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Optimization of Inulinase Production by a Fungal Species Isolated from Rotten Garlic Samples

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Abstract

Introduction: Inulinases are β-fructohydrolase enzymes that catalyze the hydrolysis of inulin. Recently, this enzyme has gained much importance mainly due to its ability to produce high-density fructose syrup using inulin as a raw material. In the current study, screening of inulinase-producing microorganisms was carried out from the rhizosphere soil of the Dahlia plant and rotten garlic samples.

Materials and Methods: The inulinase activity was detected with the help of 3,5-dinitrosalicylic acid (DNSA) and Seliwanoff's method, and the organism showing the highest potential was selected for further optimization studies.

Results: The optimum culture conditions for inulinase production, by the test fungal culture, were observed when 5% inoculum was added to the minimal medium (pH 5.5) containing 1% inulin/ costus root powder as a carbon source and 0.15% $NaNO_3$/ NH_4Cl as a nitrogen source, and incubated at 30°C for 48h under shaker conditions (200 rpm). Maximum enzyme activity was observed at pH level of 5 and temperature level of 45°C, with thermal stability noted between 35°C-55°C. The I/S value of the crude enzyme was calculated to be 0.45 indicating true inulinase activity. It showed no significant inhibition in the presence of metal ions such as Zn^{2+}, Mg^{2+}, and Fe^{3+}. The Ca^{2+} ions showed partial inhibition whereas Cu^{2+} ions showed an enhancement in the enzyme activity.

Conclusions: These factors may present the test fungal culture isolated in the present study to be a potential candidate for the production of thermo-tolerant and metal resistant inulinase enzyme in order to be used for various biotechnological processes.

Keywords: Enzyme Activity, Inulinase, I/S Value, Optimization, Seliwanoff's Method

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Introduction

The biochemical reactions form the basis of all life processes occurring in living things and catalytic enzymes are the key components of this setting. Recently, numerous advances have been made in the food-enzyme technology sector, which offers benefits like higher product quality, lower manufacturing costs, reduced energy consumptions and less waste productions.[1] In addition, it may also help the food technologists to address global health concerns related to food safety viz., obesity, malnutrition and toxicity.[2]

Among the enzymes that hydrolyze polymers into monomers i.e., simple sugars, inulinase has attracted much attention in recent years. These enzymes can hydrolyze inulin- a complex polymer that serves as reserve carbohydrates in plants mainly belonging to the Asteraceae and Campanulaceae family.[3] Inulin consists of a linear ß-2-1 linked poly-fructose chains displaying a terminal glucose unit. Examples of plants containing inulin in large quantities include Jerusalem artichoke *(Helianthus tuberosus* L.*)*, chicory (*Cichorium intybus*), dahlia (*Dahlia pinnata* Cav), burdock (*Arctium lappa*), costus (*Saussurea lappa*), cardon (*Cynara cardumculus*), yacon (*Smallanthus sonchifolius*), dried tubers, Rye grass (*Lolium multiflorum*), onion (*Allium cepa*) and garlic (*Allium sativum*).[4-6] Some of these plants contain up to 50% inulin by weight.[6,7]

Inulinases are designated as 2-1-β-D fructan hydrolase (EC 3.2.1.7), and most inulinases are ß-fructo-furanosidase that hydrolyze fructose moieties at the terminal ß-2-1 position.[8] They may show endo-acting or exo-acting hydrolysis action on inulin. Exo-acting inulinases act by successively splitting off terminal fructose units in sucrose, raffinose, and inulin to liberate fructose (i.e., a monosaccharide) as the main end product, and oligosaccharides are produced in low amounts. In contrast, the endo-acting inulinases produce oligosaccharides like inulotriose, inulotetraose and inulopentaose as the main end product, and monosaccharides in minor amounts.[9-11] In general, the inulinases isolated from fungal species are mostly extracellular and exo-acting type.[12]

The hydrolyzation of inulin by inulinase mainly results in the formation of fructose (almost 95%), and a small amount of glucose. These ultra-pure fructose syrups are 1.5-2 times sweeter than sucrose, safe for consumption and can be utilized by diabetics. Hence, it is extremely useful in the food industry.[3] Conventional fructose production from starch needs at least

three steps, including alpha-amylase, amyloglucosidase and glucose isomerase action, yielding approximately 45% fructose solution. Fructose formation from inulin, on the other hand, is a single step reaction and yields up to 95% pure fructose.[13] Fructose is present in numerous products including soft drinks, fruit drinks, sports drinks, baked goods, candies, jams, yoghurts, condiments, canned and packaged foods, and other sweetened food products. In certain commercial juices, the use of fructose instead of glucose or sucrose for sweetening purposes provide fewer calories, better flavour preservation, and avoid the bitter taste of artificial sweetener like saccharin. It also improves the appearance of the finished products. This is due to the formation of melanoidin and its decomposition into 'hydroxymethyl furfural', which readily condenses with amino acids in proteins and peptides to give attractive colour and flavour. Moreover, since fructose is much more soluble in water and alcohol than sucrose or fructose, bulk handling is easier, and syrups containing fructose are less liable to crystallization. Furthermore, fructose is utilized normally even when blood sugar metabolism is abnormal. Interestingly, studies have shown that small amounts of oral fructose may improve glycemic control in people with diabetes.[7]

Although inulinases were first isolated from plants, the tedious purification processes have discouraged the use of plant-based inulinases for industrial processes. The feasible production of these enzymes, to be used at the industrial level, is possible with the help of microorganisms that act as bio-reactors for the production of the same. Both intracellular and extracellular types of inulinases are known.[3] On a commercial level, the fungal strains of *Aspergillus* sp. and yeast strains belonging to *Kluyveromyces* sp. (diploid yeast) are the most common and preferred choice for inulinase production.[12] It is not recently, that the microbes are exploited in the food industry. The common example includes soy sauce production with the help of *Aspergillus* sp.[14] Other industrial applications of inulinases include the production of ethanol, acetone, butanol, brandy, single cell protein etc. A complete fermentation of crude inulin extract into ethanol can be obtained using *Kluyveromyces fragilis* in complete anaerobiosis. Also, continuous ethanol and single cell protein production are possible using suitable microbial cells.[15]

The current study was carried out with an aim to determine the optimum culture conditions for inulinase production by test organisms isolated from natural sources, and to investigate the kinetic parameters for inulinase activity.

Materials and Methods

Enrichment and Isolation of Inulinase Producers

One gram of soil collected from the rhizosphere region of Dahlia plant and a rotten garlic bulb were used as samples for the enrichment of inulinase producers. A 0.5 g of the above sample was mixed with 30 mL sterile minimal medium [composition in g/L: $(NH_4)_2SO_4$ (0.05), $MgSO_4.7H_2O$ (0.2), KH_2PO_4 (3), $NaNO_3$ (1.5), KCl (0.5), $FeSO_4.7H_2O$ (0.01), inulin (3), pH 7] containing inulin as a sole source of carbon. It was then incubated at 30°C for 24 hours under shaker conditions. After incubation, 1.5 mL of the above enriched medium was transferred to another flask containing 30 mL of fresh medium and incubated further at 30°C for 24 hours under shaker conditions. A 100 µL sample of this enriched medium was isolated on solid minimal medium and checked for inulinase activity by the 3,5-Dinitrosalicylic acid method.[16]

Qualitative and Quantitative Screening of Inulinase Producers

The inulinase activity of the obtained isolates was assayed using a protocol described by Miller in 1959.[17] The cell density of bacterial isolates and fungal spores were adjusted to 1×10^8 cells/mL using the Browns opacity tube and hemocytometer respectively. A 1.5% inoculum size of test isolates was then suspended in 30 mL minimal medium and was allowed to grow at 30°C. Sample aliquots were collected after 24, 48 and 72 hours intervals, and centrifuged at 5000 rpm for 15 minutes. This cell-free supernatant (CFS) was used as a crude enzyme for determining the inulinase activity.

Qualitative assay for screening of inulinase production was carried out by the Selivanoff method. In this assay, a solution was prepared by adding 2mL of 0.2% inulin to 2 mL of acetate buffer solution (0.01 M, pH 4.5). This solution was mixed with 0.5 mL CFS and incubated at 50°C for 20 minutes. After incubation, the tubes were kept in boiling water bath for 10 minutes to inactivate the enzyme and then cooled at room temperature. The development of red colour on the addition of 0.5 mL of above solution to 5 mL of Seliwanoff's reagent, and boiling it for 10 minutes, confirmed the presence of fructose (i.e ketose sugar).

The reaction mixture was prepared similarly for quantitative assay except that after the inactivation of the enzyme, 1mL of DNSA reagent was added to 1 mL of the above mixture and kept in boiling water bath for 10 minutes. The resulting mixture was then cooled and 6 mL of distilled water was added to it. Finally, the absorbance of the supernatant was measured at 575 nm using a spectrophotometer. The heat-inactivated crude enzyme prepared by boiling CFS at 100°C for 10 minutes was used as a blank. A calibration curve was plotted using fructose as a standard sugar (100-1000 µg/mL) and the enzyme activity was calculated in U/mL. One unit of inulinase activity was defined as the amount of enzyme that produced one µmole of fructose per minute under assay conditions.

Optimization of Culture Conditions for the Production of Inulinase Enzyme

Optimization of culture conditions for inulinase production was studied in 25 mL minimal medium containing 1% inulin as a sole carbon source and was incubated at 30°C for 48 hours under shaker conditions. These conditions were investigated by varying one parameter at a time while keeping the others constant. The varying parameters included the inoculum size (1%, 2%, 5% and 10%), pH (3.5, 4.5, 5.5, 6.5, 7, 7.5, 8.5 and 9), aeration condition i.e., static or shaker (200 rpm). In addition, the effect of different carbon sources was studied by replacing 1% inulin in the minimal medium with 1% of different carbon sources (viz., glucose, sucrose, fructose, inulin, dry

garlic powder, dry onion powder and crude costus root powder). Similarly, the effect of 0.15% of different nitrogen sources (viz., $NaNO_3$, $[NH_4]_2SO_4$, Peptone, NH_4Cl and yeast extract) were also studied by incorporating one at a time in the minimal medium.

At the end of the incubation period, the CFS was collected and the inulinase activity was assayed using the DNSA method.

Determination of I/S Value of Inulinase

Since inulin has no fixed molecular weight, its Km value cannot be determined. Furthermore, an increasingly smaller residual inulin chain is presented as a substrate to the enzyme by gradual hydrolysis (i.e removal of fructose end group); thus influencing the rate of hydrolysis. Hence, inulinase shows non-hyperbolic kinetics and differs from the standard Michaelis-Menten kinetics for enzymatic activity.[18] For this reason, the ratio between these two activities is commonly expressed as the I/S ratio i.e.,

$$I/S = \frac{\text{Total units of inulinase activity}}{\text{Total units of invertase activity}}$$

Where the inulinase unit is the amount of enzyme hydrolyzing 1 µmol fructose/min and the invertase unit is the amount of enzyme hydrolyzing 1 µmol sucrose/min.

The I/S value for real invertases are extremely low values. Conversely, the I/S value for true inulinase are high. Hence, I/S values are generally presented in studies related to inulinase enzymes.

In our study, the enzyme activity of inulinase was checked by incubating 2 mL of 0.2% inulin in 0.01M acetate buffer (2 mL) and 0.5 mL of CFS (precipitated enzyme) at 45°C for 20 minutes and was then assayed for the presence of reducing sugars by the DNSA method. Similarly, the enzyme activity of invertase was checked by replacing 0.2% inulin with 1% sucrose. The calibration curve of inulinase and invertase was prepared with 180-1800 µg/mL of fructose and 100-1000 µg/mL glucose solution respectively. The I/S value was then calculated.

Kinetic Studies of Inulinase Enzyme

The kinetic studies were carried out using CFS as a crude enzyme. It was assayed to determine the optimum conditions of pH (4-9) and temperature (30°C, 35°C, 40°C, 45°C, 50°C, 55°C) for optimum enzyme activity. In addition, the effect of metal ions (viz., $FeSO_4$, $MgSO_4$, $CaCl_2$, $CuSO_4$, $ZnSO_4$) were also studied. For this purpose, 0.5 mL of 0.01M solutions of different salts and 2 mL of inulin (0.2%) in 0.01M acetate buffer (2 mL) was taken into tubes to which 0.5 mL of enzyme solution was added to give a final concentration of 0.001M in an assay system containing salts. This solution was incubated at 45°C for 20 minutes. Control was kept without the addition of any salts. Inulinase activity was assayed at the end of the incubation period by the DNSA method described above.

A Comparative Study Between Submerged and Solid State Fermentation

Commercial quality wheat bran, rice bran and corn flour were purchased from the local market and were used as substrates for solid state fermentation (SSF). The substrates were supplemented with acidified mineral solution (composition in g for 100 g dry substrate: KH_2PO_4 (3.5); $MgSO_4.7H_2O$ (0.5); $MnSO_4.7H_2O$ (0.0028); $FeSO_4.7H_2O$ (0.0087); $ZnSO_4.7H_2O$ (0.0025); $CaCl_2$ (3.5)) and sterilized by autoclaving it at 15 psi for 20 minutes. The initial pH and moisture of the substrate were set at 5.5%-6% and 60% respectively. Fermentation was carried using 25 g autoclaved substrate in 250 mL conical flask inoculated with 5% (v/w) spore suspension (i.e 2.5×10^6 spores per mL). Each flask was incubated at 30˚C. The samples were withdrawn after 48 hours of incubation. For determination of enzyme activity, a weighed quantity of the fermented matter (10 g) was treated with 50 mL distilled water and mixed thoroughly on a magnetic stirrer for 30 minutes. The whole content was then filtered through a muslin cloth and the filtrate was then collected and used as a crude enzyme. The enzyme assay was performed using the DNSA method as described above.

The submerged state fermentation (SmF) was carried out using a 50 mL sterile minimal medium containing inulin, inoculated with 5% (v/w) spore suspension and incubated at 30°C for 48 hours under shaker conditions. The CFS collected after incubation time was used as a crude enzyme and the enzyme assay was performed using the DNSA method.

Results and Discussion

Enrichment and Isolation of Inulinase Producers

In the present study, three cultures were successfully isolated from the enriched medium. These included a gram-negative, non-motile cocco-bacillary rod-shaped bacteria with polar arrangement isolated from rhizosphere soil of dahlia plant (IS-1); a gram-positive, non-motile cocci shaped bacteria, isolated from rotten garlic bulb (IS-2) and a fungal culture showing black spores isolated from rotten garlic bulb (IS-3). Qualitative screening of inulinase activity was confirmed in all three isolates by the Selivanoff method. However, the fungal isolate showed the best enzyme activity, comparatively, determined by the DNSA method and was used for further studies. The enzyme activities observed by the cultures isolated in our study are presented in Figure 1.

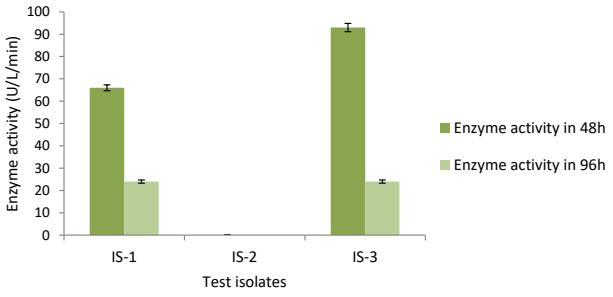

Figure 1. Enzyme Activity Observed by the Inulinase Producers.

Optimization of Culture Conditions for the Production of Inulinase Enzyme

Recent research has greatly increased our understanding of the ways in which the growing cell can control its synthetic processes by mechanisms such as end-product inhibition and restriction of enzyme formation by gene controlled repression.[12] It is, therefore, necessary to optimize culture condition to obtain maximum production of the enzyme. The Figures 2-10 indicate the optimum parameters for inulinase production and activity. In this study, the optimum inulinase production was observed when 5% inoculum was added to the minimal medium (pH 5.5) containing inulin as a sole carbon source and incubated at 30°C for 48 hours under shaker conditions.

Studies on the effect of inoculum level on inulinase production (Figure 2) showed that 5% inoculum size resulted in the highest yield (118.8 U/L/min) of inulinase enzyme at the end of 48h incubation. The production of enzyme showed a drastic decrease with any further increase in the inoculum level. Another recent study carried out on inulinase production from *Aspergillus niger* ATCC 20611, reported that optimum inulinase, as well as biomass production, was achieved when the inoculum size was adjusted to 6% (v/v).[19] Previous studies carried out on *A. niger* strain van Tieghem and AUP19 has reported an optimum inoculum size of 5% and 10% respectively for optimum inulinase production using pure inulin as substrate.[20,21]

Higher levels of inoculum may cause depletion of nutrients within a short span of time adversely affecting the enzyme production. Also, the lower levels of inoculum size may result in a longer lag phase and a slow initial growth rate; thus increasing the time of incubation. Hence, optimization of inoculum size is the prime consideration in production related studies.[22]

Aeration is another parameter which affects the production of enzymes. Agitation allows aeration of the medium due to rotary movement of the shaker, and uniform mixing of nutrients and oxygen. This helps in optimum biomass production due to proper nutrient utilization and ultimately optimum enzyme production. In the present study, increased aeration under shaker conditions supported better enzyme production (120 U/L/min) as compared to static condition (46 U/L/min) presented in Figure 3. Similar observations have also been reported by other researchers. Inulinase production from *A. niger* ATCC 20611 using inulin, and *Kluyveromyces marxianus* YS-1 using root tubers of *Asparagus officinalis* was found to be optimum at 150 rpm agitation speed respectively.[19,22]

Figure 4 shows the effect of pH on inulinase production by the test isolate. In our study, the pH range of 5.5-6.5 was found to be suitable for the growth of test culture and enzyme production, with an optimum inulinase production at pH 5.5 (120 U/L/min). Extremely low levels of enzyme production (26 U/L/min) were observed at pH 3.5 and 7 respectively. No increase in biomass and hence enzyme production was noted below pH 3.5 and above pH 7 in the present study. Another similar study reported optimum enzyme production by *Bacillus* sp., at pH 6 and temperature of 37°C using Dhalia as raw inulin source.[23] A recent study reported optimum inulinase production by *Penicillium oxalicum* BGPUP-4, using carrot pomace, in a SSF between pH 5 and 7.[24]

Figure 5 presents the effect of incubation time on inulinase production by the test culture. It showed optimum inulinase production in 48 hours (93 U/L/min), and a decrease in the same with a further incubation period. By the end of 72h, 50.53% reduction in enzyme production was observed. In contrast to our findings, an incubation period of 72 hours and 60 hours was found to be optimum for inulinase production by *A. niger* AUP19 and *K. marxianus* YS-1 respectively.[21,22]

Apart from the physical parameters discussed above, the

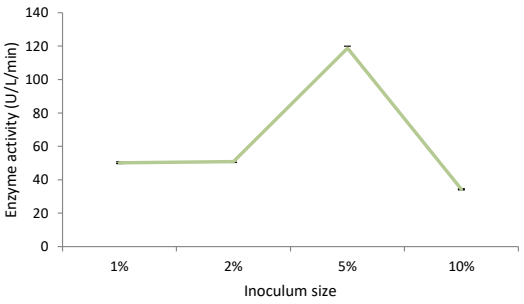

Figure 2. Optimization of Inoculum Size for Inulinase Production by the Test Culture.

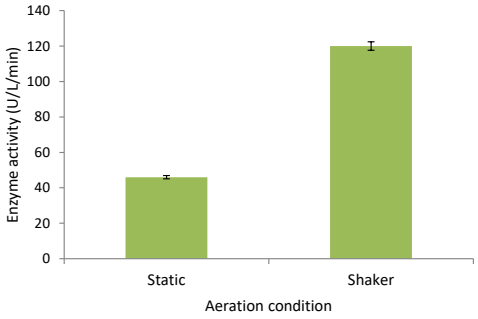

Figure 3. Optimization of Aeration Condition for Inulinase Production by the Test Culture.

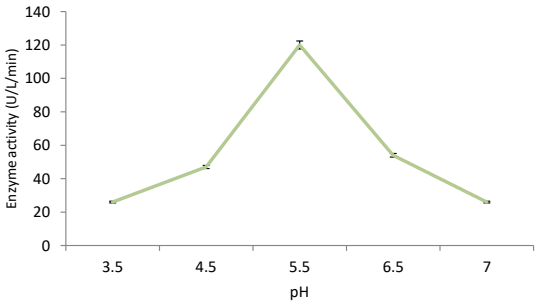

Figure 4. Optimization of pH for Inulinase Production by the Test Culture.

source of carbon and nitrogen in the growth medium greatly affects the enzyme production by microbes. It is generally observed that the media which are low in free sugar or high in complex polysaccharides favour inulinase production. Also, the addition of inulin as a carbon source in the growth medium is usually a prerequisite for the enzyme production.[12] However, many crude sources of inulin are studied for inulinase production by micro-organisms. The optimization of carbon sources for enzyme production may further help us study catabolite repression in microbial cultures and suitable inducers for inulinase biosynthesis.[25] It has been observed in the case of many organisms that inulin serves as the best inducer for inulinase production followed by sucrose, fructose and glucose.[18] It has also been reported in general that inulin serves as the best substrate for microbial strains showing only inulinase activity, and sucrose serves as the best substrate for microbial strains showing additional invertase activity.[26] Similarly, studies of the effect of various nitrogen sources on inulinase production are equally important since individual fungal species are most likely to show a preference for a specific nitrogen source for optimum inulinase production and activity.[21]

In the present study, pure inulin was found to be the best source of carbon (120 U/L/min) followed by Costus root powder (66 U/L/min) as presented in Figure 6. A considerable amount of inulinase (46 U/L/min) was produced using garlic powder as a source of carbon. Sucrose and glucose minimally supported the growth of test culture giving a poor enzyme yield of 26 U/L/min, whereas onion powder completely failed to support any growth in the medium. Similarly, among the nitrogen sources (Figure 7), $NaNO_3$ and NH_4Cl proved to be most effective for the production of inulinase with an enzyme yield of 120 U/L/min followed by peptone (72 U/L/min). Yeast extract showed low enzyme production of 46 U/L/min.

In contrast to our findings, inulinase production by a Bacillus sp. was found to be highest in medium containing onion as a carbon source in a previous study (25.9 U/mL).[23] A recent study reported the use of carob extract for inulinase production from *Aspergillus niger*.[27] In an earlier study, 0.5% chicory powder was reported to be the best carbon source for inulinase production by *Xanthomonas* sp.[28] Another study reported 3% chicory roots and corn steep liquor as best carbon and nitrogen sources for inulinase production by *Aspergillus tamarii* AR-IN9.[29] In another study, *K. marxianus* showed optimum inulinase production using 20% Dahlia extract as a carbon source and 2% yeast extract as a nitrogen source in 96 hours at 28°C and 120 rpm.[30] Another study reported maximum inulinase production by *A. niger* AUP19 (176/U/mL) in a medium containing 5% (w/v) inulin and galactose as an additional carbon source, and corn steep liquor and $(NH_4)H_2PO_4$ as nitrogen sources.[21] *K. marxianus* YS-1 showed maximum inulinase production in a medium containing raw inulin (3.5%) and beef extract (2%) as a carbon and nitrogen source respectively.[22] Another study has also reported a significant relationship between C/N ratio and inulinase production and how its optimization can lead to higher biomass production and I/S ratio.[31] However, further studies are required to confirm these findings.

Partial Purification of Inulinase Enzyme

In our study, the enzyme precipitate was not obtained and hence CFS was used as a crude enzyme this study. This failure may be due to the low concentration of enzyme or extremely sensitive nature of enzyme produced which could not survive the salt precipitation and temperature conditions used in this study. In previously published studies, successful purification of inulinase from *A. niger* van teighem was carried out and 83% enzyme was recovered by acetone purification method.[26] In another study, the mycelial, as well as extracellular inulinase

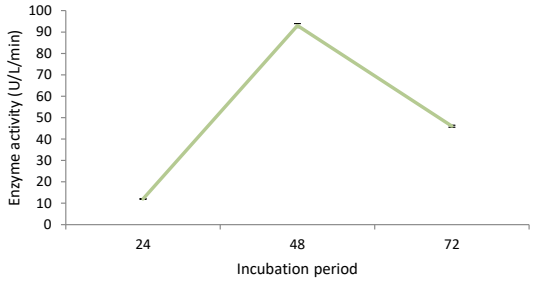

Figure 5. Optimization of the Incubation Period for Inulinase Production by the Test Culture.

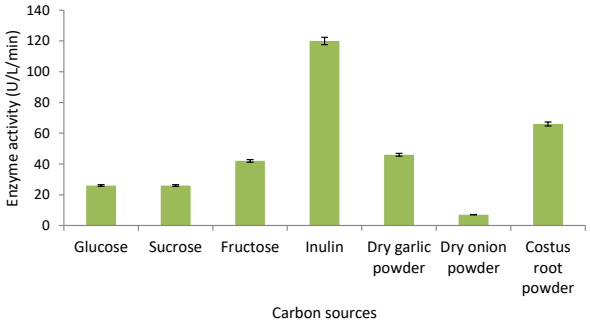

Figure 6. Optimization of Various Carbon Sources for Inulinase Production by the Test Culture.

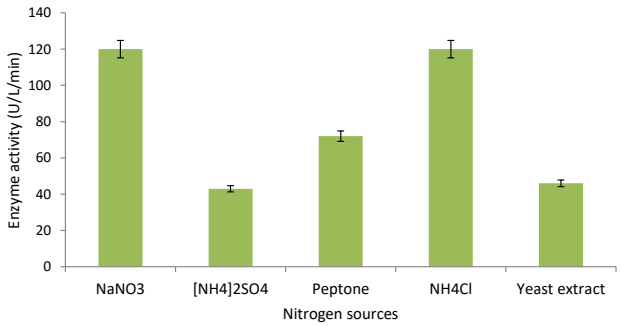

Figure 7. Optimization of Various Nitrogen Sources for Inulinase Production by the Test Culture.

enzymes, were partially purified by Sephadex G-100 column chromatography and the molecular weight of both inulinases was found to be around 300 000.[32] A semipurified inulinase was obtained from *Kluyveromyces fragilis* with about 55%-65% recovery yield.[18] A 45KDa molecular weight of inulinase enzyme isolated from *Achromobacter* sp. has also been reported previously.[33]

Determination of I/S Value of Inulinase

The activity on inulin and sucrose was found to be 184 U/L/min and 407 U/L/min respectively. Hence the calculated I/S value of inulinase was estimated to be 0.45 indicating a moderate-high activity of inulinase enzyme in our study. In general, the I/S ratios in the range of 0.02-7.9 have been reported in previously published studies.[34] An I/S ratio of 0.9947 was reported by inulinase produced by *Penicillium oxalicum* BGPUP-4 in a solid-state fermentation.[24] *K. marxianus* CBS 6556 also showed significant inulinase activity with sucrose, raffinose, stachyose, and inulin as substrates and exhibited an S/I ratio of 15 under standard assay conditions.[35] A recent study reported I/S ratio of 0.97 by inulinase produced by *Aspergillus niger*.[27]

Kinetic Studies of Inulinase Enzyme

The study of kinetic parameters is crucial in determining the enzyme activity and stability. These studies help us in understanding the limitations of the enzyme and hence modify and control suitable conditions when it is utilized on an industrial level, to avoid the chances of its denaturation. The pH and temperature are the two major factors affecting the significance of an enzyme. For instance, low pH range has been reported to be advantageous in the industrial preparation of sugar syrups because of the reduced color formation. Similarly, thermo-stability offers an added advantage for industrial use as it can improve the solubility of substrates and combat microbial contamination of reactors. For this reason, firstly the effect of pH (Figure 8) and temperature (Figure 9) was determined on the enzyme activity of the crude enzyme (CFS) in our study.

Inulinase obtained from the test culture showed optimum enzyme activity at pH 5 (120 U/L/min) and temperature of 45°C (184 U/L/min). It also showed thermal stability in the range of 35-55°C. An extracellular inulinase from *Penicillium* sp. strain TN96 showed optimum inulinase activity at pH 5.5 and 40°C.[36] Another extracellular acetone precipitated inulinase from *A. niger* van Tieghem UV11 grown on costus root powder/corn steep liquor medium showed a pH optimum of 5.5 and temperature optimum of 50°C.[26] In another study, a crude inulinase enzyme obtained from *Aspergillus fumigatus* showed optimum activity at pH 5.5 in acetate buffer and temperature 45°C.[37] The mycelial and extracellular inulinases produced by *Fusarium oxysporum* grown on *Cichorium intybus* root extract showed optimum pH 5.8 and 6.2; and optimum temperature 30°C and 37°C respectively.[33] Generally, the microbial inulinases are described as stable and active between pH 3.5 and 6.5.[38] Specifically, the fungal inulases show pH optima between 4.5 and 7.0, yeast inulinases between 4.4 and 6.5, and bacterial inulinases between 4.8 and 7.0.[12]

The effect of different metal ions was also studied on the enzyme activity of inulinase produced by the test culture (Figure 10). The inulinase obtained from test culture in our study showed no inhibition of enzyme activity at the concentration of 0.001M solutions of metal ions like Zn^{2+}, Mg^{2+} and Fe^{3+}. The Ca^{2+} metal ions showed partial inhibition (46 U/L/min) and Ca^{2+} showed a positive effect on enzyme activity (165 U/L/min). In case of inulinase obtained from *A. niger* van Tieghem UV 11, Mg^{2+} and Ca^{2+} were found to be weak activators, while Fe^{3+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} were

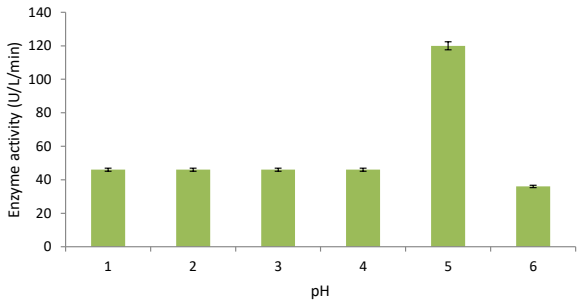

Figure 8. Effect of pH on the Enzyme Activity of Inulinase Produced by the Test Culture.

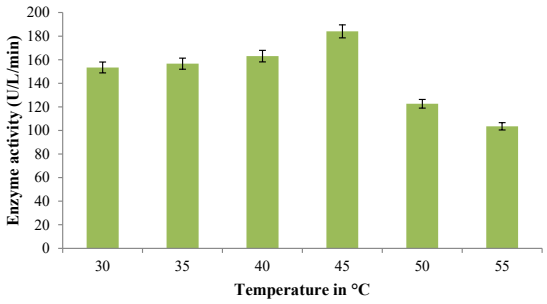

Figure 9. Effect of Temperature on the Enzyme Activity of Inulinase Produced by the Test Culture.

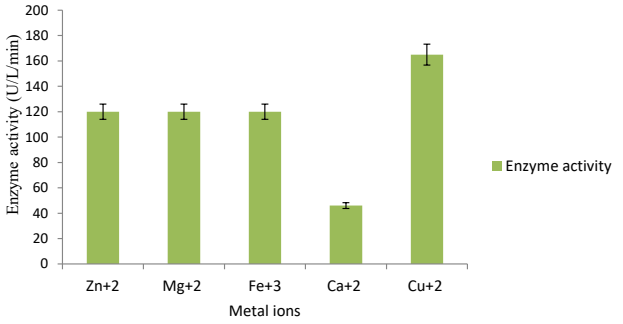

Figure 10. Effect of Metal Ions on the Enzyme Activity of Inulinase Produced by the Test Culture.

found to be inhibitors of the enzyme.[26] Also, the enzyme obtained from *A. fumigatus* was completely inhibited by 1mM Ag^{+} and Hg^{2+}, and activated in the presence of 1mM Ca^{2+} and Mn^{2+}. In addition, it was reported that 10% glycerol protected the enzyme from deactivation at 45°C and 50°C for 30min.[37] Another study reported stimulation of the inulinase activity by Mn^{2+} and Ca^{2+} and complete inactivation by Hg^{2+} in Rhizopus sp. strain TN-96.[36]

A Comparative Study Between Submerged and Solid State Fermentation

Among the three substrates used for SSF, only wheat bran showed slight inulinase production of 7 gds. The SmF, on the other hand, showed an enzyme activity of 184 U/L/min. Corn flour and rice bran failed to show any enzyme production. Hence, it can be concluded that SmF is more effective for inulinase production by the test fungal culture isolated in our study. Effective SmF is also reported for the production of inulinase by *A. niger* NK-126 grown in media containing dandelion tap root extract.[39] In another study, SSF was carried out for the production of inulinase from *Staphylococcus* sp. RRL-1 and *K. marxianus* ATCC 52466 using wheat bran, rice bran, coconut oil cake and corn flour as substrates. The wheat bran medium supported both cultures effectively producing 107.64 U/gds (*Staphylococcus* sp.) and 122.88 U/gds (*K. marxianus*) of inulinase in 48 and 72h respectively.[40] *P. oxalicum* BGPUP-4 also showed cost-effective production of inulinase enzyme (322.10 IU/gds) using carrot pomace in SSF.[24] Similarly, a recent study reported inulinase production from *Mucor circinelloides* (411.3 U/gds) using apple pomace in SSF.[41] *K. marxianus* NRRL Y-7571 successfully showed inulinase production in both SSF and SmF. The optimum pH and temperature for enzyme activity were reported to be 4.5 and 55°C for SmF, and 5.0 and 55°C for SSF respectively. It was further reported that the inulinase obtained by SmF was less susceptible to pH changes and that obtained by SSF is more resistant to higher temperatures.[42]

Conclusions

An inulinase producing fungal culture isolated from a rotten garlic sample in the current study showed a promising potential for industrial applications in various biotechnological processes. Under optimum conditions, a significant amount of enzyme production was observed. Moreover, the crude enzyme showed tolerance to changes in temperature over a wide range, with optimum activities observed at 45°C. This eliminates the necessity to strictly monitor temperature conditions and at the same time helps to increase substrate solubility and keeps the contaminants in control to a large extent. The tolerance to metal ions also adds to the favorability of use of this enzyme, since metal contamination is a commonly faced problem in industrial levels.

Authors' Contributions

All authors have contributed equally to this study.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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