



ORIGINAL ARTICLE

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Synthesis and characterization of microbial fructosyltransferase (FTase) from endophytic *Bacillus stercoris* S1 for fructooligosaccharide production

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Abstract

Purpose Presently, there is a rising awareness of the additional health benefits and market potential for functional foods. Among various functional foods short chain Fructooligosaccharides (FOS) have made its prestige in food industry. Therefore, FOS producing enzymes seems very promising as in the future, FOS markets are expected to thrive worldwide. Keeping in view the potential of microbial Fructosyltransferase in FOS production, an attempt has been made to produce FTase of microbial origin followed by its partial purification and characterization.

Methods *Stevia rebaudiana* was exploited to isolate Fructosyltransferase (FTase) enzyme producing endophytic bacteria. Preliminary screening to select FTase producers was done by Triphenyl Tetrazolium Chloride (TTC) plate assay. Fructosyltransferase producer was identified by morphological, biochemical techniques followed by 16S rRNA gene technique. Fructosyltransferase production of *B. stercoris* S1 was enhanced by optimization of inoculum size, incubation time, temperature, pH of medium, carbon source concentration by following one variable at a time method. Partial purification of FTase was achieved by ammonium sulphate precipitation at 30–60%. Partially purified enzyme was characterized for its temperature, pH and shelf stability.

Results In total 07 bacterial isolates were isolated. The bacterial isolate S1 was selected as it exhibited maximum zone of hydrolysis (22 mm) in TTC plate assay. Quantitative screening was done in terms of transfructosylating activities. Isolate S1 exhibited 50.06 U/ml. Maximum Fructosyltransferase activity 119.55 U/ml was recorded in nutrient broth supplemented with 60% sucrose at 72 h with an optimized pH of 6.0 at 40 °C. FTase titres after partial purification were 161.25 U/ml with specific activity 497.68 U/mg, purification fold and recovery percent 1.73 and 73.6% respectively. Partially purified FTase were found active in a temperature range 30°C to 80°C and in pH range of 5.0 to 9.0. FTase was found stable at -20°C for 45 days.

Conclusion The results obtained showed that the *B. stercoris* S1 represents a promising source for FTase enzyme that can be efficiently utilized for FOS production.

Keywords Fructosyltransferase, Prebiotics, Fructooligosaccharides, *Bacillus stercoris*, Characterization

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Background

Presently, there is a rising awareness of the additional health benefits and market potential for functional foods. Among various functional foods, short chain Fructooligosaccharides (FOS) are in great demand in food industry. The global fructooligosaccharides market size is projected to reach USD 20.71 Billion by 2030 growing at a CAGR of 8.8 % from 2023-2030 (Correa et al., 2024). FOS are carbohydrates that have health beneficial effects include activation of the human immune system, resistance to infection, enhanced mineral absorption in the gastrointestinal tract, synthesis of vitamin B-complex, lowering serum cholesterol etc. Furthermore, they beneficially affect the health of host by selectively stimulating the growth and/or activity of *Bifidobacterium* and *Lactobacilli* (Yadav et al. 2022).

FOS are substituent for sugar and fat in food products. Low sweetness of FOS is useful in those foods where the use of sucrose is restricted. These can also be used as fat replacers because they enhance the stability of foams, emulsions and mouthfeel by increasing unctuousness in food. As food ingredient, FOS and inulin are extensively employed in infant formula to stimulate the infant microbiota and to enhance the quality, taste and texture of these products. They are also added in dairy products, beverages, bakery products, meat products (Nobre et al. 2022; Correa et al., 2024). Fructooligosaccharides are defined as a combination of three sugar molecules: 1-kestose (GF2), nystose (GF3) and frutofuranosylnystose (GF4) where the units Fructosyltransferase (F) are combined with sucrose (GF) at the β position ($2 \rightarrow 1$) (De la Rosa et al. 2022). Fructooligosaccharides represent one of the main oligosaccharide classes with prebiotic characteristics. Prebiotics are defined as a substrate that is selectively utilized by host microorganisms conferring health benefit. FOS fulfills the criteria for prebiotic classification as these are resistance to gastric acidity to hydrolysis by mammalian enzymes, and to gastrointestinal absorption, fermented by intestinal microflora; and these selectively stimulate the growth and activity of those intestinal bacteria that contribute to health and well-being (Chen et al. 2020). FOSs are naturally found in commonly consumed fruits and vegetables (De La Rosa et al. 2022). The enzyme Fructosyltransferase (FTase) is responsible for FOS production (Pereira et al., 2025). In industries plant based and microbial based FTase are utilized for the production of FOS. The production of FOS using Fructosyltransferase (FTase) derived from microorganisms has attracted attention in last decade. Considering the current global environmental concerns the use of microbial enzymes FTases or microbial cells as whole-cell biocatalysts for FOS production is of significant interest (Dias et al., 2025). Fructosyltransferase (FTases EC 2.4.1.9.) possess transfructosylating

activity acting on the β -1-2 link of sucrose and transfer a fructose molecule to an acceptor such as other sucrose molecule leading to generation of FOS and release glucose in the reaction (Muñiz-Márquez et al. 2016). Plant Fructosyltransferases are known to have various region selectivity and each of them could produce oligosaccharides with different structure. The production of plant FTases is limited by seasonal conditions and the production yield of FOS prepared by these enzymes takes a longer production path as it acts as a series of enzymes that work together to synthesize FOS in relatively small amount. Whereas, FTases of microbial origin remain unaffected by seasonal conditions and only single enzyme produces FOS. Although, there are many constraints in the industrial production of FOS using microbial fructosyltransferase (FTase). Many natural FTase-producing microbes, such as *Aspergillus* species, secrete only small amounts of the enzyme. Insufficient enzyme production limits the economic viability of FOS production at an industrial scale. Another challenge is the sensitivity of FTase to temperature, pH shifts, and metal ions. The free enzyme denatures quickly during prolonged reactions. Many authors (Cunha et al. 2019) also reported about instability of FTase, according to them FTase in the soluble form deactivates quickly during the reaction and cannot be easily separated from the reaction medium, which limits the scaling of FOS production and increases their production cost. A further issue is its hydrolytic activity. FTase catalyzes both transfructosylation (FOS synthesis) and hydrolysis, but high hydrolytic activity reduces FOS yield (Wei Xu, et al. 2024). This undesired activity may be addressed through optimization strategies aimed at suppressing hydrolytic activity while enhancing transfructosylation. Enzyme immobilization also presents challenges, as FTase can lose activity upon immobilization. Therefore, optimization of the support material is essential and should be considered in future studies. Finally, purification and downstream processing remain major bottlenecks in the industrial-scale production of FOS. Therefore, there is a dire need to develop bioprocesses to stabilize microbial FTase for longer period. Microbial FTase are extracellular as well as intracellular. However, until now a complex information about properties and preparation of Fructosyltransferases is still missing. To address knowledge gap about FTase production objective of present study was isolation and screening of FTase producing microorganisms followed by partial purification and characterization of FTase enzyme. The present study aimed to address several fundamental constraints in fructooligosaccharide (FOS) production. A potential fructosyltransferase (FTase)-producing bacterial isolate was identified from *Stevia rebaudiana*. Optimization of the bioprocess for enzyme production using the One Variable At A Time (OVAT) approach resulted

in a significantly higher yield of FTase. The enzyme was then partially purified and characterized. It exhibited activity over a wide temperature range (30°C to 80°C) and pH range (5 to 9), and remained stable for up to 45 days at −20°C. These findings provide strong technical support for the potential application of bacterial FTase in FOS production.

Materials and methods

Isolation of fructosyl transferase producer

A fructosyltransferase-producing bacterial strain was isolated from the medicinal plant *Stevia rebaudiana* (Stevia). Isolation was performed using the spread plate method on Nutrient Agar under aerobic conditions. Distinct bacterial colonies that developed were subsequently purified by repeated streaking to obtain pure cultures. The purified isolates were preserved at −20 °C in Nutrient Broth supplemented with 30% (v/v) glycerol and stored in a deep freezer for long-term maintenance.

Preliminary screening of fructosyltransferase producing microorganisms

Preliminary screening of isolated bacterial samples were done by Triphenyl Tetrazolium Chloride (TTC) plate assay (Ojwach et al. 2020).

Streak plate method

A 24-hour-old bacterial culture was streaked onto Czapek Dox Agar (CDA) plates supplemented with 3% (w/v) sucrose as the sole carbon source. The plates were incubated at 37 °C for 48 hours. Following incubation, the plates were sprayed with 2,3,5-triphenyltetrazolium chloride (TTC) dye. A red coloration developed around the streaked areas, indicating the reduction of TTC in the presence of fructosyltransferase (FTase), thus confirming enzyme activity.

Well diffusion method

Growth of bacterial isolates

A bacterial isolate of 1.0 OD was inoculated into 50 mL of Nutrient Broth supplemented with 3% (w/v) sucrose. The cultures were incubated at 37 ± 2 °C for 48 hours in shaker incubator at 100 rpm. After incubation, the cultures were centrifuged at 10,000 rpm for 20 minutes at 4 °C. The supernatant was carefully collected into sterilized test tubes, while the cell pellets were discarded. The supernatant served as the crude enzyme extract for further analysis.

Assay

Wells of 7 mm diameter and 5 mm depth were made in Czapek Dox Agar plates supplemented with 3% (w/v) sucrose using a sterile cork borer. Each well was filled with 200 µL of the culture supernatant (crude enzyme

extract). The plates were incubated at 37 °C for 24 hours. Following incubation, the plates were sprayed with 2,3,5-triphenyltetrazolium chloride (TTC) dye. The appearance of clear reddish-colored zones around the wells indicated fructosyltransferase activity, due to the reduction of TTC. Isolates producing distinct red zones were selected based on the diameter of the hydrolysis zone.

Quantitative screening of fructosyltransferase

FTase assay

The reaction mixture contains 1 ml culture supernatant and 2 ml of 5% sucrose in citrate phosphate buffer (0.1M, pH 6.5). The reaction mixture was incubated at 50°C for 20 minutes. After incubation 1 ml of reaction mixture was added to 3 ml of DNSA reagent and tubes were boiled in water bath for 15 minutes. With the exception of the enzyme, a control experiment was conducted. When the solution was still warm, 1 ml of sodium potassium tartrate (Rochelle Salt) was added and tubes were cool to ambient temperature. The O.D. was measured at 540 nm using a spectrophotometer and compared to the reagent blank, which consisted of 1 ml of distilled water, 3 ml of DNSA reagent and 1 ml of sodium potassium tartrate (Rochelle Salt). In order to create the standard curve, a stock solution of glucose (1 mg/ml) was used. The enzyme activity was expressed in terms of U/ml and specific Activity U/mg (Ojwach et al. 2020). U/ml of enzyme activity represents µ moles of glucose released/min/ml of enzyme. Specific Activity (SA) represents µ moles of glucose released/min/mg of protein

Determination of protein concentration

Proteins were measured by using Bovine Serum Albumin, for molecular biology Nuclease and Protease Free (Hi-Media Make) as a standard protein (Lowry et al. 1951)

Statistical analysis

The data recorded on microbiological studies were statistically analysed by using MS-Excel and OPSTAT packages. The mean values of data were used for the analysis of variance (ANOVA) by using Complete Randomized Design (Sheoram et al. 1998). To improve reliability and accuracy of results three replications were done with each experiment.

Molecular identification of isolate exhibiting FTase activity

The selected isolate S1 identified at molecular level using 16S rRNA technique. The identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. On the basis of 16S rRNA gene technique S1 was identified *Bacillus stercoris*. The sequences so

obtained were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number.

Optimization of process parameters for fructosyltransferase by screened enzyme producing isolates by one variable at a time approach (OVAT)

Different growth parameters viz. inoculum size, incubation time, temperature, pH and sucrose concentration were studied for *B. stercoris*S1 to monitor their effect on FTase enzyme production.

Effect of inoculum size

Bacillus stercoris S1 was cultured separately in 50 mL of nutrient broth and incubated at $37 \pm 2^\circ\text{C}$ for 24 hours to attain substantial growth. Following incubation, 10 mL inoculum at different optical densities ($\text{OD}_{600} = 0.5, 1.0, 1.5, 2.0, \text{ and } 2.5$) was added to 250 mL Erlenmeyer flasks containing 90 mL of nutrient broth supplemented with 10% (w/v) sucrose. The flasks were incubated at 37°C with shaking at 100 rpm for 24 hours. After incubation, the culture broth from each flask was harvested by centrifugation at 10,000 rpm for 15 minutes at 4°C . The resulting supernatants were collected and stored at 4°C for subsequent analysis. Quantitative assays were performed using the collected supernatants. Fructosyltransferase (FTase) activity and total protein content were measured as previously described.

Effect of incubation time

A total of 90 mL nutrient broth was inoculated with 10 mL of *Bacillus stercoris* S1 culture ($\text{OD}_{600} = 1.0$). The flasks were incubated at 100 rpm and 37°C for varying durations: 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h. At each time point, samples were withdrawn to determine colony-forming units (CFU) and fructosyltransferase (FTase) activity. For enzyme activity analysis, culture contents were centrifuged at 10,000 rpm for 15 minutes at 4°C , and the resulting supernatants were collected and stored at 4°C until further analysis. Quantitative assays for FTase activity were performed using the supernatants as described previously.

Effect of temperature

Flasks containing *B. stercoris* S1 (10% inoculum, $\text{OD}_{600} = 1.0$) were incubated at various temperatures — 30°C , 35°C , 40°C , 45°C , 50°C , and 55°C with shaking at 100 rpm. The cultures were incubated for 72 hours under these conditions. After incubation, the culture contents were processed as described previously: centrifuged at 10,000 rpm for 15 minutes at 4°C , and the supernatants collected and stored at 4°C for further analysis. Quantitative assays for enzyme activity were conducted using the supernatants.

Effect of pH

To study the effect of pH on enzyme production, 90 mL of nutrient broth was dispensed into each of six separate flasks, and the pH was adjusted to 3, 4, 5, 6, 7, and 8, respectively, using appropriate buffers or acid/base solutions. Each flask was then inoculated with 10 mL of *B. stercoris* S1 inoculum ($\text{OD}_{600} = 1.0$) and incubated at 100 rpm for 72 hours. After incubation, cultures were centrifuged at 10,000 rpm for 15 minutes at 4°C , and the supernatants were collected and stored at 4°C . Enzyme activity assays were performed on the supernatants as described previously.

Effect of sucrose concentration

To evaluate the effect of sucrose concentration on enzyme production, nutrient broth was supplemented with varying concentrations of sucrose: 3%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, and 70% (w/v). Each medium was inoculated with 10% (v/v) of *B. stercoris* S1 inoculum ($\text{OD}_{600} = 1.0$) and incubated under previously optimized conditions. After incubation, cultures were centrifuged at 10,000 rpm for 15 minutes at 4°C , and the supernatants were collected and stored at 4°C for further analysis. Quantitative enzyme assays were performed using these supernatants, as described earlier. Kinetic parameters such as maximum reaction rate (V_{max}) and Michaelis constant (K_m), and catalytic constant (K_{cat}) were determined by plotting the lineweaverburk plot.

Partial purification of fructosyltransferase (FTase) enzyme

B. stercoris S1 having OD 1.0 was inoculated with 10% concentration in nutrient broth containing 60% sucrose, pH 6.0. Inoculated flasks were kept at 40°C , 100 rpm for 24 h and 72 h respectively. Different concentrations of ammonium sulfate i.e. 0–10%, 10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70–80%, 80–90% were evaluated to attain saturation point for FTase from *B. stercoris*S1. The preparations were kept at 4°C for overnight and centrifuged at 10,000 rpm for 20 min at 4°C that resulted in separation of pellets and supernatants. FTase was precipitated at 30–60% level of saturation of ammonium sulfate. Precipitates of each fraction so obtained were dissolved in phosphate buffer (0.1 M, pH 7.2) and were refrigerated until further use. Enzyme activity and protein concentration was measured as given above.

Characterization of partially purified FTase of *Bacillus stercoris*S1

Effect of pH on activity of partially purified FTase of *Bacillus stercoris*S1

1 ml aliquot of partially purified FTase enzyme was added to 2 ml of freshly prepared appropriate buffers of different pH in the range 4, 5, 6, 7, 8, 9 and 10 viz. citrate phosphate buffer 0.1 M for pH 4.0–6.0, phosphate buffer

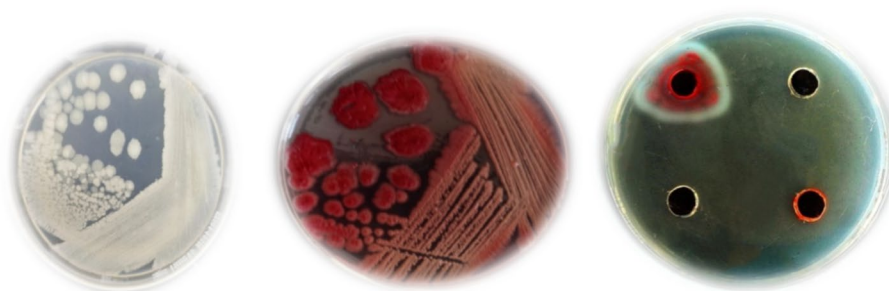


Fig. 1 Preliminary screening of FTase producing bacterial isolate S1 by triphenyl tetrazolium chloride (TTC) plate assay

0.1M for pH 7.0, Tris HCL 0.1 M for pH 8.0 and Glycine-Sodium Hydroxide buffer 0.1 M for pH 9.0–10.0.0.0. These preparations were incubated for 20 minutes at 50°C. Enzyme assay and protein concentration was measured as described above.

Effect of Temperature on activity of partially purified FTase of *Bacillus stercoris* S1

1 ml of partially purified FTase preparation was added into 2 ml of citrate phosphate buffer pH 6.5 in sterilized test tube separately. Each test tube then treated at different temperature of 30°C, 40°C, 50°C 90°C for 20 minutes. These preparations were incubated for 20 minutes at 50°C. Enzyme assay and protein concentration was measured as described above.

Shelf stability

The partially purified FTase was stored at –20°C and stability was determined at intermittent intervals viz. 15, 30, 45 days.

Fructooligosaccharide (FOS) production

FOS production was carried out using 60% sucrose w/v. Partially purified FTase 1 ml each from *B. stercoris* S1 were mixed with 9 ml of sucrose and incubated at 55 °C in an incubator for 24 h.

Analysis of reaction products

Fourier transform infra-red spectroscopy (FTIR)

End products of sucrose bio transformation were analyzed using FTIR. Samples were subjected to FTIR analysis (Shimadzu 8400S FTIR Spectrometer, equipped with KBr beam splitter) Spectrophotometer was operated at a special range of 4000–400 cm^{–1} with a maximum resolution of 0.85 cm^{–1}

Results and discussion

Isolation, screening and identification of FTase producing bacterial isolate

In total 07 isolates were isolated from *Stevia rebaudiana* which is also called as sweet leaf. Screening of FTase producing bacterial and fungal isolates have been done by

Table 1 Morphological and biochemical characteristics of FTase producer

Cell morphology	S1
Color	White
Texture	Rough
Form	Irregular
Elevation	Flat
Margin	Irregular
Gram Staining	
Gram ±	+ve
Shape	Rod
Biochemical Assay	
Catalase	+
Fermentation with glucose	+
Casein hydrolysis	+
Citrate utilization	-
Indole	-
MR-VP	+
H ₂ S production	-
Urease	-
Nitrate reductase	+
Growth conditions	Aerobic

Triphenyl Tetrazolium Chloride (TTC) plate assay. Maximum zone of hydrolysis was recorded for isolate S1 i.e. 22 mm, rest 06 isolated bacteria showed zones of hydrolysis with in 5 mm to 7 mm. Therefore, S1 was selected for further studies. The zones of hydrolysis by an enzyme in the agar plate containing Czapek dox agar with 5% (w/v) sucrose as carbon source pH 7.0 stained with TTC reagent after overnight incubation at 37°C (Figure 1). In the presence of reducing sugars, the TTC is reduced to a red water-insoluble compound, Triphenyl formazan. The appearance of zones of hydrolysis around the well and color intensity indicated positive FTase activity (Ojwach et al 2020, Gautam & Puneet, 2025). The zone of hydrolysis developed after TTC staining recorded for FTase production have been done and reported by many authors.

Selected isolate was identified on the basis of their phenotypic, biochemical characteristics. (Table 1) is depicting morphological and biochemical characteristics of S1. Further identification was done by using 16S rRNA gene technique. The identification of isolates was carried

out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune Maharashtra. Sequences of isolate S1 so obtained were submitted to NCBI database and matched with already existing sequences. Sequence similarity search was done by using Basic Local Alignment Search Tool (BLAST). Isolate S1 showed 99 % of homology with nucleotide sequence of *Bacillus stercoris* 1200 BP with accession number JCM30051. The 16S rRNA sequences of S1 have been registered to Gene bank data base with accession number OR553400. Bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. A phylogenetic tree of *Bacillus stercoris* and related species was constructed by Maximum Likelihood Estimation method as displayed in (Figure 2). This method was chosen to explore different tree topologies and branch lengths to find the one with the highest likelihood. The bootstrap values for critical nodes, including the clade containing *Bacillus stercoris* S1 and *Bacillus stercoris* JCM30051, were maximally supported 86- 92 %. So, we classify S1 as *Bacillus stercoris* S1. To best of researcher's knowledge this is the first report of FTase production by isolate *B. stercoris* S1. Although, In literature other studies pertaining to FTase production by *Bacillus* sp. viz. *Bacillus cereus*, *B. subtilis*, *B. coagulans*, *B. amyloliquifaciens* etc. have been reported by Babu et al., (2008) and Fan et al., (2021).

Quantitative assay for fructosyltransferase production

Quantitative assay for FTase production was carried out by FTase enzyme assay (Ojawach et al. 2020). The culture supernatant was taken with 5% sucrose solution as a substrate at pH 6.5 (0.1 M citrate phosphate buffer). The mixture was incubated at 50°C for 20min. DNSA was added followed by boiling. Rochelle salt was added and O.D was measured at 540 nm. The enzyme activity was expressed in terms of U/ml (μ moles of Glucose released/min/ml of enzyme). *Bacillus stercoris* S1 showed highest FTase activity i.e. 51.62 U/ml with 78.09 U/mg specific activity. Authors (Belorkar et al. 2015) measured enzyme activity quantitatively FTase activity i.e. 36.88 U/mg for *Aspergillus niger*, 21.45 U/mg for *Aspergillus flavus* and 23.78 U/mg for *Aspergillus versicolor*. EI-Beih (2009) reported (37.04 U/ml) extracellular FTase enzyme by *B. cereus* in submerged fermentation. Babu et al., (2008) reported 78.92 U mL⁻¹ FTase production by *Bacillus subtilis* NCIM 2439.

Optimization of process parameters for fructosyltransferase (FTase) by screened hyperenzyme producing isolates by one variable at a time (OVAT) approach

Effect of inoculum size

The effect of inoculum size on FTase production by the selected bacterial isolate *Bacillus stercoris* S1 was

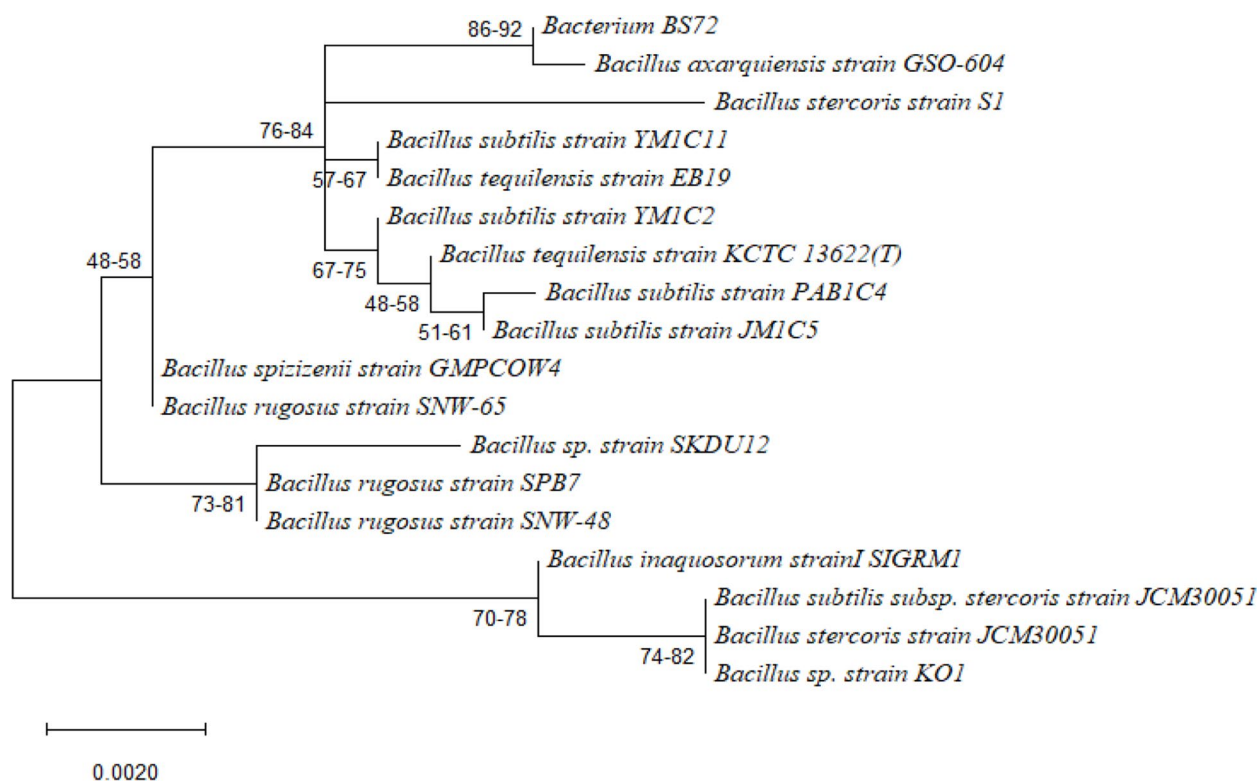


Fig. 2 Phylogenetic tree of *B. stercoris* S1 constructed by Maximum Likelihood based on 16S rRNA sequence

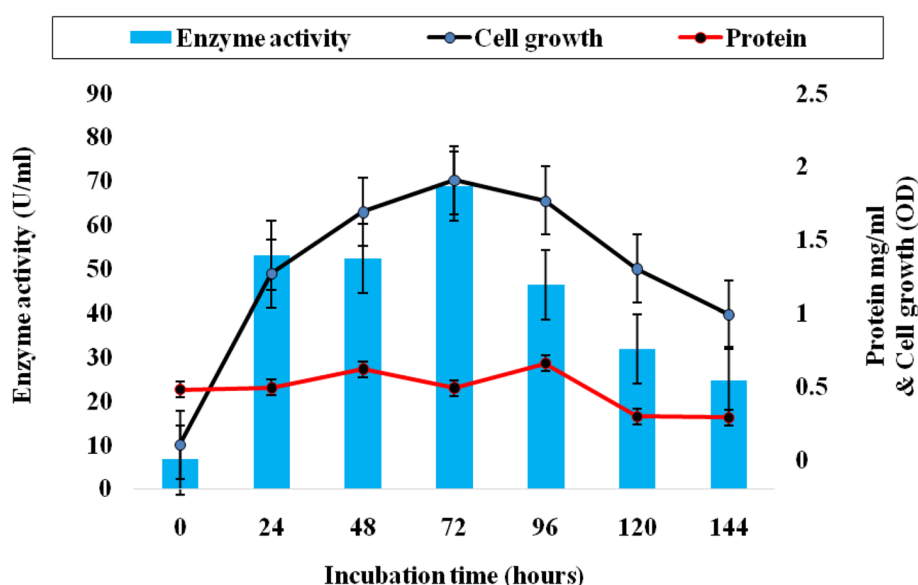
Table 2 Effect of inoculum size on FTase production by *B. stercoris* S1

Inoculum size OD _(600 nm) (10%)	Fructosyltransferase (FTase)		Protein (mg/ml)
	Enzyme activity (U/ml)*	Specific activity (U/mg)**	
0.5	45.41 ± 1.15	109.95 ± 0.57	0.413 ± 0.00
1.0	50.06 ± 0.57	71.92 ± 0.57	0.696 ± 0.05
1.5	47.63 ± 0.57	73.73 ± 0.57	0.646 ± 0.05
2.0	44.77 ± 0.57	72.09 ± 0.57	0.621 ± 0.05
2.5	42.57 ± 0.57	77.96 ± 0.57	0.546 ± 0.00
CD _{0.05}	2.30	1.81	0.14
SE (Mean)	1.6	1	0.00

Data presented along with mean (±) Standard Error

*Enzyme activity (U/ml): μmoles of Glucose released/ml of enzyme

**Specific activity: enzyme activity/mg of protein

**Fig. 3** Effect of incubation time on FTase production by *B. stercoris* S1

investigated. Various inoculum sizes, corresponding to optical densities (OD₆₀₀) of 0.5, 1.0, 1.5, 2.0, and 2.5, were tested at a fixed inoculum concentration of 10% (v/v) to evaluate their impact on enzyme activity. The results (Table 2) revealed that the highest FTase production was achieved at an inoculum size of OD₆₀₀ = 1.0, with a maximum enzyme activity of 50.06 ± 0.57 U/ml. In contrast, the lowest enzyme activity (42.57 ± 1.15 U/ml) was recorded at an inoculum size of OD₆₀₀ = 2.5. Lower inoculum size requires longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. However, excessive inoculum size can lead to increased intraspecific competition among microbial cells, resulting in nutrient depletion and subsequently reduced enzyme production. An optimal enzyme production can be achieved by balancing increasing biomass with accessible nutrients. Similar findings were reported by Ganaie et al. (2017), who evaluated the effect of inoculum size on FTase production based on spore count.

They observed that increasing the spore concentration led to enhanced FTase activity (197.11 ± 2.02 U/gds). These findings underscore the importance of optimizing inoculum size to ensure efficient fermentation and maximal FTase yield.

Effect of incubation time

The effect of incubation time on FTase production by the selected bacterial isolate *Bacillus stercoris* S1 was studied. Various incubation periods—0, 24, 48, 72, 96, 120, and 144 hours—were assessed to determine enzyme activity. Colony-forming units (CFU/ml) were measured at 24-hour intervals alongside FTase activity. The results showed that maximum FTase production (68.86 U/ml) was achieved at 72 hours of incubation, with a corresponding specific activity of 140.53 U/mg (Figure 3). In contrast, the minimum FTase activity was observed at 0 hours (6.65 U/ml), with a specific activity of 13.85 U/mg. Incubation time plays a crucial role in microbial

Table 3 Effect of temperature on FTase production by *B. stercoris* S1

Temperature degree celsius	Fructosyltransferase (FTase)		Protein (mg/ml)
	Enzyme activity (U/ml) *	Specific activity (U/mg) **	
30°C	42.51 ± 0.57	75.23 ± 1.15	0.565 ± 0.00
35°C	58.15 ± 0.57	81.55 ± 0.57	0.713 ± 0.00
40°C	73.27 ± 0.57	133.70 ± 1.15	0.548 ± 0.00
45°C	62.07 ± 0.57	105.20 ± 0.57	0.590 ± 0.00
50°C	61.49 ± 0.57	99.82 ± 0.57	0.616 ± 0.00
55°C	46.19 ± 0.57	65.89 ± 1.15	0.701 ± 0.00
CD _{0.05}	1.77	2.81	0.00
SE (Mean)	1	2.5	0.00

Data presented along with mean (±) Standard Error

*Enzyme activity (U/ml): µmoles of Glucose released/ml of enzyme

**Specific activity: enzyme activity/mg of protein

enzyme production, as it allows the microorganisms to grow, divide, and accumulate biomass, thereby enhancing enzyme synthesis (Rolfe et al. 2012). Similar trends were observed by Do Nascimento et al. (2019), who reported the time course of FTase synthesis by *Aspergillus carbonarius* PC-4 under optimized culture conditions. In that study, FTase production was carried out using pineapple crown (1.0% w/v) as a carbon source and soybean protein (0.2% w/v) as a nitrogen source (Nascimento, et al., 2019).

Effect of temperature

The effect of different incubation temperatures on the production of fructosyltransferase (FTase) by the selected bacterial isolate *Bacillus stercoris* S1 was studied. Different incubation temperature viz., 30°C, 35°C, 40°C, 45°C and 55°C were used for assessing enzyme activity. The enzyme activity data under these conditions are presented in Table 3. Maximum FTase activity for *B. stercoris* S1 was observed at 40°C, yielding 73.27 ± 0.57 U/ml with a specific activity of 133.70 U/mg. In contrast, the lowest FTase production occurred at 30°C, showing an activity of 42.51 U/ml and a specific activity of 75.23 ± 1.15 U/mg. Temperature plays a crucial role in influencing cellular metabolism and enzyme kinetics. It affects reaction rates, molecular collisions, strength of molecular interactions, and other physicochemical properties (López-Trujillo, et al., 2023). A similar study on the effect of temperature on FTase production by *Lactobacillus plantarum* was conducted by Naganandhini et al. (2014). In their study, inoculated flasks were incubated at temperatures ranging from 28°C to 37°C for 48 hours. The highest FTase activity (160.54 ± 12.6 U/ml) was recorded at 32°C.

Effect of pH

The effect of varying pH levels on FTase production by the bacterial isolate *Bacillus stercoris* S1 was evaluated using batch culture fermentation. The pH levels tested

Table 4 Effect of pH on FTase production by *B. stercoris* S1

pH	Fructosyltransferase (FTase)		Protein (mg/ml)
	Enzyme activity (U/ml) *	Specific activity (U/mg) **	
3	41.51 ± 0.57	76.16 ± 0.57	0.545 ± 0.00
4	45.41 ± 1.15	109.95 ± 0.57	0.413 ± 0.00
5	51.55 ± 0.57	93.05 ± 1.15	0.554 ± 0.00
6	77.27 ± 1.15	124.02 ± 1.15	0.623 ± 0.00
7	59.03 ± 0.57	106.93 ± 0.57	0.552 ± 0.00
8	53.48 ± 0.57	107.17 ± 0.57	0.499 ± 0.00
CD _{0.05}	2.51	2.51	0.01
SE (Mean)	2	2	0.00

Data presented along with mean (±) Standard Error

*Enzyme activity (U/ml): µmoles of Glucose released/ml of enzyme

**Specific activity: enzyme activity/mg of protein

were 3, 4, 5, 6, 7, and 8. Enzyme activity corresponding to each pH condition is presented in Table 4. Maximum FTase activity for *B. stercoris* S1 was observed at pH 6.0, with an enzyme yield of 77.27 ± 1.15 U/ml and a specific activity of 124.02 ± 1.15 U/mg. The lowest FTase production occurred at pH 3.0, where the enzyme activity was 41.51 ± 0.57 U/ml and the specific activity was 76.16 ± 0.57 U/mg. The pH of the fermentation medium significantly influences microbial growth and enzyme production, as it affects nutrient availability, enzyme stability, and metabolic pathways. Deviation from the enzyme's optimum pH could interfere with amino acid structure of an enzyme leading to a significant decrease in its activity. Several previous studies have reported similar findings. For instance, Youssef et al. (2021) observed that an initial pH of 6.0 yielded the highest levansucrase activity (39.5 U/ml) and maximum polysaccharide production (28.2 g/l). Enzyme activity declined significantly at pH values above or below this optimum. Notably, the enzyme activity remained relatively stable within the pH range of 6.0 to 8.0.

Effect of sucrose concentration and determination of kinetic parameters

The influence of varying sucrose concentrations on FTase production by *Bacillus stercoris* S1 was evaluated using batch culture fermentation. The sucrose concentrations tested were 3%, 5%, 10%, 20%, 40%, 50%, 60%, and 70%. The corresponding FTase activity at each concentration is presented in Table 5. Maximum FTase activity was observed at a sucrose concentration of 60%, yielding 115.11 ± 0.57 U/ml with a specific activity of 178.18 ± 0.57 U/mg. In contrast, the lowest enzyme production was recorded at 3% sucrose, with an activity of 36.66 ± 0.57 U/ml and a specific activity of 68.01 ± 0.57 U/mg. High sucrose concentrations have been reported in the literature to enhance transfructosylating activity, as FTases catalyze the formation of fructooligosaccharides (FOS) more efficiently under such conditions. FTases

Table 5 Effect of sucrose concentration on FTase production by *B. stercoris* S1

Sucrose Concentration %	Fructosyltransferase (FTase)		Protein (mg/ml)
	Enzyme activity (U/ml)*	Specific activity (U/mg)**	
3	36.66±0.57	68.01±0.57	0.539±0.00
5	61.30±0.57	108.11±0.57	0.567±0.00
10	75.83±0.57	199.95±0.57	0.539±0.00
20	91.66±0.57	239.32±0.57	0.383±0.00
30	95.61±0.57	221.83±0.57	0.431±0.00
40	97.33±0.57	228.47±0.57	0.426±0.00
50	104.89±0.57	232.05±0.57	0.452±0.00
60	115.11±0.57	178.18±0.57	0.646±0.00
70	111.64±0.57	248.64±0.57	0.449±0.00
CD _{0.05}	1.71	1.71	0.01
SE (Mean)	1	1	0.00

Data presented along with mean (±) Standard Error

* Enzyme activity (U/ml): µmoles of Glucose released/ml of enzyme

** Specific activity: enzyme activity/mg of protein

utilize sucrose both as a fructosyl donor and acceptor, facilitating the elongation of glycosidic chains and FOS synthesis (Rustiguel et al., 2021). The enzyme cleaves the β-1,2 glycosidic linkage of sucrose and transfers the fructosyl moiety, resulting in the production of FOS. Therefore, sucrose concentration is a critical factor in optimizing FTase yield for industrial applications.

Kinetic parameters

The enzyme kinetic data was analyzed using both non-linear regression of the Michalis-Menten model and Lineweaver-Burk double reciprocal plot. The Lineweaver-Burk plot, which represents a linearized form of the Michalis-Menten equation, produced a straight-line relationship between $1/v$ and $1/[S]$ Figure 4a. The intercepts of this line were used to estimate the kinetic parameters, resulting in $V_{max} = 118.8$ U/ml and $K_m = 56.4$ mg/ml. In contrast, the nonlinear curve fitting in MATLAB provided a smooth fit to the experimental substrate-velocity data Figure 4b. Xu et al (2015) obtained values of K_m and V_{max} equal to 56.05 g L⁻¹ and 800.1 U mg⁻¹ for sucrose as substrate, respectively, for the purified FTase of *Penicilliumoxalicum*.

The statistical analysis of the study

Analysis was performed using analysis of variance (ANOVA) to evaluate the significance of differences among various inoculum sizes, incubation times, temperatures, pH levels, sucrose concentrations on FTase production by *B. stercoris* S1. Each experiment was conducted in triplicate (three replications per treatment) to ensure reliability and statistical robustness (Table 6).

Partial purification of fructosyltransferase enzyme from *B. stercoris* S1

Maximum FTase activity was observed with 30–60% concentration of ammonium sulphate. The enzyme activity of partially purified FTase was 161.25 U/ml with specific activity of 497.68 U/mg, Purification fold was 1.73 with recovery 73.86 % (Table 7). The results are in close conformity with FTase from *A. oryzae* S719 where partial purification of enzyme was achieved at 95% of ammonium sulphate precipitation and enzyme activity was 310000 U with specific activity 156, purification fold 2.4 and recovery % was 85% (Han et al. 2020). Ammonium sulfate is a perfect salt for the fractionation of enzymes owing to its high solubility, low toxicity and lesser cost and preservative action. As the saturation of the salt increases, different enzyme proteins precipitate from solution and can be recovered.

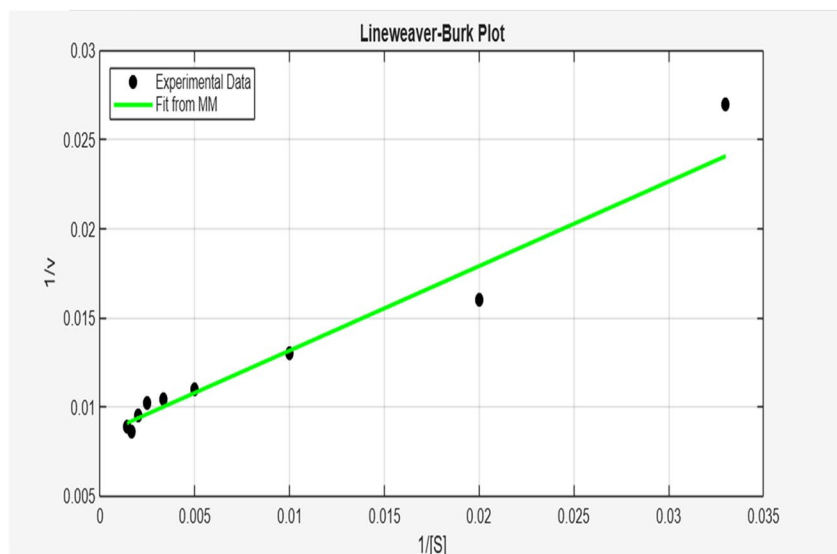
Characterization of partially purified fructosyltransferase (FTase) enzyme

Effect of temperature

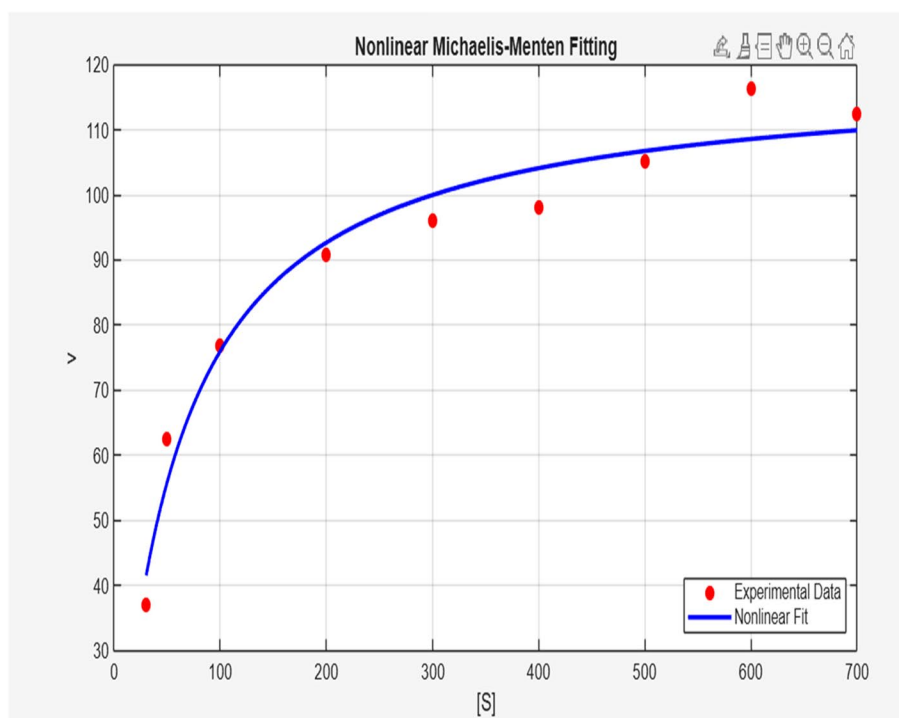
The effect of temperatures regime on the enzyme activity of partially purified FTase has been presented in Figure 5 ranging from 30°C, 40°C90°C for 20 min. It was found that the enzyme exhibited maximum activity at 50°C for *B. stercoris* S1 i.e. 161.25 U/ml. The specific activity at 50°C for *B. stercoris* S1 is 497.68 U/mg. Minimum enzyme activity for *B. stercoris* S1 was 39.71 U/ml with specific activity 136.46 U/mg at temperature 80°C. As the temperature rises from 30 to 50°C enzyme activity increases as molecules move faster, leading to more frequent and energetic collisions. When the temperature increased above 50°C the activity of the enzyme was affected negatively and gradually reduced. As the denaturation of the enzymatic protein occurs at elevated temperatures therefore, after certain level of temperature above 50°C, the enzyme activity decreased rapidly. In nutshell, the enzyme was most efficient at 50°C as compared with other tested temperatures. Purified FTase showed thermal stability over a wide range of temperature 40 °C and 60 °C (Ojawach et al.2020).

Effect of pH on activity of partially purified FTase from *Bacillus stercoris* S1

Figure 6 exhibits the impact of varying pH on the partially purified FTase activity with pH values ranging from (4, 5, 7...9). It was observed that pH variations had a significant impact on the enzyme's activity. Within the range of 6.0 to 8.0, the enzyme showed considerable activity. The highest enzyme activity was found at pH 7.0 161.25 U/ml *B. stercoris* S1 with specific activity 462.03 U/mg. The minimum enzyme activity was obtained at pH 4.0 i.e. 1.06 U/ml for FTase from *B. stercoris* S1 with specific activity 4.90 U/mg. The enzyme activity declined when



(a)



(b)

Fig. 4 **a** Lineweaver–burk (double reciprocal) plot for the determination for sucrose hydrolysis by FTase of *B. stercoris* S1, generated in MATLAB. **b** Nonlinear regression of experimental data to the michaelis–menten model, generated using MATLAB

the pH of enzyme medium altered on either side of optimum range from acidic to alkaline, a decline in enzyme activity was observed. The majority of enzyme exhibit activity at limited pH range and if this pH is exceeded or lowered, the enzyme activity decreases. At extreme pH levels the enzyme's active site can change its shape. This change prevents the formation of enzyme-substrate complex and eliminating enzyme activity. Suboptimal

pH levels can lead to change in tertiary structure which causes the inactivity of enzyme. Similar reports have been reported in literature by many authors. Partially purified FTase from *A. niger* showed optimum activity at pH 6.0 (Ojawach et al. 2020). The relative activity declined below pH 4.0 and above pH 7.5 (Silva et al. 2021).

Table 6 Statistical significance of factors affecting FTase production by *B. stercoris* S1

Factor	Significant difference observed ($p \leq 0.05$)	Most effective condition	CD _{0.05}
Inoculum Size	Yes	1.0 OD(50.06 U/ml)	2.30
Incubation Time	Yes	72 h (68.86 U/ml)	1.62
Temperature	Yes	40°C (73.27 U/ml)	1.77
pH	Yes	6.0 (77.27 U/ml)	2.51
Sucrose Concentration	Yes	60% (115.11 U/ml)	1.71

Storage stability assays of partially purified FTase from *Bacillus stercoris* S1

The partially purified FTase was stored in 0.1 M phosphate buffer pH 7.2 at -20°C in deep freezer. Enzyme was stored in the absence of substrate. The enzyme activity was checked at intervals after a time duration of i.e. 15, 30, 45 days. The enzyme was stable upto 45 days. The enzyme activity for *B. stercoris* S1 on 15, 30 and 45 was 161.07, 160.98 and 160.62 U/ml with the specific activity 508.10, 507.82 and 508.29 U/mg respectively as shown in Table 8. The shelf stability of enzyme is an important character for enzyme activity. Enzymes have limited stability during long term storage. This is due to the

deleterious effects of the environmental moisture and microbial contamination. Also, due to a variety of intramolecular and intermolecular chemical reactions including hydrolysis, aggregation, deamidation, oxidation, β -elimination, and changes in conformation enzymes in aqueous solutions are inherently unstable. This may result in a loss of its biological activity Fernandes ((Fernandes, and Fernandes, 2010)).

Analysis of FTase and sucrose reaction products by fourier transform infra-red spectroscopy (FTIR)

FTIR spectra was used to identify preliminary structures in the functional groups of oligosaccharides. The functional group of oligosaccharides based on peak vibration values in the region of infra red radiations. The FTIR spectra demonstrated that the graphs of oligosaccharides were consistent with typical carbohydrate vibrational bands. Data pertaining to FTIR analysis has been depicted in Table 9 and Figure 7. The FTIR spectra of the sample was recorded in the range of 3559 to 689 cm^{-1} and the peaks were compared. Starting from 3300 – 3200 cm^{-1} , bands have shown the presence of intermolecular bonded O-H stretching, which indicates the presence of hydroxyl groups, carboxylic acids and water. Sample exhibit visible peaks at 3300 – 3200 cm^{-1} , which indicates

Table 7 Partial Purification of FTase from *B. stercoris* S1

Steps	Volume (ml)	FTase Enzyme activity [#] (U/ml)	Total activity	Specific activity ^{**} (U/mg)	Protein (mg/ml) [*]	Purification fold ^{***}	Recovery ^{****} (%)
Crude culture supernatant	200	119.11	23,822	287.01	0.415	1	100
Ammonium sulphate precipitation (30–60%)	15	161.25	2418	497.68	0.324	1.73	73.86

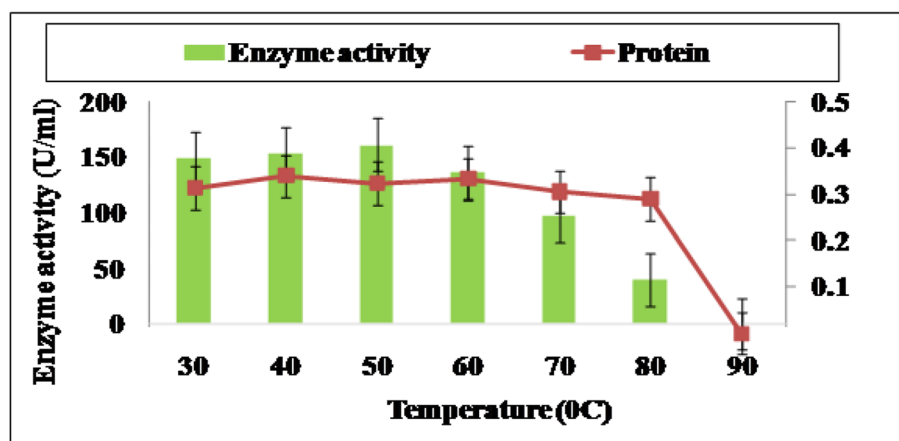
[#]Enzyme activity (U/ml): μmoles of Glucose released/ml of enzyme

^{*}Protein concentration was determined by Lowry's method

^{**}Specific activity is the activity unit/protein

^{***}Purification fold is increase in specific activity

^{****}Recovery % is remaining protein concentration as % of the initial protein

**Fig. 5** Effect of temperature on activity of partially purified FTase from *B. stercoris* S1

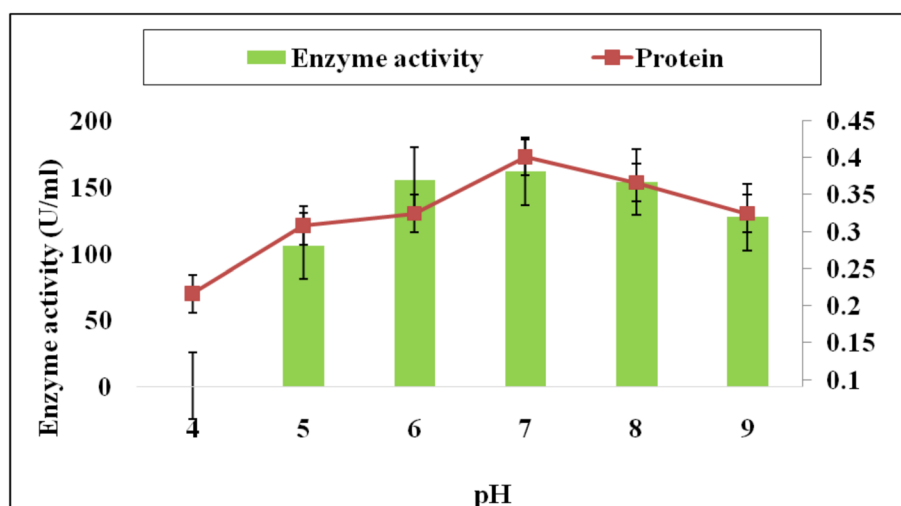


Fig. 6 Effect of pH on activity of partially purified FTase from *B. stercoris* S1

Table 8 Shelf stability of partially purified FTase from *B. stercoris* S1

Days	<i>Bacillus stercoris</i> S1		
	Enzyme activity (U/ml)*	Specific activity (U/mg)**	Protein concentration (mg/ml)***
15	161.07 ± 0.57	508.10 ± 0.57	0.317 ± 0.00
30	160.98 ± 0.57	507.82 ± 0.59	0.317 ± 0.00
45	160.62 ± 0.57	508.29 ± 0.57	0.316 ± 0.00
CD _{0.05}	1	1	1
SE (Mean)	1	1	0.00

Data presented along with mean (±) Standard Error

*Enzyme activity (U/ml): μmoles of Glucose released/ml of enzyme

**Specific activity is the activity unit/protein

***Protein concentration was determined by Lowry's method

Table 9 Analysis of FTase and sucrose reaction products by fourier transform infra-red spectroscopy

Wave numbers	S1	Functional group	Compound
829	0.12	=C-H out-of-plane	Alkene
900–100	0.14	bending	
1049–1043	0.25	C-O bending	Hydroxyl
1140–1090	0.13	C-O stretching	Phenols
1600–1300	0.15	Coupled stretching and bending	Carbohydrates
1670–1600	-	NH ₂ bending and C=O stretching	Primary amide and Secondary amide
3300–3200	0.17	O-H Stretching	Carboxylic acids, Water

the presence of hydroxyl O-H stretching. The spectrum between 1600 and 1670 cm⁻¹ represents NH₂ bending and C=O stretching, which shows the presence of primary, secondary amides and carbohydrates indicated the presence of binding peptides in these oligosaccharides (Yang et al. 2020). The absorption between 1300 and 1600

cm⁻¹ depicts the coupled stretching and bending of carbohydrates. The absorption between 1090–1140 indicates sucrose consumption and glycosidic bond formation due to transfructosylation due to FTase action. This region is called fingerprint region of sugars (Santos et al. 2014). The wavelength at 650–1000 represents =C-H out-of-plane bending and depicts the presence of alkene compound. Similar observations regarding FTIR spectra for FOS generation by FTase have been reported by many researchers in literature. In one of the report (Choukade, and Kango, 2019), transformed sucrose to FOS by using FTase from *Aspergillus tamari* NKRC 1229. Authors characterized FOS using FTIR. They revealed that evolution of peaks at 996, 1025 and 1122 cm⁻¹ during 1–24 h of FTase action indicated sucrose consumption and glycosidic bond formation due to transfructosylation.

Conclusion

In the present investigation an attempt has been made to isolate most efficient FTase producing potential bacteria from stevia its screening, identification, optimization to enhance the maximum enzyme production, partial purification followed by its characterization. The present study thus evaluates the feasibility of using partially purified extracellular FTase of bacterial origin in the enzymatic synthesis of biofunctional fructooligosaccharides due to its stability, high activity at varied temperature and pH range. The enzyme FTase is partially purified in this study that can be further purified and immobilized for improved FOS generation and can be extensively useful in food industries because of their functional properties. In this study sucrose has been utilized for FTase and FOS production which can be replaced by low cost substrates viz., agriculture/horticulture waste. By utilizing these waste to produce microbial based FTase enzymes would be a cost-effective, and scalable method for producing

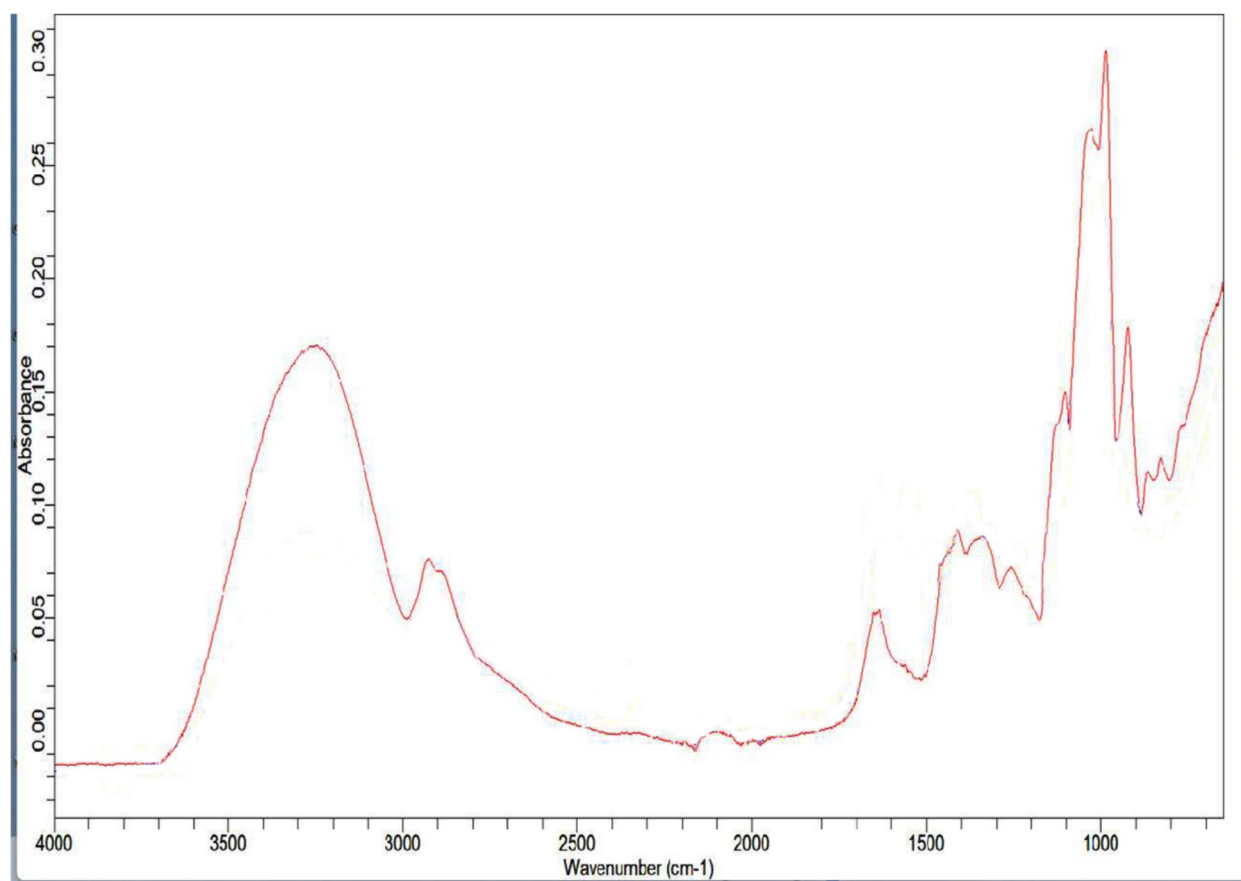


Fig. 7 Analysis of FTase and sucrose reaction products by fourier transform infra-red spectroscopy

health-promoting prebiotic FOS. This strategy supports waste valorization and aligns with circular economy principles. Future work will focus on the ongoing optimization of purification protocols and the detailed characterization of Fructooligosaccharides (FOS) using high-performance liquid chromatography (HPLC). These experiments aim to refine separation efficiency, improve detection sensitivity, and establish a more comprehensive profile of the purified FOS fractions, contributing to a deeper understanding of their structural and functional properties.

Authors' contributions

Conceptualization: Dr Neha Gautam; Methodology: Dr Neha Gautam; Formal analysis and investigation: Puneet Kumar; Dr Neha Gautam, Vikas Kumar and Shruti Gupta; Writing original draft preparation Dr Neha Gautam and Stuti Sharma; supervision: Dr Neha Gautam.

Funding

No funding.

Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Consent for publication

All of the authors consent to the publication of this manuscript in *Annals of Microbiology*.

Competing interests

The authors declare no competing interests in publishing this manuscript.

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Received: 13 January 2025 / Accepted: 4 November 2025

Published online: 28 November 2025

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