Production and Statistical Optimization of Invertase-Free Exoinulinase from *Glutamicibacter arilaitensis* using Goat Dung as Ideal Feedstock

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Inulinase is an inulin degrading enzyme that exhibits versatility in disparate bioresource and bioprocess industries. In this study, invertase-free exoinulinase was initially produced from *Glutamicibacter arilaitensis* strain ALA4 using diversified inexpensive substrates under solid state fermentation. Strain ALA4 revealed maximum production of inulinase using goat dung as quintessential feedstock. Inulinase activity of strain ALA4 was further optimized by one-factor-at-a-time method, followed by response surface methodology, which showed enhanced inulinase activity of 4678.34±34.67 U/g at 96 h using goat dung medium of pH 8.0 with 100% of moisture content. Furthermore, crude inulinase was not only thermo-alkali stable but also exhibited tolerance towards varied metal ions, organic solvents, surfactants, and inhibitors with satisfactory residual activities. Additionally, fructose produced due to the hydrolysis of inulin present in goat dung was analyzed by osazone and HPTLC tests which further confirmed exoinulinase nature of enzyme. In a nutshell, the study evidenced the first report on invertase-free exoinulinase production from *G. arilaitensis* using goat dung as proficient feedstock and demonstrated its quiescent applications in bioprocessing industries in future.

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INTRODUCTION

Inulinase has a wide range of applications not only in the preparation of high fructose syrup and fructooligosaccharides but also the production of disparate indispensable metabolites such as ethanol, lactic acid, sorbitol, 2,3-butanediol, and citric acid (Neeraj *et al*. 2017). It is an industrially important versatile enzyme, which is known to produce inulo-oligosaccharides, fructose, and glucose as decisive products by acting on β-2,1 linkages of inulin (Rawat *et al.* 2016). Based on the biocatalytic mechanism of action, exoinulinases (EC 3.2.1.8; β-2-1-D-fructan fructohydrolase) are in one of the most substantial categories of inulinase; they are known to remove the terminal fructose residues from the non-reducing end of inulin (Singh and Chauhan 2017; Khosravi *et al*. 2023). In general, inulin is a foremost storage carbohydrate after starch in 36000 species of plants (Chi *et al.* 2011). It is a polyfructan linked with β-2-1 fructofuranosidic linkages, which is used as a carbon source as well as an inducer for exoinulinase production (Singh *et al.* 2016; Riva *et al*. 2023).

Diverse groups of plants, animals, fungi, and bacteria are considered as conspicuous sources of exoinulinase (Rawat *et al*. 2016). Interestingly, bacteria are preferred over plant, animal, and fungal sources as promising producers of exoinulinase due to their high productivity and easy cultivation (Singh *et al.* 2016). So far, inulinases have been successfully characterized from *Bacillus* spp., *Marinimicrobium* spp., *Pseudomonas* spp., *Arthrobacter* spp., *Streptococcus* spp., *Staphylococcus* spp., *Clostridium* spp., *Sphingobacterium* spp., *Sphingomonas* spp., and *Streptomyces* spp. with moderate yield (Aruna and Hati 2014; Ramapriya *et al*. 2018; Elsoud *et al*. 2023). Though this enzyme has been extensively studied in bacteria, relatively few investigations have been reported on invertase-free exoinulinase production. It should be noteworthy that similar to inulinase, invertase (EC 3.2.1.26; β-D-fructofuranoside fructohydrolase) belongs to glycoside hydrolases-32 family which catalyzes the hydrolysis of carbohydrates into D-glucose and D-fructose (Lincoln and More 2017), which can certainly mimic the inulinase activity of bacteria. Hence, it is necessary to identify invertase-free exoinulinase producing new bacterial strain for its exemplary applications in bioprocess industries.

Hyper production of enzymes from bacterial strains depends on the medium's complexity, particularly the substrate used (Khusro and Aarti 2015). Substrates affect the metabolism of the strain, which improve the biosynthesis of desirable products, followed by the production of enzymes (Khusro *et al*. 2017). Inulin/inulin-rich plant extracts, sucrose, and starch have been used as sole carbon source for exoinulinase production from bacteria (Singh *et al*. 2017). Unfortunately, these substrates are costly, making the downstream process expensive, and thus an inexpensive substrate is needed. Recently, goat dung has shown potential in the production of extracellular enzymes from bacteria under solid state fermentation (SSF) (Aarti *et al*. 2017, 2018). Goat dung contains distinct nutrients such as carbohydrates, crude proteins, ash, carbon, nitrogen, phosphorus, potassium, calcium, magnesium, manganese, iron, copper, and zinc (Mnkeni and Austin 2009), thereby showing its integrity as propitious substrate for bacterial growth.

This study investigated invertase-free exoinulinase production from *Glutamicibacter arilaitensis* using goat dung as ideal no cost feedstock and then evaluated the stability of crude exoinulinase at varied conditions for industrial applications.

EXPERIMENTAL

Chemicals, Reagents, and Solvents

All chemicals, reagents, and solvents used in this investigation were obtained from HiMedia, India and stored at specific temperature for further experimental analyses.

Bacterium of Interest and Growth Conditions

Glutamicibacter arilaitensis strain ALA4, isolated previously from soil sample (Aarti *et al*. 2017), was used in this study. Unless otherwise mentioned, strain ALA4 was cultured at 37 °C for 24 h in 250 mL Erlenmeyer flasks containing 50 mL of basal broth (% w/v: peptone 1.0; yeast extract 0.5; KH_2PO_4 0.1; $MgSO_4.7H_2O$ 0.02; Na_2CO_3 1.0; NaCl 0.3; and pH 7.0) medium for varied experimental purposes.

Inulinase Production (Plate Assay)

The extracellular inulinase or exoinulinase secretion from strain ALA4 was determined as per the modified protocol of Li *et al*. (2011). Overnight grown strain ALA4

was inoculated into freshly prepared and sterilized basal broth medium constituting 1% (w/v) inulin, and incubated at 37 °C. After 24 h, the culture was centrifuged at 8000 *g* for 10 min, and the supernatant (crude inulinase) was collected. Inulin agar medium (%w/v: inulin 1.0; agar 1.8; and pH 7.0) was prepared freshly and autoclaved. The inulin agar medium was poured aseptically into the sterile petriplate and allowed to cool for the agar well diffusion assay. One hundred microlitres of culture supernatant were transferred into the well created on inulin agar medium and plates were incubated at 37 °C for 24 h. Plates were then flooded with Lugol's iodine solution [1.5% (w/v) potassium iodide and 1% (w/v) iodine] and observed for a clear zone of hydrolysis. The appearance of clear zone around the wells indicated the production of exoinulinase from the strain.

Inulinase Assay (Quantitative Analysis)

Inulinase activity of strain ALA4 was determined as per the modified protocol of Miller (1959). The crude inulinase (1 mL) was added into 1 mL of 1% (w/v) inulin and incubated at 60 °C for 10 min. One millilitre of dinitrosalicylic acid (DNS) reagent was mixed into the solution for stopping the reaction. The solution was further incubated in boiling water bath for 5 min. The solution was allowed to cool, and 3 mL of sterile distilled water was added into it. The solution was centrifuged at 6000 *g* for 10 min, and the supernatant was used for reading absorbance at 540 nm using Ultraviolet-visible (UV-Vis) spectrophotometer. Fructose solution was used as standard. One unit of inulinase activity was calculated as the quantity of enzyme released 1 µmol of fructose per minute from inulin under standard conditions. Total protein content was assessed using the Bradford method (Bradford 1976).

Invertase Assay (Quantitative Assay)

Invertase production from strain ALA4 was quantified as per the method of Singh and Chauhan (2017) with slight modifications. Strain ALA4 was grown in the medium constituting 1% (w/v) sucrose and incubated at 37 °C for 24 h. After a required period of incubation, the culture was centrifuged at 8000 *g* for 10 min, and the supernatant was collected. The supernatant (1 mL) was added into 1 mL of 1% (w/v) sucrose solution and incubated at 60 °C for 10 min. The reaction was inactivated by adding 1 mL of DNS reagent. The solution was further incubated in boiling water bath for 5 min, allowed to cool, and added into 3 mL of sterile distilled water. The solution was centrifuged at 6000 *g* for 10 min and the supernatant was used for reading absorbance at 540 nm using UV-Vis spectrophotometer. Mixture of glucose and fructose solution was used as standard. One unit of invertase activity was determined as the amount of enzyme released 1 µmol of reducing sugars per minute from sucrose under standard conditions.

Solid State Fermentation

Feedstocks used

Banana peel, wheat flour, rice flour, wheat bran, corn cob, rice bran, goat dung, and sugarcane bagasse were used as feedstocks for SSF. Feedstocks other than goat dung were obtained from the local market, dried at room temperature for 7 to 10 days, and powdered using grinder. Goat dung was collected locally in the morning, washed with water, dried completely for few days, and then powdered. All the powdered feedstocks were stored at room temperature for further processing.

Solid state fermentation and inulinase assay

Approximately 25 g of powdered feedstock were weighed and initially sterilized by dry heat sterilization, followed by UV-irradiation in laminar air flow. The moisture level (100% w/v) of sterilized feedstocks was adjusted using 0.1 M Tris–HCl buffer (pH 8.0). Feedstock was autoclaved and after cooling, bacterial inoculum was inoculated and incubated at 37 °C for 48 h under static conditions. The obtained product was further mixed with 50 mL of sterile distilled water, and the enzyme was extracted by shaking the content for 30 min at 150 rpm. The mixture was filtered, centrifuged at 8000 *g* for 15 min, and the collected supernatant was used as enzymatic source. Inulinase activity of strain ALA4 in the presence of disparate feedstock was quantified according to the method discussed earlier in this context.

Factors Screening Using One-Factor-at-a-time (OFAT) Method

The OFAT-based method was initially implemented for screening the effect of diversified variables on inulinase production from the strain using goat dung as proficient substrate. About 25 g of powdered goat dung were added in 20 mL of Tris-HCl buffer (pH 8.0; 0.1M). Diversified non-nutritional and nutritional parameters such as incubation period (12 to 96 h), pH (6.0 to 11.0), temperature (32 to 60 °C), inocula volume (7.31 to 54.82 log cfu/mL), moisture content (60 to 120%), carbon sources (1% w/w - glucose, sucrose, maltose, inulin, xylose, mannose, and lactose), and nitrogen sources (0.5% w/w - peptone, casein, yeast extract, beef extract, tryptone, potassium nitrate, ammonium sulphate, and ammonium chloride) were optimized for estimating inulinase yield. The SSF and inulinase activity were determined as mentioned before in this study.

Two Level Full Factorial Matrix

Considering the OFAT-based optimization, five factors affecting significantly ($P<0.05$) inulinase production were further optimized using 2^5 full factorial design. Variables were used at two levels- high (+) and low (-), and a total of 32 experiments were performed to estimate inulinase activity (Y) using goat dung as substrate. Analysis of variance (ANOVA) depicted the statistically significant ($P<0.05$) parameters. The impact of all the selected parameters on the response value was analyzed using first-order polynomial model equation as mentioned below,

$$Y = \alpha_0 + \sum_{i}\alpha_i x_i + \sum_{ij}\alpha_{ij} x_i x_j + \sum_{ijk}\alpha_{ijk} x_i x_j x_k + \sum_{ijkl}\alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm}\alpha_{ijklm} x_i x_j x_k x_l x_m \quad (1)$$

where Y is the response (inulinase activity), α_0 is the intercept, α_i is the i^{th} linear coefficient, and α_{ij}, α_{ijk}, $\alpha_{ijkl,}$ and α_{ijklm} are the interaction coefficients. The goodness of fit for the first-order polynomial model was determined by coefficient of determination (R^2). The desirability was set at maximum and F test was recorded to observe the significance level.

Central Composite Design (CCD) and Experimental Validation

The physical factors and nutritional components that significantly ($P<0.05$) affected the inulinase activity after two-level factorial design were further optimized using CCD of response surface methodology (RSM). Each variable in the design matrix was analyzed at five different levels ($-\alpha$, -1, 0, +1, and $+\alpha$) involving 6 central points with an alpha (α) value being ±2. The total number of experimental combinations was $2^k + 2k + n$, where 'k' is the number of variables and 'n' is the number of repetition of experiments at the central point. The experimental design consisted of 20 runs of three factors for improving the inulinase

activity (*Y*). All parameters were maintained at a central coded value of zero. The potency of selected factors to the response was estimated using a second-order polynomial equation,

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (2)$$

where *Y* is dependent variable (inulinase activity), β_0 is intercept, β_1, β_2, and β_3 are linear coefficients, β_{11}, β_{22}, and β_{33} are squared coefficients, β_{12}, β_{13}, and β_{23} are interaction coefficients, and A, B, C, A^2, B^2, C^2, AB, AC, and BC are variables levels. The R^2 value revealed the accuracy of polynomial model. The interdependent interaction between two parameters towards inulinase activity was observed using three-dimensional (3D) response plots. The authenticity of the design to enhance the inulinase production was carried out considering optimum ranges of variables selected from CCD. Inulinase activity was estimated as per the method discussed before in this investigation and compared with the predicted response value.

Characterization of Inulinase

Effect of pH and temperature

For evaluating the influence of various ranges of pH on the stability of inulinase, the crude inulinase (100 µL) was mixed with 900 µL of sodium phosphate (0.1M; pH 6.0 to 7.0), Tris-HCl (0.1M; pH 8.0 to 9.0), carbonate-bicarbonate (0.1M; pH 10.0), and glycine-NaOH (0.1M; pH 11.0) buffer solutions. The residual activities of inulinase were determined up to 4 h of incubation as per the methodology described earlier. The thermal stability of inulinase was estimated by incubating the enzyme at varied temperature (35 to 85 °C) for 4 h (Aarti *et al*. 2017). The residual activities of inulinase were calculated according to the methodology discussed earlier in this investigation.

Effect of metal ions

The residual activities of inulinase in the presence of varied divalent metallic ions (10 mM: Mg^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Co^{2+}) were determined as per the method of Aarti *et al*. (2018).

Effect of organic solvents

The residual activities of inulinase in the presence of different organic solvents (40% v/v: *n*-hexane, *n*-butanol, benzene, toluene, ethanol, methanol, ethyl acetate, chloroform, acetone, and propanol) were estimated as per the methodology of Aarti *et al*. (2018).

Effect of surfactants and inhibitors

The enzyme solution was mixed with various surfactants [1% (v/v); Tween 20, Tween 40, Tween 60, Tween 80, and SDS] and inhibitors (1mM; EDTA, hydrogen peroxide, and mercaptoethanol). Residual activities of inulinase were estimated against the control as discussed before in this context.

Analytical Assay for Fructose Formation

The formation of fructose in the hydrolyzed product of goat dung by the catalytic action of exoinulinase was confirmed by two tests as follows:

Osazone test

The osazone test was performed according to the methodology of Hassid and McCready (1942) in order to confirm the presence of reducing sugar, particularly fructose in the hydrolyzed product of goat dung. The hydrolyzed product (3 mL) was added into the mixture of glacial acetic acid (1 mL), sodium acetate (0.1 g), and phenylhydrazine hydrochloride (0.5 g). The mixture was boiled in water bath and allowed to cool. The crystal formed was spread as smear on clean glass slide and observed under light microscope. Needle-shaped yellow feathery osazone crystals formation within 2 to 3 min indicated the presence of fructose in the fermented broth. Standard fructose was used as control for osazone test.

High performance thin layer chromatography (HPTLC)

The hydrolyzed product formation was confirmed through HPTLC too using chloroform: acetic acid: water (30: 35: 5) as solvent system (Kamble *et al.* 2018). The plate was loaded with 2 µL of samples (hydrolyzed product), glucose (1% w/v), sucrose (1% w/v), and fructose (1% w/v). The developed plate was dried, scanned at 254 nm, and then 3D chromatogram was interpreted.

Software Used and Statistical Analyses

Design Expert Version 11.0.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) was used for statistical optimization. All the experiments were carried out in triplicate and data were represented as mean±standard deviation (mean±SD). ANOVA was implemented to analyse the significance ($P \leq 0.05$) level.

RESULTS AND DISCUSSION

Inulinase Assay

Strain ALA4 revealed the production of extracellular inulinase by showing 28.3±0.6 mm of zone of hydrolysis on inulin agar medium plate (Fig. 1). The total inulinase activity was estimated as 985.32±32.3 U/g using inulin as carbon source. Interestingly, the strain showed a lack of invertase activity, indicating the production of invertase-free exo-inulinase from the isolate.

Diverse groups of bacteria *viz*. *Streptococcus* spp. (Takahashi *et al.* 1985), *Clostridium* spp. (Drent and Gottschal 1991), *Xanthomonas* spp. (Naidoo *et al.* 2009), *Achromobacter* spp. (Angel *et al.* 2012), *Nocardiopsis* spp. (Lu *et al.* 2014), *Bacillus* spp. (Kwon *et al*. 2003; Meenakshi *et al.* 2013; Ramapriya *et al*. 2018), and *Streptomyces* sp. (Beroigui *et al*. 2023) have been identified as efficient inulinase producers in the past. However, the isolation of invertase-free exoinulinase-producing bacteria from natural resources is very limited and requires comprehensive investigation.

Previously, Ghadbaan and Aldeen (2015) demonstrated invertase-free exoinulinase production from rhizosphere soil-associated *Bacillus* sp. In the present investigation, *G. arilaitensis* strain ALA4 (a soil isolate) showed preponderant production of invertase-free exoinulinase. In general, plethoras of microbes have the potentiality to catalyze sucrose by secreting invertase and produce D-glucose and D-fructose as end products, similar to exoinulinase acting on inulin (Lincoln and More 2017). The inulinase (I) / invertase (S) ratio determines the inulinase or invertase nature of enzyme produced. I/S ratio $>10^{-2}$ indicates an inulinase nature of the enzyme, while the ratio $<10^{-4}$ represents an invertase

nature of the enzyme produced (Ettalibi and Baratti 1987). The present context showed a lack of invertase production from strain ALA4, thereby revealing this as the first study on invertase-free exoinulinase production from *G. arilaitensis.*

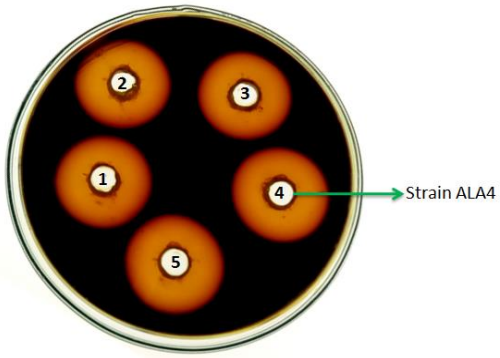


Fig. 1. Exoinulinase production from strain ALA4, showing zone of hydrolysis on inulin agar medium

Inulinase Production Using Disparate Feedstocks

Inulinase production from strain ALA4 using diversified feedstock is shown in Fig. 2. The strain depicted a maximum inulinase activity of 1685.32±31.6 U/g using goat dung as ideal substrate. The production of inulinase using other substrates was estimated comparatively lower than that of goat dung in the order of wheat flour (985.32±27.5 U/g) > wheat bran (877.31±32.5 U/g) > rice bran (818.32±28.4 U/g) > banana peel (680.32±26.6 U/g) > sugarcane bagasse (632.66±32.6 U/g) > corn cob (540.32±31.3 U/g) > rice flour (476.34±25.4 U/g).

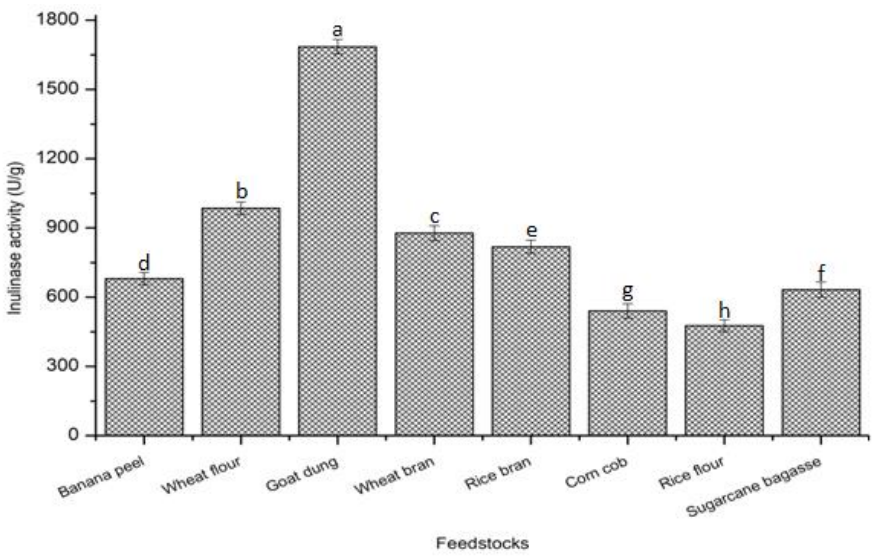


Fig. 2. Inulinase production from strain ALA4 in the presence of disparate feedstocks. Maximum inulinase activity was estimated in the presence of goat dung. ^{abcdefgh}Values with different letters are significantly (P<0.05) different.

Solid state fermentation is one of the most effectual and distinctive process technologies to produce disparate industrial enzymes at wider scale. The SSF process has plentiful advantages due to the exploitation of inexpensive substrates, obtaining high productivity, lower energy requirement and water consumption, and an eco-friendly

approach during downstream bioprocess (Pandey 2003). In the recent years, SSF has become prime choice for inulinase production from bacteria. Several agro-residues such as dahlia tuber extract, wheat bran, garlic peels, onion peels, and Jerusalem artichoke tubers extract have been successfully used for the production of inulinases from diversified bacterial species (Singh *et al.* 2017). The present investigation revealed the first significant effort on the use of goat dung as ideal no cost substrate under SSF to produce exoinulinase from bacteria. The maximum production of inulinase from strain ALA4 using goat dung as substrate might be because of the high moisture holding traits as compared to other feedstocks used in this context. Findings suggest that despite maintaining the cost-effective downstream process, the use of goat dung for inulinase production will also lead to sustain environment friendly cleaner ecosystem.

OFAT Method-based Optimization

Medium composition and various culture parameters are known to affect the metabolic pathway of bacteria, thereby influencing the enzyme productivity. Incubation period is an imperative non-nutritional factor that plays crucial role in enzyme yield. In this study, maximum inulinase activity was estimated at 72 h of incubation. The strain exhibited a significant ($P<0.05$) increment in inulinase activity from 12 h (310.52±25.5 U/g) to 72 h (2034±30.3 U/g) and decreased thereafter (figure not shown). This might be due to the reduced availability of nutrients after 72 h, and thus, causing decreased growth and metabolic activities of strain. In contrast, previous studies reported optimum inulinase activities from *Stenotrophomonas maltophilia* (Navraj *et al.* 2016) and *Bacillus cereus* (Ghadbaan and Aldeen 2015) at 24 h and 48 h, respectively. The variation in the incubation period of our investigation with respect to prior studies could be due to the complexity of goat dung, which took certain time for the fermentation process and subsequent production of inulinase.

Bacteria are sensitive to minor changes in hydrogen ion (pH) concentration, which causes conformational variations in the shape of enzyme and affects its activity. In this study, the isolate exhibited pronounced inulinase activity at pH 8.0 (2145.37±33.3 U/g) and reduced at acidic (pH 6.0 - 1153.45±25.5 U/g) as well as high alkaline conditions (pH 11.0 - 432.31±28.3 U/g) (figure not shown). The reduction in inulinase activity beyond the optimum pH corresponds to the alterations in the states of amino acids in this very protein. In addition, 8.0>pH<8.0 might have altered the shape and charge characteristics of the substrate used, thereby causing improper binding with the active site (Aruna and Hati 2014). Results parallel to the present findings were reported by Li *et al.* (2012) and Laowklom *et al*. (2012), where both research teams reported optimum pH for inulinase activity from *Marinimicrobium* sp. and *Streptomyces* sp., respectively at alkaline pH (pH 8.0). However, maximum inulinase activities from diverse groups of bacteria were also estimated at neutral (Kwon *et al.* 2003) and acidic pH (Sharma and Gill 2007).

Temperature is a crucial abiotic factor that affects enzyme productivity by modifying the physical characteristics of bacterial cells. In this context, the maximum yield of inulinase was obtained at 42 °C (2336.25±26.6 U/g). Further increase in the temperature showed significant ($P<0.05$) reduction in the enzyme activities, estimating the minimum inulinase activity at 60 °C (338.42±24.5 U/g) (figure not shown). A significant ($P<0.05$) reduction in inulinase activity of this strain at higher temperature might be because of the enzymatic denaturation and reduced viability of cells. The results were similar to those Park and Yun (2001) who demonstrated maximum inulinase activity of *Pseudomonas* sp. at 42 °C. In general, the optimum temperature for inulinase production from bacteria is

obtained between 30 and 55 °C (Singh *et al.* 2016).

The inocula volume showed no significant (P>0.05) effect on inulinase activity of isolate, exhibiting maximum enzyme activity of 2398.35±26.6 U/g using 18.27 log cfu/mL of inoculum (figure not shown). Reduction in inulinase activity at lower inoculum volume might be due to the reduced biomass and insufficient viability of cells. Likewise, higher inocula volume of strain ALA4 exhibited reduction in inulinase activity, which is attributed to the excess biomass, thereby causing rapid depletion of nutrients essential for bacterial growth and other metabolic processes in a limited period (Ghadbaan and Aldeen 2015).

Moisture is another decisive parameter that affects the production of enzymes extensively under SSF. In the present study, the effect of distinct moisture levels estimated a maximum enzyme activity of 2448.53±32.5 U/g at 100% moisture level. Lower and higher levels of moisture showed significant (P<0.05) reduction in inulinase activities, ranging from 446.64±29.3 to 1432.34±24.4 U/g (figure not shown). To the best out knowledge, previous reports did not determine influence of moisture levels on inulinase production from any bacterial source; hence, comparative information cannot be discussed here.

Among diverse carbon sources incorporated in goat dung medium for inulinase production, strain ALA4 revealed maximum enzyme activity of 2598.54±31.3 U/g in the presence of inulin. Other carbon sources did not exhibit much influence on inulinase yield from the isolate (figure not shown). The present results agree with the reports of Cho *et al*. (2001), Meenakshi *et al*. (2013), and Muslim *et al*. (2015), who identified inulin as the best carbon source for inulinase production from *Xanthomonas oryzae*, *Bacillus* sp., and *Acinetobacter baumannii*, respectively. In contrast, Zherebtsov *et al*. (2002) demonstrated sucrose and starch as prominent carbon sources for inulinase production from *Bacillus* spp. Variation in the findings of the present study with prior reports indicate the existence of varied regulatory processes in types of bacterial strains used.

In addition to carbon sources, the incorporation of different nitrogen sources into the production medium also affects the enzyme yield from bacteria. As a matter of fact, nitrogen sources are considered secondary energy sources for bacteria, which not only favour the bacterial growth but also up- or down-regulate the enzymes productivity. In the present study, among different nitrogen sources supplemented into the goat dung medium, maximum inulinase activity of 2603.23±32.4 U/g was observed using yeast extract. No significant (P>0.05) differences in inulinase activities of strain ALA4 were reported due to the supplementation of other organic and inorganic nitrogenous sources (figure not shown). Similar observations were reported by Laowklom *et al.* (2012) and Meenakshi *et al.* (2013) too, who observed maximum inulinase activities from *Streptomyces* sp. and *Bacillus* sp., respectively in the presence of yeast extract. Besides, peptone and tryptone had also been observed as effectual nitrogen sources towards maximum inulinase production from discrete bacterial strains (Li *et al*. 2012; Muslim *et al*. 2015). Generally, organic nitrogen sources favour the extensive yield of enzymes because this is prerequisite for synthesizing a variety of proteins.

Optimization of Parameters using 2^5 Full Factorial Design

The preliminary screening of diversified parameters using the OFAT method showed significant (P<0.05) influence of five factors (incubation period, pH, temperature, moisture level, and inulin). Hence, these variables were selected for 2^5 full factorial matrix-based optimization. Different ranges of these selected variables are illustrated in Table 1.

Table 1. Variables and Their Levels for 2^5 Factorial Design

Variables	Code	Units	Range and Levels	
			-1	+1
A	pH	-	7.0	8.0
B	Temperature	°C	37	42
C	Incubation period	h	60	72
D	Moisture	%	90	100
E	Inulin	%	1	1.5

The experimental designs (32 runs) for the optimization of non-nutritional and nutritional parameters using two-level full factorial matrix are shown in Table 2. Maximum inulinase activity of 3218.32±32.6 U/g (Run no. 20) was estimated in the presence of goat dung medium of pH 8.0 with 100% moisture, supplemented with 1.5% (w/w) inulin, and being incubated at 37 °C for 72 h. The experimental data of inulinase activities at various runs were observed close to the predicted values.

Table 2. Screening of Variables for Inulinase Production by 2^5 Factorial Design

Run order	pH (A)	Temperature (B)	Incubation Period (C)	Moisture (D)	Inulin (E)	Inulinase activity (U/g)	
						Experimental	Predicted
1	8	42	60	90	1.5	2678.32	2782.30
2	8	42	60	100	1.5	2495.56	2450.72
3	7	42	60	90	1.5	2145.65	2058.73
4	8	42	72	90	1	2445.31	2649.37
5	7	37	60	100	1	2368.43	2238.86
6	7	37	72	90	1	2488.86	2522.18
7	7	37	60	100	1.5	2276.32	2332.60
8	8	42	60	100	1	2554.56	2672.69
9	8	42	72	100	1.5	2132.34	2190.96
10	7	37	60	90	1.5	1224.43	1160.27
11	8	37	72	90	1.5	2767.32	2777.70
12	8	37	72	100	1	3112.34	2962.60
13	7	37	60	90	1	1668.32	1648.78
14	8	37	60	100	1	2212.21	2303.13
15	7	42	60	90	1	1888.48	1891.70
16	7	42	72	90	1	2376.32	2266.76
17	7	42	60	100	1	2213.12	2216.70
18	8	37	60	90	1	1025.32	1162.19
19	7	37	72	100	1	1688.37	1799.32
20	8	37	72	100	1.5	3218.32	3129.69
21	8	42	72	100	1	2976.54	2679.48
22	7	42	72	100	1	2835.76	2789.39
23	8	42	60	90	1	2442.29	2422.01
24	7	42	60	100	1.5	2354.88	2278.01
25	7	42	72	90	1.5	2672.23	2700.33
26	8	42	72	90	1.5	2865.71	2743.11
27	7	37	72	100	1.5	2032.12	2159.61
28	8	37	72	90	1	2645.32	2716.39
29	8	37	60	100	1.5	2754.34	2736.71
30	7	42	72	100	1.5	2832.43	3117.25
31	7	37	72	90	1.5	2414.98	2300.21
32	8	37	60	90	1.5	1543.23	1490.06

Analysis of variance results for inulinase activity using two-level full factorial design are shown in Table 3. Model F-value of 15.38 implies that the model was significant (P<0.05). Values of "Prob>F"<0.05 represent the significant (P<0.05) model term. Here, pH (A), temperature (B), incubation period (C), and moisture level (D) were statistically significant (P<0.05). The supplementation of inulin (E) into the goat dung medium revealed no significant (P=0.1557) effect on inulinase activity. In a like manner, the interactive factors such as BC, BD, CD, ABC, ABD, ABE, ACE, BCD, and BDE were observed as significant (P<0.05) model terms. The R^2 value (0.9492) of this model indicated closeness to the Adj-R^2 value (0.8875). The Pred R^2 of 0.7345 was in reasonable agreement with the Adj R^2 value. "Adeq Precision" represents the signal to noise ratio, which is considered desirable if the value is >4. In this model, a ratio of 15.196 indicated an adequate signal, thus suggesting the navigation of the design space. The model equation for inulinase activity is shown below:

Inulinase activity (Y) = 2354.68 + 134.14A + 139.66B + 239.34C + 148.92D + 45.83E – 57.65AB – 91.60BC – 93.87BD – 139.41CD – 155.84ABC – 130.42ABD – 77.89ABE – 66.64ACE – 59.57ADE + 136.55BCD – 86.00BDE – 52.27ABCD (3)

Table 3. Analysis of Variance for Inulinase Yield by 2^5 Factorial Design

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	7.807E+06	17	4.592E+05	15.38	< 0.0001	significant
A-A	6.018E+05	1	6.018E+05	20.16	0.0005	
B-B	6.242E+05	1	6.242E+05	20.91	0.0004	
C-C	1.833E+06	1	1.833E+06	61.40	< 0.0001	
D-D	7.097E+05	1	7.097E+05	23.77	0.0002	
E-E	67218.86	1	67218.86	2.25	0.1557	
AB	1.064E+05	1	1.064E+05	3.56	0.0800	
BC	2.685E+05	1	2.685E+05	8.99	0.0096	
BD	2.820E+05	1	2.820E+05	9.44	0.0083	
CD	6.219E+05	1	6.219E+05	20.83	0.0004	
ABC	7.771E+05	1	7.771E+05	26.03	0.0002	
ABD	5.443E+05	1	5.443E+05	18.23	0.0008	
ABE	1.941E+05	1	1.941E+05	6.50	0.0231	
ACE	1.421E+05	1	1.421E+05	4.76	0.0467	
ADE	1.135E+05	1	1.135E+05	3.80	0.0715	
BCD	5.966E+05	1	5.966E+05	19.98	0.0005	
BDE	2.367E+05	1	2.367E+05	7.93	0.0138	
ABCD	87444.57	1	87444.57	2.93	0.1091	
Residual	4.180E+05	14	29856.12			
Cor Total	8.225E+06	31				

R^2 – 0.9492, Adj R^2 – 0.8875, Predicted R^2 – 0.7345, CV – 7.34%, Adeq precision – 15.196, df - degree of freedom, Significant – P<0.05, Non-significant – P>0.05.

Central Composite Design-based Optimization

Considering the significant (P<0.05) impact of pH, incubation period, and moisture as per 2^5 full factorial design, these three parameters were optimized further to enhance the yield of inulinase using CCD of RSM. Selected factors and its ranges for CCD are summarized in Table 4. A total of 20 experiments were performed with appropriate ranges of selected factors by keeping other parameters unchanged, which showed the maximum inulinase activity of 4678.34±34.67 U/g at Run no. 17 constituting goat dung medium of pH 8.0 as well as moisture content of 100%, and being incubated for 96 h (Table 5). The

production of inulinase from the bacteria was estimated at about 2-fold with respect to OFAT approach-based optimization. All the actual response values for each experiment were estimated as being close to the predicted values.

Table 4. Variables with Their Ranges and Levels for CCD

Variables	Code	Range and Levels				
		-α	-1	0	+1	+α
pH	A	6	7	8	9	10
Incubation period (h)	B	48	60	72	96	120
Moisture (%)	C	80	90	100	110	120

Table 5. Experimental and Predicted Values of Inulinase Production through CCD

Run Order	pH (A)	Incubation Period (B)	Moisture (C)	Inulinase activity (U/g)	
				Experimental	Predicted
1	0	0	+ α	4048.54	4002.85
2	0	0	- α	3976.64	3951.62
3	+ α	0	0	3876.34	3901.49
4	1	-1	+1	3176.62	3207.42
5	0	0	0	3412.34	3422.56
6	0	0	0	3465.43	3422.56
7	- α	0	0	3552.23	3456.37
8	1	1	1	3987.92	3964.93
9	0	- α	0	2476.45	2417.58
10	0	0	0	3402.23	3422.56
11	-1	-1	1	3688.31	3729.12
12	0	+ α	0	3623.45	3635.53
13	0	0	0	3398.24	3422.56
14	-1	1	1	3367.89	3436.65
15	-1	-1	-1	2872.62	2945.60
16	1	-1	-1	2965.43	2946.67
17	0	1	0	4678.34	4687.53
18	1	1	-1	3712.32	3644.65
19	0	0	0	3378.68	3422.56
20	-1	1	-1	3617.28	3636.48

Table 6 shows ANOVA for inulinase quadric model of CCD. Model F-value of 118.92, which indicates that the model was significant ($P<0.05$). Values of "Prob>F"<0.05 represent that the model terms were significant ($P<0.05$). In this investigation, A, B, AB, AC, BC, A^2, B^2, and C^2 were significant ($P<0.05$) model terms. The "Lack of Fit F-value" of 5.55 implies that the Lack of Fit was non-significant ($P>0.05$) relative to the pure error. Non-significant lack of fit indicates model to be fit. The value of R^2 (0.9907) showed strong correlation between observed and predicted response values, and thus suggests the accuracy of the model. In this context, a low coefficient of variation (1.78%) represented the authenticity of the experiments. The "Predicted R^2" of 0.9431 was in reasonable agreement with the "Adj R^2" of 0.9824. "Adeq Precision" ratio of 51.012 indicated an adequate signal, and thus, suggested the navigation of design space.

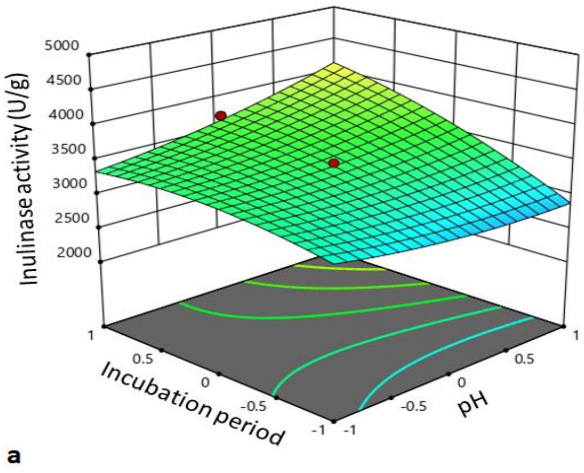
The model equation of inulinase activity is shown below:

$$\text{Inulinase activity } (Y) = 3422.56 + 132.34A + 362.10B + 15.23C + 262.50AB - 130.69AC - 245.84BC + 90.64A^2 - 141.01B^2 + 196.11C^2 \quad (4)$$

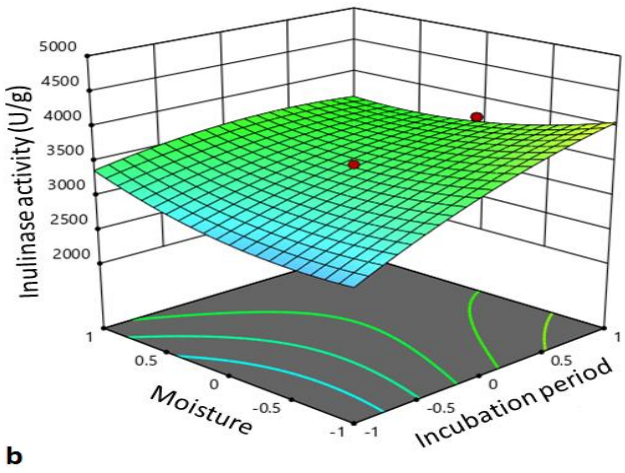
Table 6. Analysis of Variance for Inulinase Yield by CCD

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	4.239E+06	9	4.710E+05	118.92	< 0.0001	Significant
A-A	2.392E+05	1	2.392E+05	60.40	< 0.0001	
B-B	1.906E+06	1	1.906E+06	481.30	< 0.0001	
C-C	3167.66	1	3167.66	0.7999	0.3921	
AB	5.512E+05	1	5.512E+05	139.20	< 0.0001	
AC	1.366E+05	1	1.366E+05	34.50	0.0002	
BC	4.835E+05	1	4.835E+05	122.09	< 0.0001	
A^2	1.165E+05	1	1.165E+05	29.41	0.0003	
B^2	2.677E+05	1	2.677E+05	67.59	< 0.0001	
C^2	5.452E+05	1	5.452E+05	137.68	< 0.0001	
Residual	39600.78	10	3960.08			
Lack of Fit	35352.78	6	5892.13	5.55	0.0596	not significant
Pure Error	4248.00	4	1062.00			
Cor Total	4.278E+06	19				

R^2 – 0.9907, Adj R^2 – 0.9824, Predicted R^2 – 0.9431, CV – 1.78%, Adeq precision – 51.012, df - degree of freedom, Significant – P<0.05, Non-significant – P>0.05.



a



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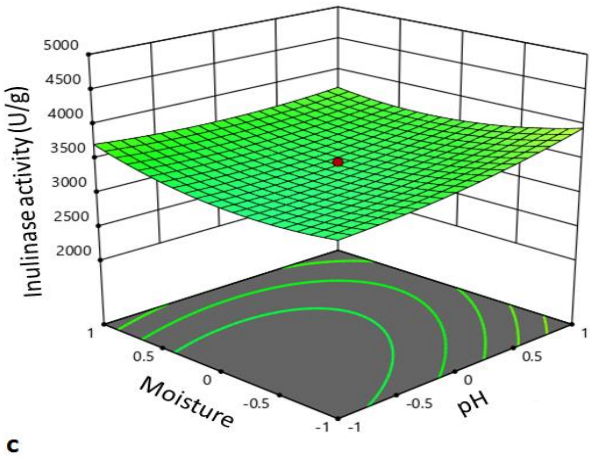
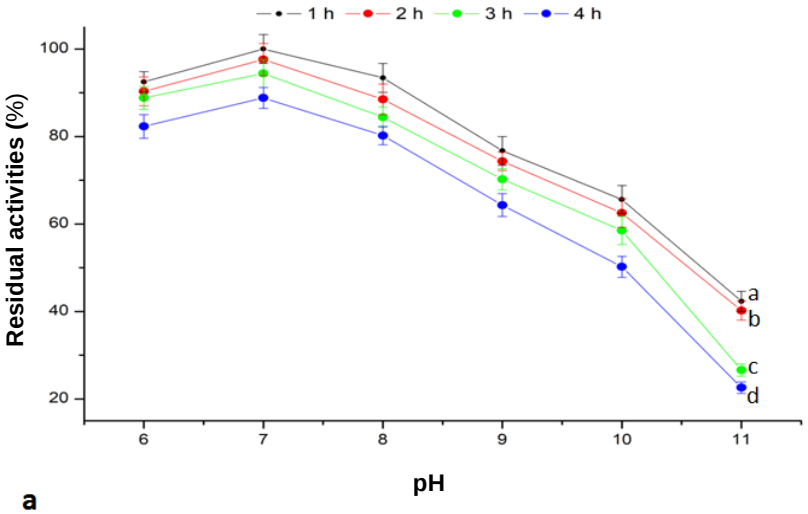


Fig. 3. 3D plots showing interdependent interaction between **(a)** pH and incubation period, **(b)** incubation period and moisture, and **(c)** pH and moisture on inulinase activity

The interactive influence of pH and incubation period, moisture level and incubation period, and pH and moisture level is illustrated in Fig. 3 (a-c). The 3D plots constituting contour plots revealed the significant ($P<0.05$) effect of pH and incubation period towards the increment of inulinase activity. Further, the statistical model was authenticated using CCD based optimized parameters, which exhibited the inulinase activity close to the predicted response value. Generally, the interactions between corresponding factors are considered significant ($P<0.05$) if the shape of the contour plot is obtained elliptical, whereas circular shape of contour plots indicate non-significant ($P>0.05$) interactions between the corresponding parameters (Khusro *et al*. 2016). Statistical tools-based optimization has been implemented extensively in the past for enhanced productivity of inulinase from diversified bacterial sources (Dilipkumar *et al.* 2013; Kamble *et al.* 2018).

Characterization of Inulinase

The effect of varied ranges of pHs on the stability of crude inulinase is depicted in Fig. 4a.



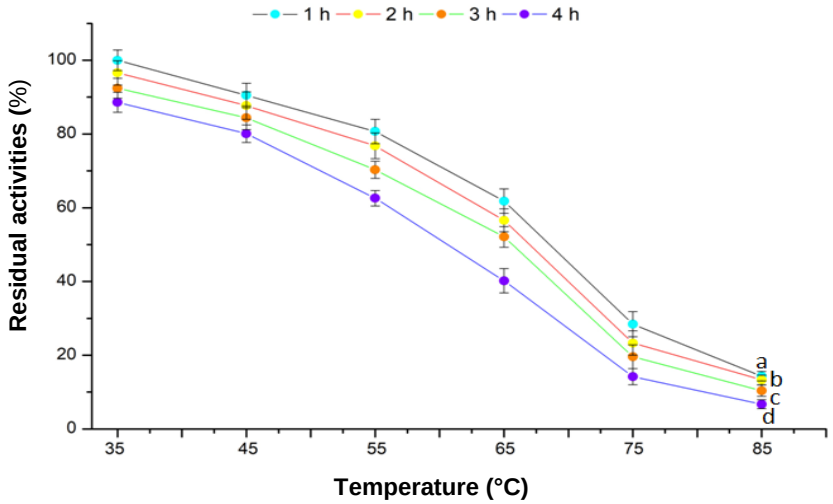


Fig. 4. Stability of inulinase at **(a)** various pHs and **(b)** temperature up to 4 h of incubation. [abcd]Values with different letters are significantly (P<0.05) different.

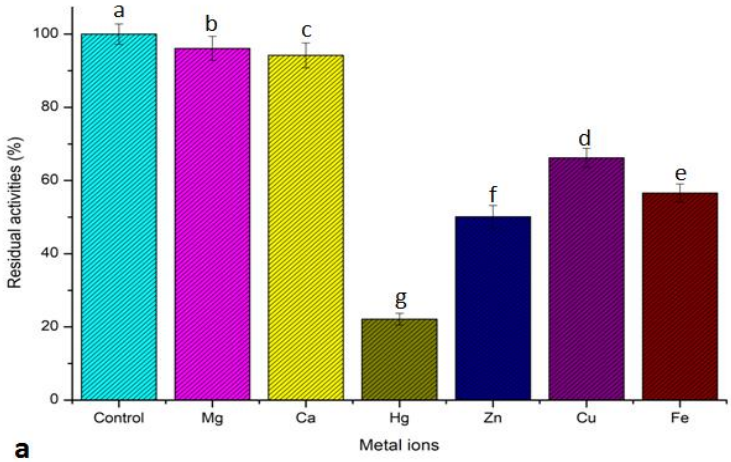
The enzyme exhibited stability up to pH 10.0 for 4 h with residual activity of 50.2±2.4%. However, the enzyme resisted high alkaline pH (pH 11.0) too for first 2 h only with residual activity of 40.2±2.2% and reduced (P<0.05) its stability for further increase in incubation period. Kang *et al.* (1998) demonstrated stability of *Arthrobacter* sp.-associated inulinase in the range of pH 5.0 to 10.5. However, most of the studies investigated in the past revealed stability of bacterial inulinase from acidic to neutral pH (Kwon *et al.* 2003; Tohamy Eman 2006; Ramapriya *et al*. 2018).

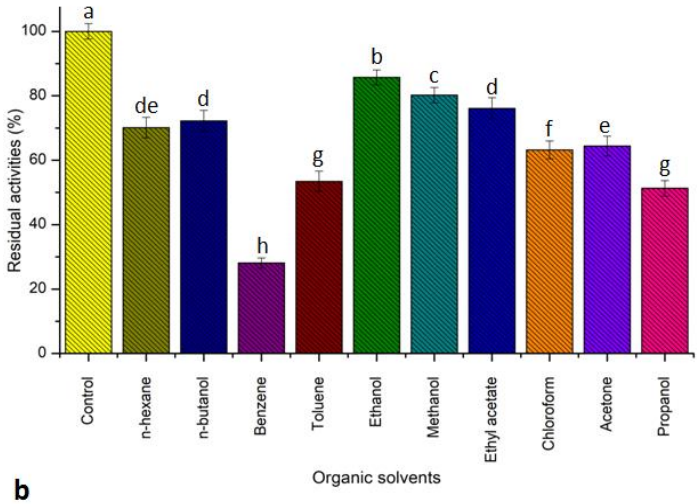
In a like manner, inulinase retained its stability up to 65 ºC with residual activity of 40.2±2.8% for 4 h of incubation period. A drastic reduction (P<0.05) in the stability of enzyme was estimated at higher temperature (Fig. 4b). Findings more or less similar to these results were observed by Aruna and Hati (2014) and Rampriya *et al*. (2018) too, who depicted stability of *Bacillus* spp.-associated inulinase between 30 and 70 ºC. In contrast, Liebl *et al*. (1998) observed stability of *Thermotoga maritime*-derived inulinase up to 90 ºC. In general, bacterial inulinases are found stable in the range of 30 to 60 ºC (Gill *et al*. 2003). Thermo-alkali stable nature of strain ALA4-associated inulinase suggested its paramount significance in the bioprocessing industries, especially in the bioconversion efficiency of lignocellulosic biomass. High temperature and pH resistant inulinase can avoid any kind of microbial contamination during product formation and will induce the degradation of lignocellulosic biomasses.

The stability of inulinase towards diversified metal ions is illustrated in Fig. 5a. The enzyme showed the highest stability in the presence of Mg^{2+} and Ca^{2+} with residual activities of 96.1±3.3 and 94.2±3.4%, respectively with respect to control. The stability of enzyme was decreased (P<0.05) in the availability of other metal ions, exhibiting the minimum residual activity of 22.1±1.6% towards Hg^{2+}. Similar to the present findings, previous studies also reported stability of bacteria-associated inulinases towards Mg^{2+} and were strongly inhibited in the presence of Hg^{2+} (Meenakshi *et al.* 2013; Markosyan and Vardanyan 2023). Tohamy Eman (2006) also observed stability of *Streptomyces grisenus*-associated inulinase towards Ca^{2+}, but the stability of enzyme was decreased after Mg^{2+} exposure. In general, divalent cations are often known to induce the enzyme activity. The adverse impact of ions on inulinase stability is mainly attributed to the direct inhibition of

the catalytic site. The reduced stability of inulinase due to Hg^{2+} supplementation might be due to the binding with thiol (-SH-) group and its interaction with tryptophan residue or the carboxyl group of amino acids (Kim *et al.* 2000). Previously, metal ions stable enzyme saccharified distinct lignocellulosic biomasses (Aarti *et al*. 2018).

The solubilizing potentials of organic solvents for hydrophobic substances are essential for their use as additives in the biomass bioconversion mechanism. Figure 5b depicts the stability of inulinase towards disparate organic solvents. In this study, maximum residual activities of 85.7±2.3 and 80.2±2.4% were estimated in the presence of ethanol and methanol, respectively. Results showed comparatively lower ($P<0.05$) stability of enzyme towards other polar and non-polar solvents in the order of ethyl acetate (76.1±3.3%) > *n*-butanol (72.2±3.3%) > *n*-hexane (70.1±3.2%) > acetone (64.4±3.1%) > chloroform (63.2±2.8%) > toluene (53.4±3.2%) > propanol (51.3±2.4%) > benzene (28.1±1.6%). As a matter of fact, it is a unique trait of this inulinase because most of the enzymes lose stability in the presence of solvents. To the best of our knowledge, bacterial inulinase stability towards diversified solvents has not been investigated in the past; hence comparative study cannot be discussed here.





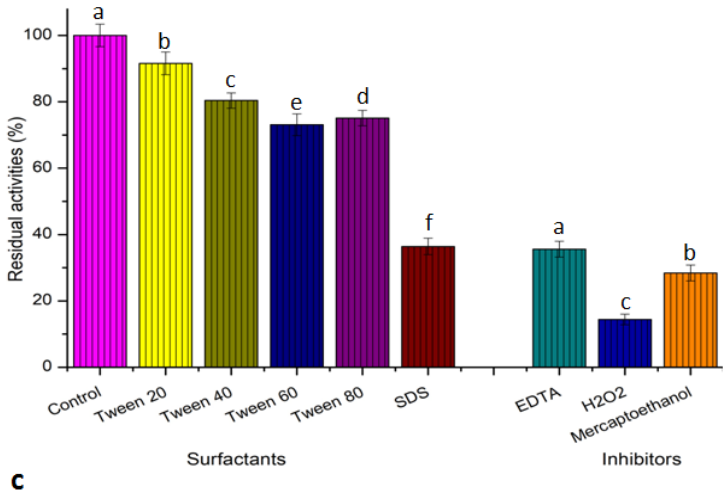


Fig. 5. Stability of inulinase in the presence of various **(a)** metal ions, **(b)** organic solvents, and **(c)** surfactants and inhibitors. [abcdefgh]Values with different letters are significantly ($P<0.05$) different.

Inulinase exhibited a promising ($P<0.05$) rate of tolerance towards various surfactants, showing the highest and the lowest residual activities of 91.6±3.4 and 36.4±2.5% in the presence of Tween 20 and SDS, respectively (Fig. 5c). In addition, the stability of inulinase in the presence of inhibitors was observed ($P<0.05$) in the order of 35.6±2.4% (EDTA) > 28.4±2.4% (mercaptoethanol) > 14.4±1.6% (H_2O_2) (Fig. 5c). Generally, enzymes resistant to surfactants and inhibitors are essential for the adequate saccharification of lignocellulosic biomasses, as discussed by Aarti *et al*. (2018).

Fructose Analyses

The qualitative analysis of reducing sugar in the hydrolyzed products of goat dung was confirmed using the osazone test, which showed the formation of osazone crystals by fructose present in the fermented product. Figure 6 shows the formation of characteristic needle-shaped crystals that are formed by the fermented products as well as the standard.

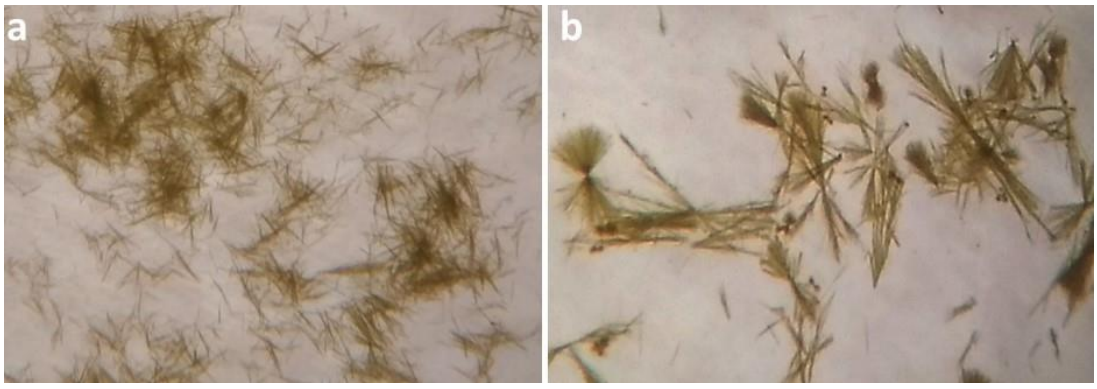


Fig. 6. Microscopic observation of osazone crystal formation in **(a)** standard fructose and **(b)** hydrolyzed product of goat dung. Needle-shaped crystal formation indicated the presence of fructose in the hydrolyzed products of goat dung.

The formation of fructose monomer in the hydrolyzed products due to the catalytic action of exoinulinase was further confirmed by HPTLC, which evidenced the liberation of fructose and showed close resemblance with the standard fructose (Fig. 7).

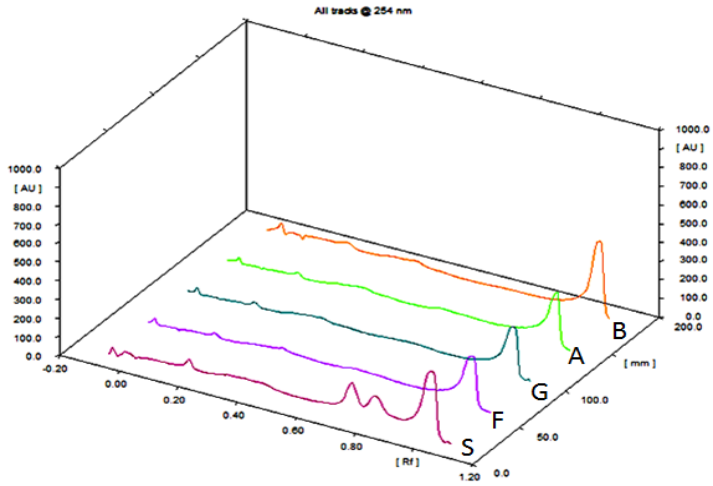


Fig. 7. HPTLC chromatogram of sucrose (**S**), fructose (**F**), glucose (**G**), and hydrolysate of goat dung in duplicate (**A** and **B**).

In this work, all the qualitative tests showed the production of fructose as major reducing sugar during the goat dung degradation process, indicating inulinase as an end group cleaving enzyme (Aruna and Hati 2014). Similar observations were reported by Li *et al*. (2012), Laowklom *et al*. (2012), Ramapriya *et al*. (2018), and Kamble *et al*. (2018).

CONCLUSIONS

1. The study evidenced the first report on invertase-free exoinulinase production from *G. arilaitensis* strain ALA4 using goat dung as plausible substrate under solid-state fermentation (SSF).
2. A two-level full factorial matrix experimental approach, following the CCD and RSM design systems showed about 2-fold increment in inulinase activity (4678.34±34.67 U/g) as compared to the conventional method of optimization.
3. Findings suggested that goat dung could be used not only as proficient substrate for inulinase production from bacteria but also to sustain a cleaner ecosystem.
4. The stability towards high temperature and pH, metal ions, organic solvents, surfactants, and inhibitors revealed quiescent applications of inulinase in bioprocessing industries.

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