers
	ET554	Pinpoint	Circular	Even	Flat	White	smooth	Transparent	Gram-positive rod
60°C	No Growth								

Note: The table 4 details colony morphology and Gram characteristics of thermotolerant bacterial isolates obtained using the enrichment technique at different incubation temperatures; Observations were made using standard microbiological methods; “Pin point” denotes colonies smaller than 1 mm in diameter; “No growth” indicates that no bacterial colonies were recovered at 60 °C; Terms such as “spore former” and “no spore” refer to observations from spore staining

Table 5 — One-way ANOVA for population of thermotolerant bacteria isolated

	Source of Variation	d.f.	Sum of Squares	Mean Squares	F-ratio	P-value
Baiting technique	Temperature	4	4269.652	1067.413	23.43453	4.59E-05
	Error	10	455.4872	45.54872		
	Total	14	4725.139			
Enrichment technique	Temperature	4	1927673	481918.3	36.4804	6.16E-06
	Error	10	132103.3	13210.33		
	Total	14	2059776			

Note: One-way ANOVA showing the effect of temperature on the population of thermotolerant bacteria isolated using baiting and enrichment techniques; Temperature had a significant influence ($p < 0.05$) on bacterial population in both methods, with a stronger effect observed in the enrichment technique

Based on the fitted response curve equations, the optimal temperature for the enrichment technique is 46.3°C, with a corresponding enzyme activity of 2.96 U/mL. For the baiting technique, the optimal temperature is 44.4°C, with an enzyme activity of 1.56 U/mL (Fig. 2).

As a method of analysing the interaction of isolation method with temperature, enzyme activity was compared across temperature for each isolation using the post-ANOVA l.s.d. (least significant difference) t-test. The l.s.d. was 0.55. Result of the comparison tests are given in Table 8.

The data presented in Table 8 shows the results of post-ANOVA comparisons of inulinase activity at three different temperature ranges (40 to 45°C, 45 to 50°C, 50 to 55°C). As the temperature increases from 40 to 45°C enzyme activity increases significantly by 0.88 U/mL in the baiting technique, and 1.83 U/mL in the enrichment technique. As temperature increases from 45 to 50°C, enzyme activity decreases significantly by 0.56 U/mL in the baiting technique; nevertheless, the decrease is not significant in the enrichment technique at this temperature range. However, when the temperature increases from 50 to 55°C, the difference in enzyme activity is not significant in either of the techniques. This can also be observed in Fig. 2.

Between these two techniques, the enrichment method revealed a 38% increase in the enzyme activity compared to the baiting method. This demonstrates that while both methods can isolate thermotolerant inulinase producers, the enrichment technique is slightly more effective at higher temperatures. Despite the decrease in microbial numbers, with rising temperatures, inulinase activity increased for both techniques from 40 to 45°C

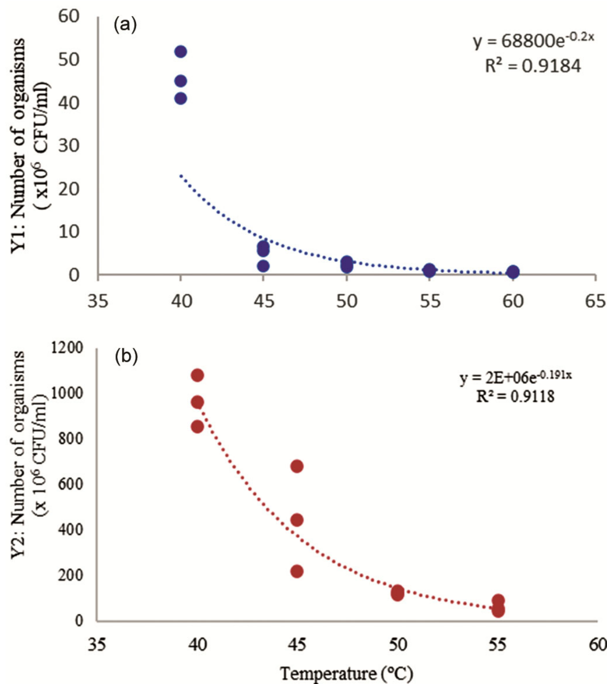

Fig. 1 — Response curve fitted for population of thermotolerant bacteria isolated by (a) baiting technique (b) enrichment technique

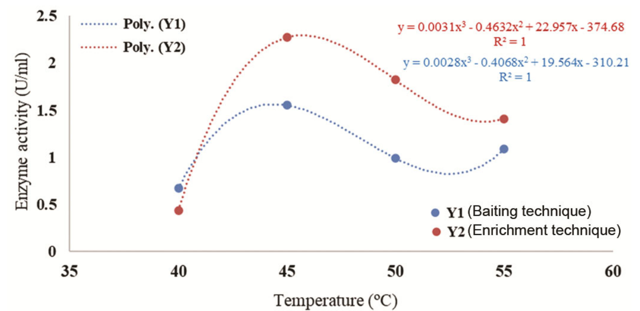

Fig. 2 — Response curve fitted for enzyme activity of thermotolerant bacteria isolated by baiting and enrichment techniques

Table 6 — Two-way ANOVA with Interaction for inulinase Activity of thermotolerant bacteria isolated by baiting as well enrichment technique at four different temperature levels

Source of Variation	d.f.	Sum of Squares	Mean Squares	F-ratio	P-value
Isolation Method (M)	1	3.009422	3.009422	8.8671	0.004097
Temperature (T)	3	16.93083	5.643609	16.6286	4.26E-08
Interaction (M x T)	3	3.094078	1.031359	3.0389	0.035295
Error	64	21.72102	0.339391		
Total	71	43.75535			

Note: Two-way ANOVA was performed to analyze the effect of isolation method (baiting vs. enrichment), temperature, and their interaction on inulinase activity of thermotolerant bacteria; Degrees of freedom (d.f.), sum of squares, mean squares, F-ratio, and p-values are presented; Significant p-values (< 0.05) indicate that both isolation method and temperature had a significant effect on inulinase activity, with a notable interaction between the two factors

Table 7 — Comparison of mean inulinase activity of thermotolerant bacteria isolated by baiting and enrichment technique at four different temperature levels

Isolation Method	Temperature				
	40ºC	45ºC	50ºC	55ºC	Mean
Baiting Technique (U/mL)	0.67	1.55	0.99	1.09	1.07
Enrichment Technique (U/mL)	0.44	2.27	1.82	1.41	1.48
Mean	0.56	1.91	1.4	1.25	—

Note: Table 7 presents the comparison of mean inulinase activity (U/mL) of thermotolerant bacteria isolated by baiting and enrichment techniques at four different temperature levels (40°C, 45°C, 50°C, and 55°C); The enrichment technique consistently exhibited higher inulinase activity across all temperatures compared to the baiting technique, with the highest activity (2.27 U/mL) recorded at 45°C by the enrichment method; Overall, the enrichment technique showed a greater mean activity (1.48 U/mL) than the baiting technique (1.07 U/mL)

Table 8 — Post-ANOVA l.s.d. t-test for Interaction of Isolation Method and Temperature on enzyme activity

Temperature comparison	Baiting technique	Enrichment technique
40°C to 45°C	1.55 − 0.67 = 0.88*↑	2.27−0.44 = 1.83*↑
45°C to 50°C	1.55 − 0.99 = 0.56*↓	2.27 −1.82 = 0.45 ns
50°C to 55°C	1.09 − 0.99 = 0.01 ns	1.82 − 1.41 = 0.41 ns

Note: l.s.d.: least significant difference, *Significant at 5%, ns: not significant, ↑: increase, ↓: decrease

(Table 8, Fig. 2). This probably suggests that while fewer organisms were present, those that remained were more efficient at producing inulinase at elevated temperatures. Although there are not many studies on thermotolerant inulinase-producing bacteria from compost, there are a couple of studies conducted on other microorganisms producing thermotolerant enzymes like protease, cellulase and amylase from compost.[35–37]

Upon comparing the two isolation techniques, the enrichment technique yielded significantly higher number of inulinase producers. Enrichment process favoured continuous adaptation and selection, leading to the isolation of highly active inulinase producers capable of thriving at high temperatures. This is in support of the review reported by Gu *et al.* (2021).[23] According to this review, selective culturing, frequent transfers and pre-enrichment treatments result in a surge of target microorganisms. Recent studies have demonstrated that enrichment techniques are highly effective in isolating a wide variety of thermotolerant microorganisms from various environmental niches, emphasizing their crucial role in microbial isolation.[38–41,47]

Although baiting is effective for isolating specific microorganisms, it performed less efficiently than enrichment in this study. This could be attributed to the composition of the bait (onion), which contains other sugars in addition to inulin. These extra sugars might have attracted non-target organisms, rather than potential inulinase producers. However, baiting technique can still be considered for isolating microorganisms with specialized metabolic roles and for those which may not thrive under general enrichment conditions.[22,42,43]

Biochemical Tests and Molecular Identification

Among the total 34 isolates obtained through enrichment and baiting technique, 3 isolates were finally shortlisted based on enzyme activity for carrying out biochemical characterization. Biochemical profiling (Table 9) showed differences in the metabolic characteristics of isolates ET503, ET552, and BT453. While ET552 was mostly negative with the exception of gelatin liquefaction, ET503 and BT453 exhibited similar reactions, with positive results for Voges–Proskauer, citrate utilization, starch hydrolysis, nitrate reduction, and gelatin liquefaction. ET552 showed a neutral butt and an alkaline slant in Triple Sugar Iron reactions, thus suggesting limited carbohydrate fermentation. Because these phenotypic traits were insufficient for precise taxonomic resolution, these isolates were further subjected to 16S rRNA gene sequencing to clarify their taxanomic position. BLAST analysis of the sequences showed that ET503 and BT453 shared 100% identity with *Bacillus sonorensis*, while ET552 was most closely related to *Aneurinibacillus thermoaerophilus* (99.57% identity).

Table 9 — Biochemical tests of three thermotolerant bacteria isolated by Enrichment and Baiting technique

Test name	ET503	ET552	BT453
Methyl red	Weakly Positive	Negative	Positive
Voges-proskauer	Positive	Negative	Positive
Citrate utilization	Positive	Negative	Positive
Triple sugar iron	Alkaline slant, Acid butt	Alkaline slant, Neutral butt	Alkaline slant, Acid butt
Starch hydrolysis	Positive	Negative	Positive
Nitrate reduction	Positive	Negative	Positive
Gelatin liquefication	Positive	Positive	Positive
Casein hydrolysis	Negative	Negative	Negative

Note: The table presents the biochemical characteristics of the three thermotolerant bacterial isolates (ET503, ET552, and BT453) obtained by baiting and enrichment techniques; Results indicate positive reactions for several tests in ET503 and BT453, whereas ET552 showed limited activity, being positive only for gelatin liquefaction; All three isolates were negative for casein hydrolysis

Phylogenetic reconstruction using the neighbour-joining method (Fig. 3) revealed distinct clustering patterns for the isolates. ET503 and BT453 were placed in a strongly supported clade with *Bacillus sonorensis* reference sequences JSNBEBT50 4C (PP086720.1), L46 (KU551167.1), CY12 (KU551137.1), and YS73 (KU551260.1), confirming their close evolutionary affiliation with this species. Other *Bacillus sonorensis* accessions (FN397516.1, HM191249.1, OR807426.1, OR268217.1, PP661233.1, DQ993679.1) were positioned more distantly, reflecting broader divergence within the genus. The concordance between molecular data and biochemical traits supports the identification of ET503 and BT453 as *Bacillus sonorensis* and highlights their potential as thermotolerant inulinase producers.

Similarly, phylogenetic analysis of ET552 (Fig. 3c) clustered the isolate within a distinct sub-clade of the genus *Aneurinibacillus*. The neighbour-joining reconstruction revealed a strong evolutionary association with *Aneurinibacillus thermoaerophilus* strain 1 (MW927322.1), supported by its close proximity to strains 2 (MW927323.1), N25 (KY433871.1), and PL-5 (PP572913.1). In contrast, *Aneurinibacillus* members such as strains LD3

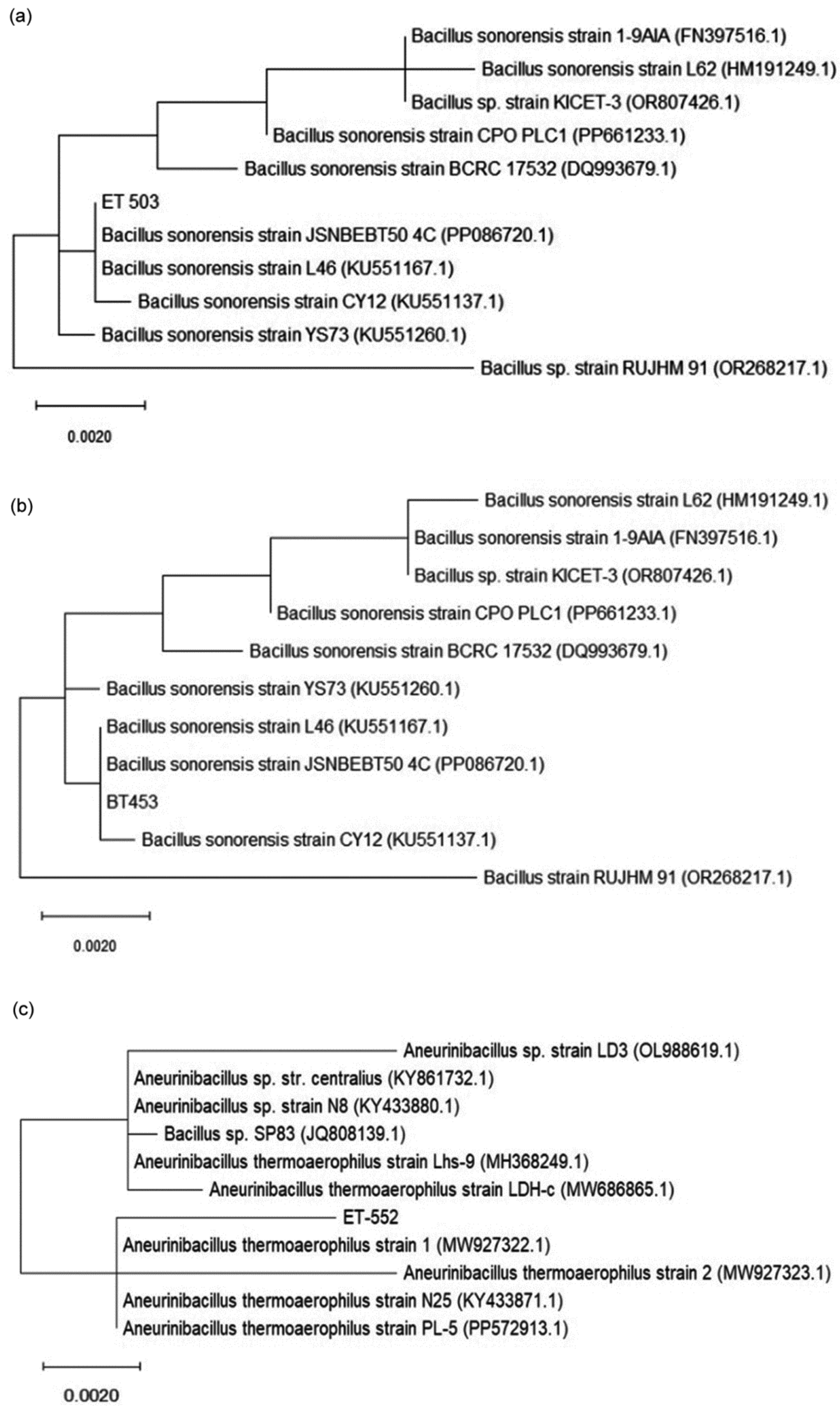

Fig 3 — Phylogenetic placement of thermotolerant bacterial isolates based on 16S rRNA gene sequences; (a) Isolate ET503, (b) Isolate BT453, and (c) Isolate ET552 showing their evolutionary relationships with closely related bacterial species

(OL988619.1), centralis (KY861732.1), and N8 (KY433880.1) formed adjacent but clearly separated branches, reflecting intra-genus diversity. The combined molecular phylogeny and observed thermotolerant enzymatic activity suggest that ET552 represents *Aneurinibacillus thermoaerophilus*, further underscoring the significance of this species as a promising inulinase producer with potential biotechnological applications.

In this study, a thermophilic bacterial isolate, *Aneurinibacillus thermoaerophilus*, capable of producing inulinase was identified and characterized. To the best of our knowledge, this is the first report of its kind. Although its enzyme activity is relatively low, the strain remains a promising candidate for inulinase production, provided its genetic makeup or nutritional requirements are optimized. At 50°C, the inulinase activity of *Bacillus sonorensis* was 2.62 IU/mL, whereas *Aneurinibacillus thermoaerophilus* exhibited an activity of 1.36 IU/mL.

Conclusions

This study clearly demonstrates that the enrichment technique is more reliable and efficient than the baiting method for isolating thermotolerant, inulinase-producing bacteria from compost. The enrichment approach selectively promoted the growth of desired microorganisms, leading to the successful isolation of *Bacillus sonorensis* (ET503 and BT453) and *Aneurinibacillus thermoaerophilus* (ET552). The optimum isolation temperatures of 46.3°C for enrichment method and 44.4°C for baiting method, arrived based on statistical inference, highlight the strong thermal adaptability of these strains. The outcome of this work also resulted in identifying *A. thermoaerophilus* as a novel thermotolerant inulinase producer. These findings point to their potential use in industrial applications such as high-temperature fermentation and the production of thermostable inulinase for food and bioprocess industries. However, as this work was based on isolates from a single compost source, broader sampling and genetic characterization are needed to capture diversity more comprehensively. Future studies can also focus on optimizing enzyme production and assessing scalability for industrial and environmental applications.

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Conflict of Interest

The authors declare that there is no conflict of interest among the authors of the manuscript.

References

1 Vela M R, Fernandes S & Jiang B, Fungal inulinases as potential enzymes for application in the food industry, *Adv Food Sci Technol*, **5(8)** (2013) 1031–1042, DOI:10.19026/ajfst.5.3201.

2 Singh R S, Singh T, Hassan M & Kennedy J F, Updates on inulinases: Structural aspects and biotechnological applications, *Int J Biol Macromol*, **164** (2020) 193–210, doi: 10.1016/j.ijbiomac.2020.07.078.

3 Vendrell-Pascuas S, Castellote-Bargalló A I & López-Sabater M C, Determination of inulin in meat products by high-performance liquid chromatography with refractive index detection, *J Chromatogr A*, **881(1–2),** (2000) 591–597, DOI:10.1016/S0021-9673(00)00351-4.

4 Gill P K, Manhas R K & Singh P, Purification and properties of a heat-stable exoinulinase isoform from Aspergillus fumigatus, *Bioresour. Technol*, **97(7)** (2006) 894–902, DOI: 10.1016/j.biortech.2005.04.034.

5 Singh R S, Chauhan K & Kennedy J F, A panorama of bacterial inulinases: production, purification, characterization and industrial applications, *Int J Biol Macromol*, **96** (2017) 312–322, DOI: 10.1016/j.ijbiomac.2016.12.004.

6 Kango N & Jains S C, Production and properties of microbial inulinases: Recent advances, *Food Biotechnol*, **25(3)** (2011) 165–212, DOI: 10.1080/08905436.2011.590763.

7 Chen H Q, Chen X M, Li Y, Wang J, Jin Z Y, Xu X M, Zhao J W, Chen T X & Xie Z J, Purification and characterisation of exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06, *Food Chem*, **115(4)** (2009) 1206–1212, DOI: 10.1016/j.foodchem.2009.01.067.

8 Singh R S, Chauhan K & Kaur R, Inulinase production in shake-flask fermentations from *Mucor circinelloides* BGPUP-9, *J App Biol Biotech*, **6(06)** (2018) 18–25, DOI: 10.7324/JABB.2018.60603.

9 Uzunova K, Vassileva A, Kambourova M, Ivanova V, Spasova D, Mandeva R, Derekova A & Tonkova A. Production and properties of a bacterial thermostable exo-inulinase, *Z Naturforsch C Bio Sci*, **56(11–12)** (2001) 1022–1028, DOI: 10.1515/znc-2001-11-1220.

10 Gao W, Bao Y, Liu Y, Zhang X, Wang J & An L, Characterization of the thermo-stable endoinulinase from a new strain *Bacillus smithii* T7, *Appl Biochem Biotechnol*, **157(3)** (2009) 498–506, DOI: 10.1007/s12010-008-8313-1.

11 Drent W J & Gottschal J C, Fermentation of inulin by a new strain of *Clostridium thermoautotrophicum* isolated from dahlia tubers, *FEMS Microbiol Lett*, **78(2–3)** (1991) 285–292, DOI:10.1111/j.1574-6968. 1991.tb04457.x

12 Olicón-Hernández D R, Guerra-Sánchez G, Porta C J, Santoyo-Tepole F, Hernández-Cortez C, Tapia-García E Y &

Chávez-Camarillo G M, Fundaments and concepts on screening of microorganisms for biotechnological applications, Mini review, *Curr Microbiol*, **79(12)** (2022) 1–8, DOI:10.1007/s00284-022-03082-2.

13 Lee H, Lee Y M, Jang Y, Lee S, Lee H, Ahn B J, Kim G H & Kim J J, Isolation and analysis of the enzymatic properties of thermophilic fungi from compost, *Mycobiology*, **42(2)** (2014) 181–184, DOI:10.5941/MYCO.2014.42.2.181

14 Martins L F, Antunes L P, Pascon R C, de Oliveira J C F, Digiampietri L A, *et al*., Metagenomic analysis of a tropical composting operation at the sa˜o paulo zoo park reveals diversity of biomass degradation functions and organisms, *PLoS One*, **8(4)** (2013) e61928, DOI: 10.1371/journal.pone.0061928.

15 Langarica-Fuentes A, Zafar U, Heyworth A, Brown T, Fox G & Robson G D, Fungal succession in an in-vessel composting system characterized using 454 pyrosequencing, *FEMS Microbiol Ecol*, **88(2)** (2014) 296–308, DOI: 10.1111/1574-6941.12293.

16 López-González J A, Suárez-Estrella F, Vargas-García M C, López M J, Jurado M M & Moreno J, Dynamics of bacterial microbiota during lignocellulosic waste composting: Studies upon its structure, functionality and biodiversity, *Bioresour Technol*, **175** (2015) 406–416, DOI: 10.1016/j.biortech.2014.10.123.

17 D'haeseleer P, Gladden J M, Allgaier M, Chain P S G, Tringe S G, Malfatti S A, Aldrich J T, Nicora C D, Robinson E W, Paša-Tolić L, Hugenholtz P, Simmons B A & Singer S W, Proteogenomic analysis of a thermophilic bacterial consortium adapted to deconstruct switchgrass, *PLoS One*, **8(7)** (2013), DOI: 10.1371/journal.pone.0068465

18 Ballhausen M, Vee J, Hundscheid M & Boer W, Methods for baiting and enriching fungus-feeding (mycophagous) rhizosphere bacteria, *Front microbiol*, **6 (**2015), DOI:10.3389/fmicb.2015.01416

19 Issa Q, AL-joburi M & Hashim A, Production of inulinase from *Rhizopus oryzae* by solid state fermentation, *J Biotechnol Res Center*, **8(4)** (2014) 46–55, DOI:10.24126/jobrc.2014.8.4.382.

20 Othman Z, Selim A, Bayoumy S & Saber W, Inulinase production from plant materials by some local yeast strains, *J Agr Chem Biotechnol*, **11(3)** (2020) 71–77, DOI;10.21608/jacb.2020.87000.

21 Knobloch S, Jóhannsson R & Marteinsson V, Co-cultivation of the marine sponge *Halichondria panicea* and its associated microorganisms, *Sci Rep*, **9(1)** (2019) DOI:10.1038/s41598-019-46904-3.

22 Macaulay B M, Boothman C, van Dongen B E & Lloyd J, A novel "microbial bait" technique for capturing Fe(III)-reducing bacteria, *Front Microbiol*, **11** March (2020) 1–15, DOI:10.3389/fmicb.2020.00330.

23 Gu J D, On enrichment culturing and transferring technique, *Appl Environ Biotechnol*, **6(1)** (2021) 1–5, DOI: 10.26789/AEB.2021.01.001.

24 Madhuri R J, Saraswathi M, Gowthami K, Bhargavi M, Divya Y & Deepika V, Recent approaches in the production of novel enzymes from environmental samples by enrichment culture and metagenomic approach, in *Recent Developments in Applied Microbiology and Biochemistry*, Elsevier Inc. (2018), DOI:10.1016/B978-0-12-816328-3.00019-2.

25 Saini A, Aggarwal N K & Yadav A, Isolation and screening of cellulose hydrolyzing bacteria from different ecological niches, *Bioeng Biosci*, **5(1)** (2017) 7–13, DOI:10.13189/bb.2017.050102.

26 Allais J J, Hoyos-Lopez G, Kammoun S & Baratti J, Isolation and characterization of thermophilic bacterial strains with inulinase activity, *Appl Environ Microbiol*, **53(5)** (1987) 942–945, DOI:10.1128/aem.53.5.942-945.1987.

27 Layh N, Hirrlinger B, Stolz A & Knackmuss H J, Enrichment strategies for nitrile-hydrolysing bacteria, *Appl Microbiol Biotechnol*, **47(6)** (1997) 668–674, DOI:10.1007/s002530050993.

28 Umesh-Kumar S, Rehana F & Nand K A, Baiting technique for the isolation of bacteria producing starch-hydrolysing enzymes, *Lett Appl Microbiol*, **8(1)** (1989) 33–35, DOI:10.1111/j.1472-765X.1989.tb00217. x.

29 Shapiro J A, The significances of bacterial colony patterns, *Bioessays*, **17(7)** (1995) 597–607.

30 Jett B D, Hatter K L, Huycke M M & Gilmore M S, Simplified agar plate method for quantifying viable bacteria, *Bio Techniques*, **23(4)** (1997) 648–650, DOI:10.2144/97234bm22.

31 Miller G L, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal Chem*, **3** (1959) 1426–1428.

32 Gavrailov S & Ivanova V, Effects of nitrogen and carbon sources on the production of inulinase from strain *Bacillus* sp. SG113, *Acta Sci Nat*, **3(1)** (2016) 69–74, DOI:10.1515/asn-2016-0010.

33 Chandna P, Nain L, Singh S & Kuhad R, Assessment of bacterial diversity during composting of agricultural byproducts, *BMC Microbiol*, **13(1)** (2013) DOI:10.1186/1471-2180-13-99.

34 Chinakwe E C, Ibekwe V I, Ofoh M C, Nwogwugwu N U, Adeleye S A, Chinakwe P O, Nwachukwu I N & Ihejirika C E, Effect of temperature changes on the bacterial and fungal succession patterns during composting of some organic wastes in greenhouse, *J Adv Microbiol*, February (2019) 1–10, DOI:10.9734/jamb/2019/v15i130075.

35 Acharya A, Joshi D, Shrestha K & Bhatta D, Isolation and screening of thermophilic cellulolytic bacteria from compost piles, *Sci World*, **10(10)** (2012) 43–46, DOI:10.3126/sw. v10i10.6861.

36 Valsange A, Evarkar S P, Tawde S V, Kareppa B M & Gujar R S, Analysis of protease activity of enzyme isolated from compost soil, *J Microbiol Biotechnol Res*, **2(6)** (2012) 1–4.

37 Ibraheem O, Screening and isolation of thermophilic fungi obtained from three selected compost waste sites, *J Microbiol Biotechnol Food Sci*, **10** (2021) 1–5, DOI:10.15414/jmbfs.3537.

38 Chamnipa N, Thanonkeo S, Klanrit P & Thanonkeo P, The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high-temperature ethanol production, *Braz J Microbiol*, **49(2)** (2018) 378–391, DOI: 10.1016/j.bjm.2017.09.002.

39 Phong H X, Klanrit P, Dung N T P, Yamada M & Thanonkeo P, Isolation and characterization of thermotolerant yeasts for the production of second-generation bioethanol, *Ann Microbiol*, **69(7)** (2019) 765–776, DOI:10.1007/s13213-019-01468-5.

40 Karakashev D, Kotay S M, Trably E & Angelidaki I A, Strict anaerobic extreme thermophilic hydrogen-producing culture enriched from digested household waste, *J Appl Microbiol*, **106(3)** (2009) 1041–1049, DOI:10.1111/j.1365-2672.2008.04071. x.

41 Roh Y, Liu S V, Li G, Huang H, Phelps T J & Zhou J, Isolation and characterization of metal-reducing *Thermoanaerobacter* strains from deep subsurface environments of the Piceance Basin, Colorado, *Appl Environ Microbiol*, **68(12)** (2002) 6013–6020, DOI:10.1128/AEM.68.12.6013-6020.2002.

42 Simpanya M F & Baxter M, Isolation of fungi from soil using the keratin-baiting technique, *Mycopathologia*, **136(2)** (1996) 85–89, DOI: 10.1007/BF00437500.

43 Ghodsalavi B, Svenningsen N B, Hao X, Olsson S, Nicolaisen M H, Al-Soud W A, Sørensen S J & Nybroe O, A novel baiting microcosm approach used to identify the bacterial community associated with *Penicillium bilaii* hyphae in soil, *PLoS One*, **12(10)** (2017) DOI: 10.1371/journal.pone.0187116.

44 Kimura M, A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences, *J Mol Evol*, **16** (1980) 111–120.

45 Kumar S, Stecher G, Li M, Knyaz C & Tamura K, MEGA X: Molecular evolutionary genetics analysis across computing platforms, *Mol Biol Evol*, **35** (2018) 1547–1549.

46 Cappuccino J G & Sherman N, *Microbiology: A Laboratory Manual*, 12th edn, Pearson Education, Hoboken (NJ), (2021).

47 Kamagata Y, Keys to cultivating uncultured microbes: Elaborate enrichment strategies and resuscitation of dormant cells, *Microbes Environ*, **30(4)** (2015) 289–290, DOI:10.1264/jsme2.ME3004rh.