Production and characterization of *Aspergillus terreus* inulinase, using a by-product of green asparagus (*Asparagus officinalis* L.)

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Abstract

The roots of asparagus are a by-product of growing asparagus and are among the richest sources of fructans. These polymers' immunostimulating and prebiotic properties make them intriguing dietary additives. The purpose of this work was to use inexpensive agricultural waste, such as asparagus roots from *Aspergillus terreus* ATCC 10020, to create a method for producing the vital enzyme inulinase, which finds several uses in the food and pharmaceutical sectors. High-titer generation of inulinase on asparagus roots (*Asparagus officinalis*) by *A. terreus* in a submerged culture was accomplished based on statistical experimental design. A three-level Box-Behnken design after a two-level Plackett-Burman design produced a high inulinase titer of 1446.82 U/ml, 1.63 fold the highest level found in the screening experiment. The optimal levels were found to be asparagus roots, 2.5 g/L; $NaNO_3$, 4 g/L; KCl, 0.3 g/L; K_2HPO_4, 0.5 g/L; (NH_4) H_2PO_4, g/L; $FeSO_4$, 0.5 g/L; $MgSO_4$, 0.3 g/L; and temperature, 35˚C and pH, 5.0. Using 70% ammonium sulphate precipitation and ultra-filtration, the produced inulinase enzyme was purified 3.60 times with 60% activity recovery. The molecular weight of the enzyme was around 60 KDa. The purified enzyme was most active at pH 6.0 and 55˚C. The temperature stability was up to 60˚C, while the pH stability range was 3-6. The enzyme activity was enhanced by Mg^{2+}, Mn^{2+}, and Cu^{2+}, whereas Hg^{2+} was shown to be the most potent inhibitor. The properties of the enzyme showed its ability to hydrolyze inulin with high efficiency. It also points to the potential of inulinase in the food and pharmaceutical sectors.

Keywords

Aspergillus terreus, Inulinase, Asparagus roots, Production, Purification, Characterization

INTRODUCTION

Numerous plant species generate inulins, a class of naturally occurring polysaccharides. The inulins are members of the fructan class of dietary fibers. Some plants employ inulin, which is usually present in roots or rhizomes, as a way to store energy. Other types of carbohydrates, such starch, are not stored by the majority of plants that produce and store inulin. Inulin is a dietary fiber component that has been authorized by the US Food and Drug Administration to increase the nutritious content of processed foods [1, 2]. Many

plant species, such as wheat, onions, bananas, garlic, asparagus, Jerusalem artichokes, agave, and chicory, naturally contain inulin, a storage carbohydrate. For these plants, inulin is employed as an energy store and for controlling cold tolerance **[3, 4]**.

A diverse group of fructose polymers make up inulin. It is made up of repeating fructosyl and chain-terminating glucosyl moieties connected by β(2,1) linkages **(Figure 1)**. Standard inulin has a degree of polymerisation (DP) between 2 and 60. High-performance inulin is the final product after the fractions with DP less than 10 are eliminated throughout the production process. Only the longer-chained molecules were referred to as inulin in certain papers, which regarded the fractions with DP less than 10 as short-chained fructo-oligosaccharides **[5]**. The β(2,1) links prevent inulin from being broken down by the enzymes in the human digestive system, which contributes to its functional qualities, which include lower calorie content, dietary fiber, and prebiotic benefits. It has minimal effect on food products' sensory qualities when color and smell are absent. Oligofructose has a sweetening characteristic comparable to sugar and is 35% as sweet as sucrose **[6]**.

The Food and Drug Administration (FDA) has authorized inulin as an additive to increase the amount of dietary fiber in processed meals **[7]**. It might have a subtle sweetness or a dull taste. It can be used in place of flour, sugar, and fat. This is beneficial since 30% of the dietary energy of carbohydrates is found in inulin. Apart from its versatility, inulin offers nutritional benefits by boosting the absorption of calcium and magnesium and encouraging the development of gut microorganisms. Inulin has little effect on blood sugar levels because of the body's limited capacity to digest fructans. It may also be useful in treating blood sugar-related conditions like metabolic syndrome **[8, 9]**. Dietary supplementation with inulin-type

fructans decreased blood levels of low-density cholesterol, a biomarker of cardiovascular disease, according to a comprehensive assessment of clinical trial studies **[10]**.

Potential negative effects include increased frequency of bowel movements when taking 20–50 g of dietary inulin daily. People with inulin allergies may experience gastrointestinal distress, bloating, flatulence, diarrhea, and stomach inflammation as a result of consistently consuming inulin **[11, 12]**.

One of the three forms of dietary fiber-soluble, insoluble, and resistant starch-is inulin. A gelatinous substance is created when soluble fiber dissolves in water. Certain soluble fibers have the potential to reduce blood glucose and cholesterol levels. Because inulin cannot be broken down by the human enzymes amylase and ptyalin, which are designed to break down starch, it travels through the digestive tract mostly undigested. Bacteria only break down inulin in the colon, releasing large amounts of hydrogen, carbon dioxide, and/or methane in the process. Foods containing inulin can be somewhat gassy, especially for people who are not used to it. For this reason, they should first be taken in moderation **[13]**.

One essential sweetener, high-fructose syrups, is made using inulinases. When used as a sweetener, fructose is a safe alternative to sucrose since it provides advantages for diabetics. The enzymatic production of fructose is beneficial since it does not produce harmful byproducts like difructose anhydride **[14]**.

Inulin is enzymatically broken down by inulinase to provide 95% pure fructose and fructo oligosaccharides. Inulinases have also been used to produce inulo-oligosaccharides, which are low-calorie saccharides that serve as growth factors for gut flora microorganisms.

Inulinases are also used in the production of bio-ethanol from inulin. Inulinases have also been used to convert lactic acid, citric acid, gluconic acid, pullulan, sorbitol, and single cell oil **[15, 16]**.

The ability of fungal strains to thrive on low-cost substrates and their stability as enzymes at high temperatures and low pH make them interesting candidates for the synthesis of inulinase enzymes. Effective manufacturers of inulinase enzymes include Aspergillus, Rhizopus, Penicillium, and Kluyveromyces **[17]**. From a functional perspective, asparagus and its byproducts may also be regarded as items of interest because of their high inulin, dietary fiber, and other phytochemicals, including phenolic components **[18]**.

The perennial flowering plant species Asparagus (*Asparagus officinalis*) is indigenous to Eurasia and belongs to the genus Asparagus. Its young shoots are utilized as a spring vegetable and are widely grown as a food crop **[19]**. Asparagus has relatively little sodium and little dietary energy. In addition to being a very good source of dietary fibre, protein, beta-carotene, vitamin C, vitamin E, vitamin K, thiamin, riboflavin, rutin, niacin, folic acid, iron, phosphorus, potassium, copper, manganese, and selenium, it is also a good source of vitamin B6, calcium, magnesium, and zinc. Additionally, it contains chromium, a trace mineral that controls how well insulin transports glucose from the bloodstream into cells. Due to the relative abundance of this chemical in the asparagus plant, the amino acid asparagine was initially isolated from asparagus, thus its name.

Notably, the root's inulin **(Figure 2)** content (16.18 g/100 g) was much greater than that of the edible spear portion (1.30 g/100 g) and the spear by-product (1.46 g/100 g) **[20]**. In this work, we used a cheap substrate, such asparagus roots, to increase the production of

inulinase enzymes from *Aspergillus terreus*. To do this, the response surface approach was used to optimize the concentrations of the important components. Additional objectives of this study were the purification and characterization of the generated inulinase enzyme.

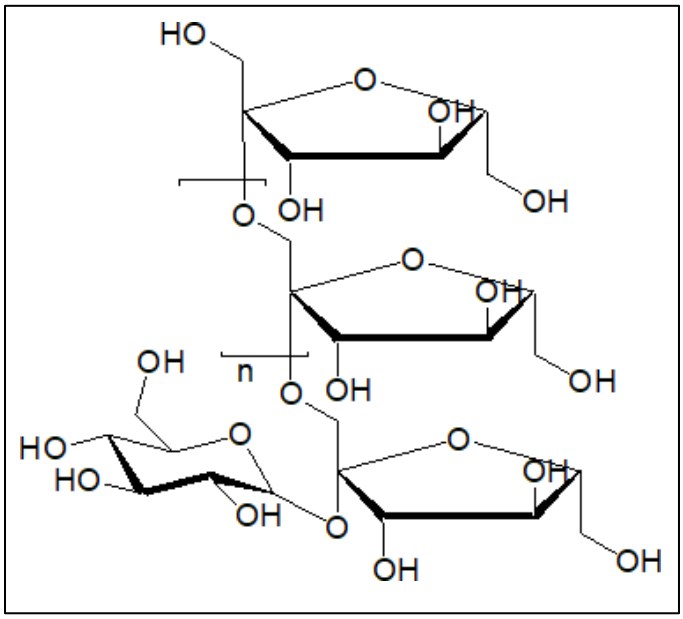

Figure 1
Structure of inulin

Figure 2
Roots of asparagus

MATERIALS AND METHODS

Chemicals and materials

The source of inulin was Techno PharmaChem, India. The roots of asparagus (*Asparagus officinalis*) were collected from the Orman Garden in Cairo, cleaned, let to air dry, and then milled into a fine powder.

Dialysis membrane -150 (HiMedia Laboratories, India), standard protein markers (IRIS11Prestained Protein Ladder (IRIS11#PMI11-0500), (3–260) KDa, Bio-Helix, USA), and ammonium sulphate were all acquired from Acros-Organics (Fisher Chemical, Egypt).

Microbial strain

Aspergillus terreus ATCC 10020 used in this study was obtained from Food Technology Research Institute, Agricultural Research Service (ARS) Culture Collection (Peoria, Illinois, USA).

Culture media

The following ingredients were utilized in the shake-flask screening culture for inulinase production: 1%, Inulin; 0.1%, K_2HPO_4; 0.05%, $MgSO_4$. $7H_2O$; 0.15%, $NaNO_3$; 0.05%, KCI; 0.01%, $FeSO_4.7H_2O$; 0.2%, $NH_4H_2PO_4$, after using 1N NaOH to bring the medium's pH down to 6.5, it was autoclaved for 20 min at 121˚C and 15 pressure to sterilize it.

Inoculum preparation

Potato-dextrose-agar (PDA) is used to cultivate *A. terreus* ATCC 10020 at 4˚C. After a 7-day PDA plate was incubated at 30˚C with 10 mL of distilled water, spores were scraped to create a spore suspension, which had a final concentration of around 2×10^6 spores/mL. Every four weeks, the spore solution and subculture are replaced.

Culture screening for enzyme production

0.3 mL of spore suspension was added to each 250 mL flask that had 50 mL of screening media. The flasks were then incubated for eight days at 30˚C with 200 rpm agitation. After the mycelia were removed by filtering, broth samples were collected on the second, fourth, sixth, and eighth days and their inulinase activities were measured.

Effect of carbon source on inulinase production by A. terreus

A variety of carbon sources, including inulin, fructose, sucrose, maltose, glucose, and asparagus roots, were investigated as carbon sources for the synthesis of the inulinase enzyme at 5% concentration.

Multi-factorial studies to maximize the production of inulinase

The production of inulinase may be significantly increased by optimizing the medium and fermentation process by choosing the ideal nutritional and environmental conditions. We used a progressive optimization approach in this investigation, with the first phase focusing on screening and determining the environmental and nutritional parameters influencing *A. terreus*'s production of inulinase. The second step was identifying the combination that produced the highest inulinase activity once the important elements influencing inulinase production were identified.

Plackett-Burman design

The first step involved screening and identifying the most important factors (medium components) affecting the production of inulinase using a Plackett-Burman design. Plackett-Burman is unable to quantify the interactions between various components. This approach is especially useful for screening a large number of variables when it is unclear which factors are most likely to affect the dependent variable. Two tiers and nine factors in this investigation, the Plackett-Burman design was used. In twelve experimental trials, nine factors-including temperature, pH, asparagus roots, K_2HPO_4, $MgSO_4.7H_2O$, $NaNO_3$, KCI,

$FeSO_4.7H_2O$, and $(NH_4)H_2PO_4$ were screened. Each component was evaluated at two levels: -1 for the low level and +1 for the high level.

The factors examined and their relative levels in the experimental design are shown in **Table 1**. Every experiment was run in triplicate for eight days, and the response (dependent variable) was determined by taking the mean of the inulinase activity. The difference between the means of inulinase activity observed in cultivations employing +1 and −1 concentrations was used to compute each factor's major effect.

To suit the Plackett-Burman design, a first-order polynomial linear model was used, as indicated by the equation below:

$Y=\beta o + \Sigma \beta iXi,$

where Xi is the coded independent variable (level of each component), βo is the model intercept, βi is the linear coefficient for each factor, and Y is the response (inulinase activity (U/mL). The effect of the relevant factor on inulinase production was indicated by the size of the coefficient βi and whether it was positive or negative. Using JMP Pro 13 (USA), the data was fitted to the model at a $P < 0.05$ significance level. The Box-Behnken design was used to better optimize the important aspects.

Table 1

Variables and their levels used in the Plackett-Burman design to screen for variables influencing *Aspergillus terreus*'s production of inulinase

Factor	Low level (-1)	High level (+1)
asparagus roots	10 g/L	20 g/L
K_2HPO_4	0.5	2
$MgSO_4.7H_2O$	0.3	1
$NaNO_3$	0.5	3
KCl	0.3	1
$FeSO_4.7H_2O$	0.05	0.5
$(NH_4)H_2PO_4$	1	4

temperature	25	35
pH	5	7

(−1) and (+1) are coded levels in Plackett-Burman design

Variables optimization using Box-Behnken design

According to **Box et al. [21]**, the Box-Behnken design comprises fifteen trials with three levels. Using the least squares approach and multiple regressions, the data were fitted to the following quadratic equation to determine inulinase production:

$$Y=\beta o + \Sigma \beta iXi + \Sigma \beta ijXi\ Xj + \Sigma \beta iiXi$$

where Y is the expected response (inulinase activity); β0, βi, βii, and β ij are the regression coefficients of the intercept; linear, quadratic, and interaction terms; and Xi and Xj are the coded independent variables.

Analytical method

According to **Miller et al. [22]**, the activity of the inulinase enzyme was assessed by adding 0.5 mL of the substrate, which is 1% inulin in 0.2 M sodium acetate buffer (pH 6.5), to 0.5 mL of buffer-diluted enzyme extract solution, and then incubating for 20 min at 50°C. To halt the reaction, the reaction mixture was submerged in boiling water for five minutes before being allowed to cool to room temperature. At 575 nm, the absorbance was measured in relation to an enzyme-free blank. By utilizing fructose as a reference and the 3, 5-dinitrosalicylic acid (DNS) reagent technique to measure the released reducing sugars, enzymatic activity was ascertained. The quantity of enzyme that converted 1 µmol of fructose per min from inulin was considered a unit of inulinase activity.

Protein concentration was measured by Bradford method [23] using a commercial protein assay kit and bovine serum albumin was used as standard protein

Enzyme purification

Ammonium sulphate, a fine and dried powder, was gradually added to the crude enzyme extracts while stirring to achieve 50 to 90% saturation at 4˚C. The mixture was stirred for 60 min and then left to stand for 24 hrs at 4˚C. The precipitate was dissolved in 10 mL of 0.2 M sodium acetate buffer (pH 6.5), the mixture was centrifuged at 10,000 rpm for 20 min at 4˚C, and the supernatant was disposed of. After dialyzing the enzyme against 0.2 M sodium acetate buffer for 48 hrs, the excess ammonium sulphate salt that had been coupled to it was eliminated. Ultra centrifugal filters (50 kDa) were used to concentrate the sample for 15 min at 4˚C and 5000 rpm.

Molecular weight determination

The molecular weight of the partly purified inulinase enzyme was calculated using the IRIS11Prestained Protein Ladder (IRIS11#PMI11-0500), (3–260) KDa, Bio-Helix, USA, and 12% sodium dodecyl sulphate-polyacrylamide gel SDS-PAGE. Coomassie Brilliant Blue [24] R-250 was used to stain the gel. The typical protein markers with known molecular weights were compared to the inulinase enzyme's molecular weight.

Determination of optimal temperature

By combining 0.5 mL of enzyme with 0.5 mL of substrate (1% inulin in 0.2 M sodium acetate buffer pH 6.5), the impact of temperature on inulinase enzyme activity was assessed. The mixture was then incubated for 20 min at various temperatures (30 to 90˚C). After five min of being submerged in boiling water to halt the reaction, the reaction mixture was

allowed to cool to room temperature. Using fructose as a reference, the DNS reagent technique was used to measure the released reducing sugars in order to quantify the enzymatic activity.

Half mL of the enzyme was incubated with 0.5 mL of 0.2 M sodium acetate buffer pH 6.5 at different temperatures (30-90˚C) for 120 min in order to assess the enzyme's thermal stability. The enzyme activity that remained was then assessed.

Determination of optimal pH

To find out how pH affected enzyme activity, 0.5 mL of crude enzyme was added to 0.5 mL of substrate (0.5% inulin in 0.02 M sodium acetate buffer produced at varied pH 3-8) and incubated for 20 min at 50˚C. Fructose was used as a standard in the DNS reagent technique to measure the released reducing sugars in order to evaluate the enzymatic activity.

The crude enzyme was incubated for 24 hrs at 4˚C in a series of sodium acetate buffers with pH values ranging from 3 to 9 in order to assess the pH stability of the enzyme. After bringing the enzyme solution's pH down to 6.5, the enzyme's remaining activity was assessed.

Effect of ions on enzyme activity

The effect of ions to enzyme activity was carried out by measuring the activity of inulinase enzyme in several ions solution: $CaCl_2$, $MnCl_2$, $MgCl_2$, $FeSO_4$, $CuSO_4$, $CoCl_2$, $ZnSO_4$, $HgCl_2$ and NaCl; each with a concentration of 10 mM. The process was done by dissolving 1 mL of 1% enzyme in the optimal pH buffer previously obtained. 10 mM ion solution was then

added along with 1 mL of the enzyme. Inulinase activity assay was then conducted as previously described.

RESULTS AND DISCUSSION

Effect of carbon source on inulinase production

Because inulinase biosynthesis is dependent on the carbon source used, its production might vary greatly. **Figure 3** compares the research of *A. terreus* cultivated on different carbon sources (5 g/L) for inulinase production. As can be observed, asparagus roots had the highest inulinase output (375 U/mL) followed by inulin (300 U/mL). The fructans polymers that make up asparagus roots range in size from one to twenty-five units of fructose. Based on its composition, it may be a suitable substrate for inulinase production. The lowest inulinase activity (90, 105, and 110) U/mL was obtained with maltose, glucose, and fructose, respectively, as the carbon source. Inulinase production has been reported to be attributed to catabolism repression by free sugars **[25]**.

The strong and specific substrate inulin stimulates the formation of the inulinase enzyme, which accounts for the high inulinase production observed with inulin and asparagus roots **[26]**. The increased synthesis of inulinase may also be explained by the fact that inulin functions as a matrix of support and a source of nutrients to encourage fungal adherence. In the shake flask cultures, *A. terreus* mycelia had grown into spherical pellets of various diameters based on the carbon sources.

Because inulinase's biosynthesis is dependent on the carbon source used, its production might vary greatly. The high inulin content in asparagus roots may be the cause of the increased inilinase activity. Fructan polymers, which range in size from 10 to 40 units

of fructose and contain a linear chain, make up asparagus roots [27]. Based on its composition, it may be a suitable substrate for inulinase production. Extracellular inulinases are the only carbon source produced by Aspergillus [28]. Aspergillus mycelia have grown into spherical pellets with different diameters in the shake-flask cultures, contingent on the carbon sources.

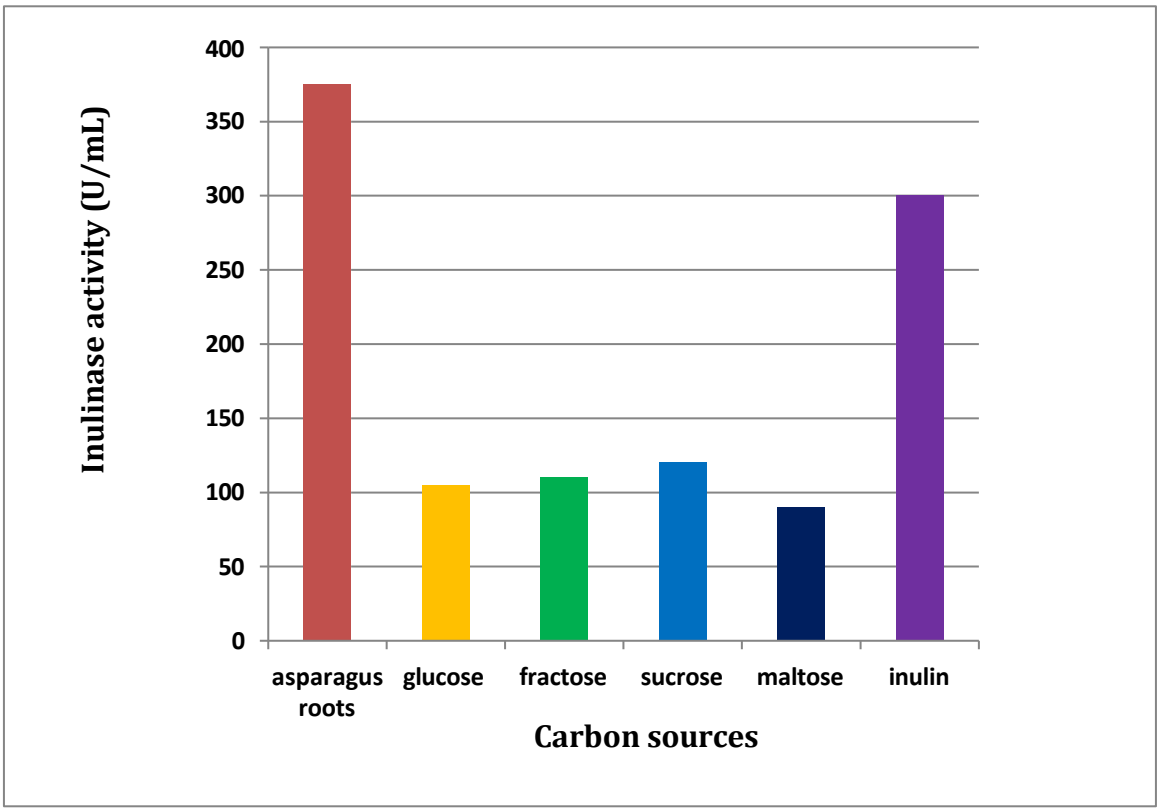

Figure 3
Production of inulinase enzyme on various carbon sources by *A. terreus* ATCC 10020

Multi-factorial designs for optimizing inulinase production Plackett–Burman design

Optimization of the growth medium for the inulinase production by selecting the best nutritional and environmental conditions is important to increase the inulinase yield. A

sequential optimization strategy was applied in this work, where the first phase dealt with screening and identifying the nutritional and environmental factors affecting inulinase production by *A. terreus* once the significant factors affecting inulinase production were determined, the second phase involved ascertaining the combination that leads to the maximum inulinase activity.

In the first phase, a Plackett-Burman experimental design was applied to reflect the relative importance of various factors. The examined levels of the nine culture variables, was applied with twelve different experiments. All the experiments were performed in duplicated, and averages of the observations were presented according to the proposed design. The importance of altering the medium composition to get increased inulinase activity is demonstrated by **Table 2**, which displays the vast range of inulinase activity recorded in the Plackett–Burman experiment, ranging from 100 to 886.43 U/mL. A linear correlation model describes the relationship between the inulinase activity and the nine parameters (temperature, pH, asparagus roots, K_2HPO_4, $MgSO_4.7H_2O$, $NaNO_3$, KCI, $FeSO_4.7H_2O$, and $(NH_4)\ H_2PO_4$).

Temperature, $NaNO_3$, $FeSO_4.7H_2O$, and other factors all had favorable impacts on inulinase synthesis, indicating that inulinase production rose as these factors' concentrations rose. In the Plackett–Burman statistical analysis, variables were considered significant if their confidence level was more than 95% ($p < 0.05$). With *p* values of 0.0022, 0.034, and 0.032, respectively, **Table 3** demonstrates that asparagus roots, KCl, and $NaNO_3$ had the greatest effects. With an R^2 value of 0.94, the Plackett–Burman model demonstrated

a reliable and fair match. Based on the Plackett–Burman design results, the concentrations of asparagus roots, KCl, and $NaNO_3$ were identified as the main relevant parameters investigated on the synthesis of fungal inulinase. These values were then selected for additional optimization using the Box–Behnken design. In the Plackett-Burman statistical analysis, variables with an 86% confidence level were considered significant factors **(Figure 4)**. With P values of 0.002, 0.033, and 0.021, respectively, asparagus roots, KCl, and $NaNO_3$ had the greatest effects. With an R_2 value of 0.86, the Plackett-Burman model was found to have an acceptable and reliable fit. Based on the Plackett-Burman design results, the concentrations of asparagus roots, $NaNO_3$, and KCl were identified as the three main relevant parameters examined on the synthesis of fungal inulinase. These were then selected for additional optimization using the Box-Behnken design.

Table 2

Plackett–Burman experimental design for screening of factors affecting inulinase production by *A. terreus*

Pattern	asparagus roots (g/L)	K_2HPO_4 (g/L)	$NaNO_3$ (g/L)	$MgSO_4$ (g/L)	KCl (g/L)	$FeSO_4$ (g/L)	$(NH_4)H_2PO_4$ (g/L)	T. °C	pH	Inulinase (U/ml)
−+−−+−+++	10	2	0.5	0.3	1	0.05	1	25	7	186.30
+++++++++	5	2	0.5	1	0.3	0.5	1	35	7	440.61
−+++−−−+−	5	0.5	3	0.3	0.3	0. 5	1	35	5	886.43
−−−+−−+−+	10	0.5	3	1	0.3	0.05	4	25	7	266
+−+++−−−+	5	0.5	0.5	1	1	0.5	4	25	5	374.86
−+−+++−−−	10	2	0.5	0.3	0.3	0.5	4	25	5	173.26
−−+−+++−−	5	2	3	0.3	0.3	0.05	4	25	7	734

−−+−−+−++	10	0.5	3	0.3	1	0.5	1	35	5	383.61
+−−+−+++−	10	2	3	1	1	0.5	4	35	7	359.93
+−−−+−−+−	5	2	3	1	1	0.05	1	25	5	586.88
+++−−−+−−	10	0.5	0.5	1	0.3	0.05	1	35	5	100
++−−−+−−+	5	0.5	0.5	0.3	1	0.05	4	35	7	353.43

Table 3

Estimated effects, *t*-ratios and *p*-values for the Plackett-Burman design

Term	Estimate	Std Error	*t* Ratio	*p*-Value
asparagus roots	-41.10	9.48	-4.23	0.002*
$NaNO_3$	132.33	37.92	3.93	0.021*
KCl	-148.80	135.44	-3.47	0.033*
K_2HPO_4	50.55	63.20	0.79	0.491
$FeSO_4$	-12.96	10.98	0.57	0.720
$MgSO_4$	31.23	31.73	0.71	0.610
$(NH_4)\ H_2PO_4$	8.24	6.12	0.27	0.850
T	6.32	9.48	0.68	0.551
pH	-27.20	47.41	-0.64	0.576

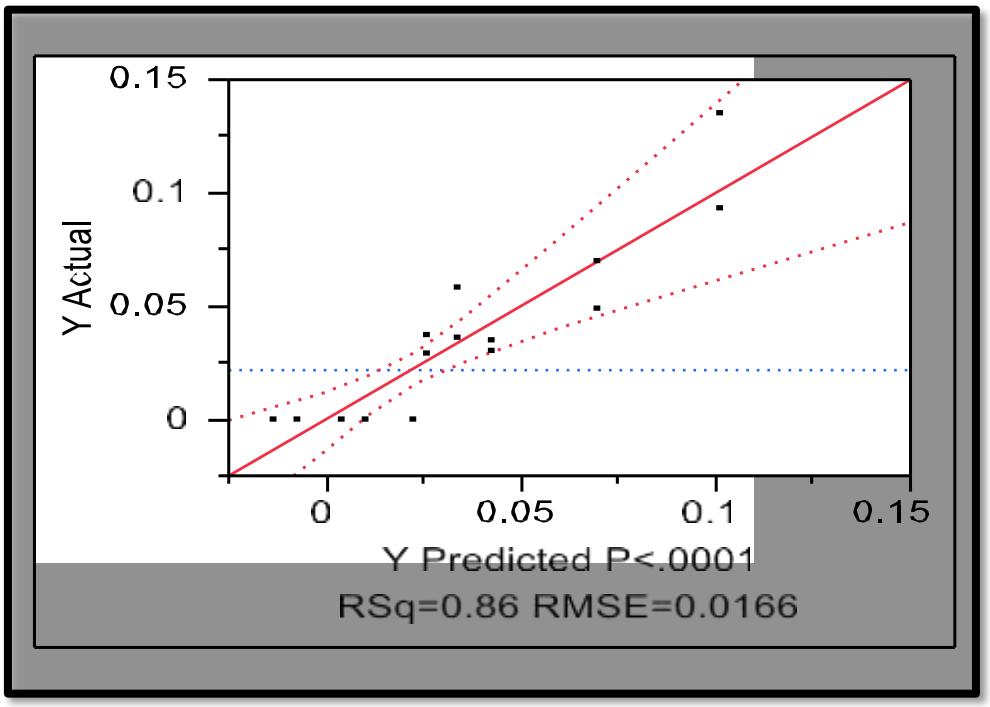

Figure 4
Comparison between predicted and experimental values for inulinase production using asparagus roots

Box-Behnken design

The Box-Behnken design matrix is displayed in **Table 4**. Following the evaluation of each component at three distinct levels (-1, 0, and 1), the experimental and predicted inulinase activities were compared. The Box-Behnken experiment's large range of inulinase activity measurements, which range from 760.40 to 1446.82 U/mL, emphasises how important varying amounts of asparagus roots, $NaNO_3$, and KCl are for increasing inulinase activity.

Table 4

Inulinase activity responses with the Box-Behnken factorial experimental design

Pattern	asparagus roots*	$NaNO_3$*	KCl*	Experimental inulinase (U/ml)	Predicted Inulinase (U/ml)
0-+	7.5	3	0.3	1146.12	1129.28
++0	2.5	5	0.2	1360.45	1333.87
+0-	2.5	4	0.1	1426.69	1405.45
00+	7.5	4	0.3	1200.56	1190.45
+-0	2.5	3	0.2	1320.75	1300.76
0+-	7.5	5	0.3	1126.42	1102.02
+0+	2.5	4	0.3	1446.82	1450.04
-0+	5	4	0.1	866.24	843.83
0-+	7.5	3	0.1	1166.56	1114.3
0-0	7.5	3	0.2	1134.16	1102.88
0++	7.5	5	0.1	1233.81	1213.75
--0	5	3	0.2	846.59	832.29
0+0	7.5	5	0.2	1126.76	1110.72
-+0	5	5	0.2	760.40	745.86
-0+	5	4	0.3	766.22	750.99

*** g/L**

Table 5

Estimated effects, *t*-ratios and *p*-values for the Box-Behnken design

Term	Estimate	Std Error	t Ratio	p-Value
Intercept	1299.2	10.19	55.30	<0.0001*
asparagus roots	255.6	9.76	15.55	<0.0001*
$NaNO_3$	43.34	8.34	-4.23	0.0091*
KCl	25.2	9.33	1.69	0.271

asparagus roots*$NaNO_3$	**72.2**	**12.33**	**4.92**	**0.0070***
asparagus roots*KCl	**7.00**	**13.44**	**0.62**	**0.511**
asparagus roots*KCl	**-32.3**	**14.33**	**-1.36**	**0.130**
asparagus roots* asparagus roots	**-83.2**	**15.98**	**-5.61**	**0.0040***

The linear, quadratic, and interaction factor coefficients are computed and shown in **Table 5**. Coefficients and the model are deemed significant if *P* is less than 0.05. It demonstrates that $NaNO_3$ and asparagus roots had substantial *P* values of 0.0070 and^0.0001, respectively. Asparagus roots*$NaNO_3$ was the only significant interaction term. The word asparagus roots*asparagus roots was a notable quadratic term. Additionally, the model *P* value was less than 0.0001, demonstrating the model's accuracy in predicting inulinase activity.

The concentrations used in each experiment are represented by the values for the variables in the equation. Inulinase activity of 1446.82 U/ml was obtained under ideal circumstances, which included asparagus roots, 2.5 g/L; $NaNO_3$, 4 g/L; KCl, 0.3 g/L; K_2HPO_4, 0.5 g/L; (NH_4) H_2PO_4, g/L; $FeSO_4$, 0.5 g/L; $MgSO_4$, 0.3 g/L; and temperature, 35˚C and pH, 5.0. Both carbon and nitrogen sources served as limiting substrates, according to the RSM data, and variations in their concentration had an impact on the formation of inulinas.

More inulinase activity was generated by *Aspergillus terreus* ATCC 10020 than by the majority of other species documented in the literature. Using 1% *Ceratonia siliqua* extract as a substrate under Plackett-Burman design optimized conditions, *A. niger* 204447 reached a maximum inulinase activity of 1565 U/mL **[16]**. On 1% Dahlia tuber, *A. tamarii* AR-IN9

produced 72.00 U/mL inulinase activities [29]. Using *Penicillium oxalicum* cultivated on 2% inulin, [30] optimized the synthesis of inulinase and obtained an activity of 12.06 U/mL.

Using Taguchi design, **Abou-Taleb et al. [31]** maximized the production of inulinase from *Candida oleophila* grown on 1% chicory root, achieving an activity of 47.29 U/mL. On 3% American agave, *A. terreus* recently produced 130.00 U/mL inulinase activities [32].

Ammonium sulfate precipitation and dialysis

Ammonium sulphate saturation is used to demonstrate the activity of inulinase enzymes in **Table 6**. The enzyme inulinase was purified by adding 70% ammonium sulphate. The reason for the decrease in enzyme activity at 70% concentration is that protein is thought to be sufficiently precipitated, meaning that only a portion of the remaining enzymes can precipitate at that concentration. Additionally, the higher concentration of ammonium sulphate can decrease selectivity in the deposition process [33]. 70% ammonium sulphate has also been shown to enhance the specific activity of inulinase. The addition of ammonium sulphate to 70% was then chosen for the next stage of dialysis. In order to preserve the stability of the enzyme during storage, dialysis uses passive and selective diffusion by semi-permeable membranes to remove tiny and undesirable components from a solution, such as salts and ions [34]. **Table 6** provides information on the purification of inulinase. The crude enzyme has 670 mL of activity. A 70% saturated ammonium sulphate solution was used to purify the enzyme, and then ultra-filtration was used. After purification, the enzyme's specific activity was 52106 U/mL with a 60% recovery rate. It had a purification fold of 3.6.

The specific activity of the enzyme in our investigation is higher than that of the majority of published literature [35].

Table 6
Purification of inulinase enzyme

Purification step	Volume (mL)	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Purification folds	Yield %
Crude enzyme	**670**	**288886**	**0.10**	**14320**	**1**	**100**
Ammonium sulphate 70% precipitation,	**425**	**231700**	**0.35**	**33093**	**2.3**	**80**
Dialysis-filtration	**80**	**173500**	**0.67**	**52106**	**3.6**	**60**

Estimation of molecular weight of inulinase enzyme produced by A. terreus ATCC 10020

The molecular weight for the purified inulinase of *A. terreus* ATCC 10020 was estimated using gel electrophoresis (SDS-PAGE) for final purification step including ultra filtration **(Figure 5)**. From the SDS-electrophoresis pattern was observed only one band, with characterized molecular weight 62.0 KDa. On SDS-PAGE, pure inulinase appeared as a single band, indicating that it was a monomer made up of a single polypeptide chain. The inulinase had a molecular weight of around 60 kDa. The findings are consistent with those of **Cho et al. [36]**, who found that the molecular weight of fungal inulinases varies from 30 to 175 kDa. With a molecular weight of 66 KDa, exo-inulinase was generated from *A. tamarii* utilizing kaolin clay as a carbon source **[25]**. **Gill et al. [37]** used inulin as a carbon source and found that the inulinase from *A. fumigates* has a molecular weight of 176.5 kDa.

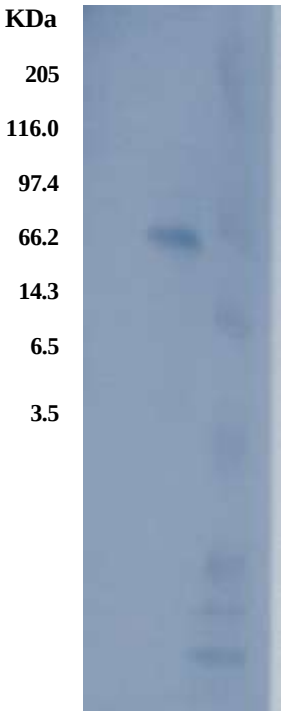

Figure 5
SDS-PAGE analysis of the purified inulinase from *Aspergillus terreus* ATCC 10020
Right lane: molecular weight markers
Lift lane: purified enzyme

Determination of optimum temperature and thermo stability of inulinase

The temperature variation used was 30-90˚C with an interval of 10˚C. The optimum temperature was determined based on the temperature that had the highest inulinase activity.

The enzyme's activity rose with temperature, peaking at 55˚C. It then decreased at 60, 70, 80, and 90˚C, reaching 80, 66, 50, and 30% of its peak activity **(Figure 6)**. The majority of reported inulinase generated by Aspergillus species has a temperature range of 50 to 60°C, which is also the observed optimal temperature. According to **Garuba et al. [25]**, the exoinulinase enzyme generated from *A. tamarii* has an optimal temperature of 60°C.

According to **Huitron et al. [38]**, the *A. niger* CH-A-2010-produced exo-inulinase exhibited its greatest activity at 50°C. Comparing our strain to inulinase from other Aspergillus species, we found that it is highly robust in terms of thermal stability, retaining 93% of enzyme activity after 30 minutes at 40˚C and around 31.9% of activity after the same period of time at 90˚C **(Figure 7)**.

A. tamarii was used by **Sabre et al. [29]** to manufacture inulinase, which exhibited 75% temperature stability at 50˚C for 90 min. *A. niger* was used to generate inulinase by **[39]**, which only maintained 21.8% of its activity after 6 hrs at 60˚C. Thermal stability is correlated with molecular interactions such as hydrophobic and electrostatic contacts, as well as intermolecular bonds like hydrogen and disulphide bonds. Thermal stability is essential in industrial applications, particularly when biomass is hydrolyzed at high temperatures. Inulinases with an ideal temperature over 50˚C are essential for the commercial production of fructo-oligosaccharides and fructose from inulin because high temperatures allow for proper inulin solubility and protect against microbial contamination **[40]**.

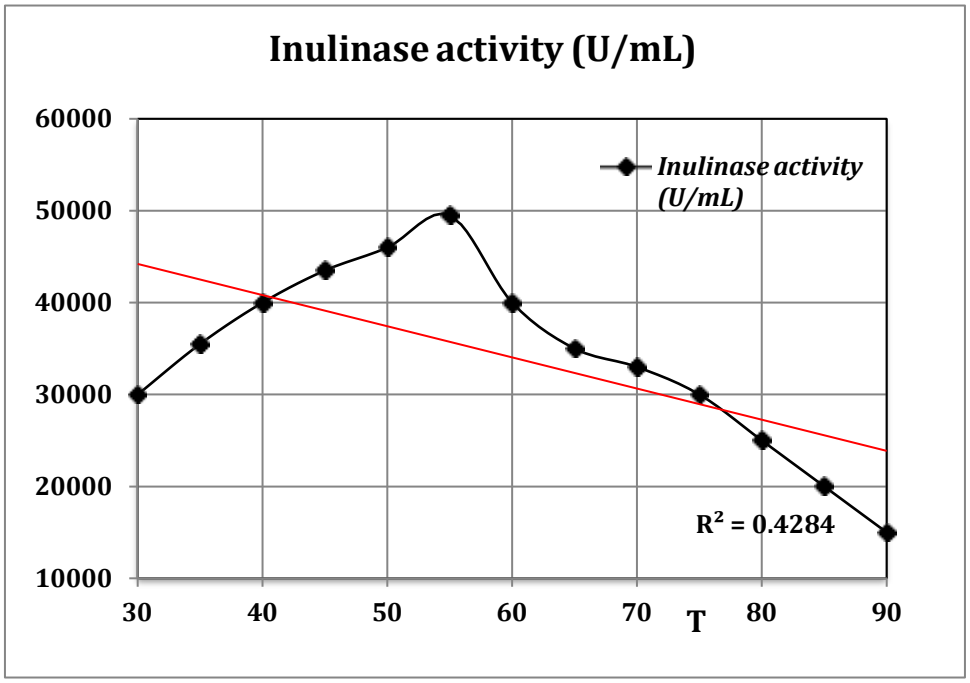

Figure 6
Optimal temperature of the purified inulinase from *A. terreus*

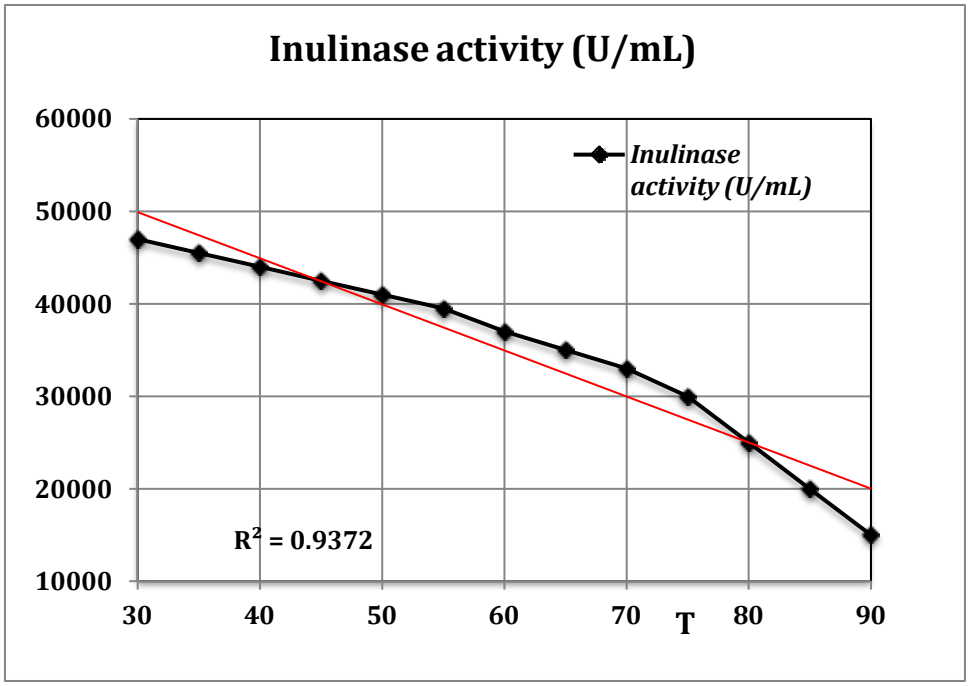

Figure 7
Thermo stability of the purified inulinase from *A. terreus*

Determination of optimum pH

Determination of optimum pH was carried out by measuring the activity of inulinase enzyme produced by the purified enzyme in the pH range 3-9. The ideal pH range for enzyme activity was between 5 and 6. Activity peaked at pH 6 and subsequently decreased at pH 7, 8, and 9, reaching 85, 53, and 31.9 percent of its maximal activity **(Figure 8)**. Multiple investigations reported the same ideal pH **[41]**. The inulinase enzyme maintained 100% activity at pH 4, but after 24 hrs, 98, 87, 65, and 40.0% of activity was still present when tested at pH 5, 6, 7, 8, and 9, respectively.

Compared to other species, the studied strain's inulinase was more stable at acidic pH values **(Figure 9)**. At pH 6–8, the inulinase generated by *A. ficuum* remained stable **[42]**. According to **Garuba et al. [25]**, *A. tamarii*-U4-produced inulinase was stable at (5 to 6). They are more favorable industrially than other microbial sources due to their low pH and superior temperature stability **[43]**. The catalytic portion of the enzyme is affected by the H^+ ion in the pH solution, which results in modifications to the enzyme's conformational structure. By altering the structure or residual charge of amino acids that aid in substrate binding, pH variations have an impact on enzyme performance. Changes in the charge of the enzyme's amino acid residues' functional groups are probably the cause of a drop in activity at a certain pH **[44]**.

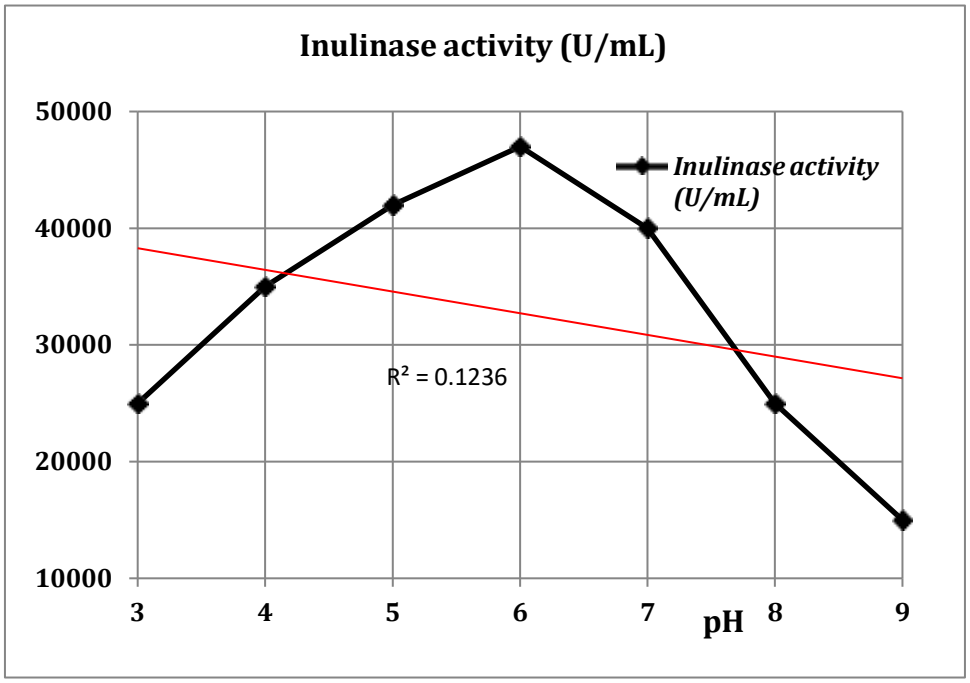

Figure 8
Optimal pH of the purified inulinase from *A. terreus*

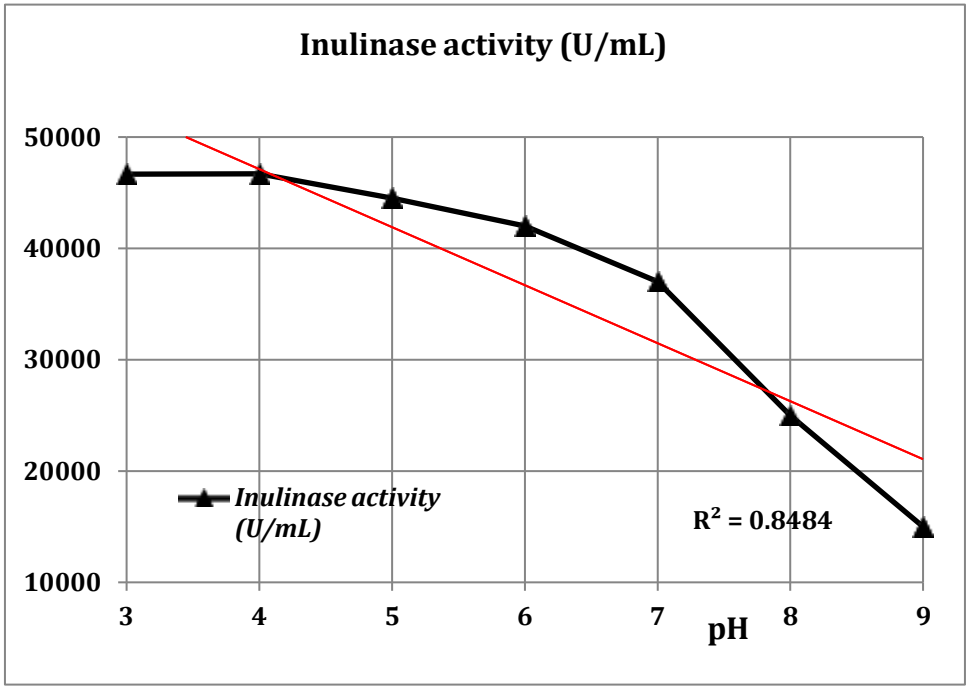

Figure 9

pH stability of the purified inulinase from *A. terreus*

Effect of ions on enzyme activity

The addition of ions was carried out to determine the effect of several ions on the activity of inulinase enzymes produced by *Aspergillus terreus* ATCC 10020. The ions used are $CaCl_2$, $MnCl_2$, $MgCl_2$, $FeSO_4$, $CuSO_4$, $CoCl_2$, $ZnSO_4$, $HgCl_2$ and NaCl with a concentration of 10 mM. The impact of metal ions on the activity of inulinase was evaluated. While Mg^{2+}, Mn^{2+}, and Cu^{2+} greatly increased the enzyme activity, we discovered that Hg^{2+}, the normal inhibitor of most enzymes, was the most powerful inhibitor **(Table 7)**. Fe^{2+}, Zn^{2+}, Co^{2+}, and Ca^{2+} were also shown to have a little inhibitory impact. Mn^{2+} has been shown to stimulate the activity of *Streptomyces* sp. **[45]** and *A. niger* (mutant strain) **[46]** inulinases.

Table 7
Effects of various ions on inulinase activity

Ion (mM)	Relative activity*(%)
None (control)	100
$CaCl_2$	92
$MnCl_2$	132
$MgCl_2$	110
$FeSO_4$	85
$CuSO_4$	104
$CoCl_2$	90
$ZnSO_4$	88
$HgCl_2$	5
NaCl	90

**Relative activities were calculated in relation to the enzyme activity without ion, which was considered to be 100%.*

Conclusions

Even though a lot of microorganisms can manufacture inulinase, a low-cost feedstock fermentation method that works well for commercial use still has to be developed. Classical methods are frequently used to optimize fermentation processes, testing one element at a time. The production of inulinase from asparagus roots (*Asparagus officinalis*) by *A. terreus* ATCC 10020 in shake-flasks can be increased by 1.63 times, from 760.40 to 1446.82 U/mL, by identifying key process variables through Plackett-Burman experiments and optimizing their levels using the Box-Behnken design. The method which uses inexpensive agricultural waste as substrates, should to have a strong chance of expanding further for use in industrial settings. Overall, the *A. terreus* ATCC 10020 strain produced thermo-stabilty inulinase, an enzyme with a low molecular weight (KDa 66). It demonstrated excellent stability at acidic pH and comparatively high temperatures. The results of this study will undoubtedly offer helpful recommendations for using asparagus roots as a substrate for pilot-scale inulinase production. The properties of the purified inulinase make it an ideal candidate in hydrolysis of inulin containing agro-wastes and other food and pharmaceutical industries. The purified inulinase's characteristics make it a perfect option for the hydrolysis of inulin-containing agricultural waste as well as other food and pharmaceutical sectors.

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