



Inulinase Production by Filamentous Fungi Indigenous to Saudi Arabian Soils

Shuroq Mohsin Al-Shahrani

King Saud University, Riyadh, Saudi Arabia,

Abstract: Inulin represents a source for the production of oligosaccharides by enzymatic hydrolysis (inulinase enzyme) that is used widely in industrial and medical applications. This research aims to identify the filamentous fungi indigenous and occupant and grow the filamentous fungi, then assess the thermotolerant inulin-degrading fungal isolates and investigate the higher yield produced by local fungal strains of inulinase enzyme, and comparing with reference ones, as well as Identify the growth parameters conditions that affecting on enzyme production local fungal strains. Fourteen agricultural soil samples were collected from the top layer (0-15 cm) from different regions of the territory of the Kingdom of Saudi Arabia (by placing 100 g of the soil sample from each region), depending on the climatic differences. Soil sample sites were Mecca, Al-Riyadh, AlQassim, AlBaha, AlGouf. The fungi produced inulinase enzymes are isolated and incubated at 28-30°C for 10 days, then DNA was isolated and tested for thermo-tolerance. In addition to this, the inulinase enzyme in liquid media (time course) was assessed and the optimum production conditions were studied (Agitation, temperature, pH, the concentration of inulin, carbon sources, nitrogen source). There are thirty fungal isolates from twelve soil samples, they belong to *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*, *Rhizoctonia*, *Mucor*, *Alternaria*, and *Rhizopus* genus. The inulinase enzyme activity ranged between 11.76-39.16 U/ml, as well as the optimum production conditions, are: agitation at 150 rpm, incubation temperature at 30°C, pH 6, 2% of inulin concentration, maltose as carbon source, and tryptone as a nitrogen source. Finally, high inulinase productivity was obtained by local fungal strain in this study.

Keywords: Enzyme, Fungi, Inulinase, Inulin, Saudi Arabia, Soil.

*Corresponding Author

Shuroq Mohsin Al-Shahrani , King Saud University, Riyadh, Saudi Arabia,



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I. INTRODUCTION

Inulin is a polysaccharide of β -(2,1)-linked fructose that can be found as a reserve carbohydrate in Compositae family plant tubers, like those of *Vernonia Herbacea* (Veil Rusby). Inulin represents a source for the production of ultra-high-fructose syrups by enzymatic hydrolysis. The enzymatic hydrolysis of the fructans is an attractive alternative to acid hydrolysis but is preferable because of lower byproduct (such as difructose anhydride) formation¹, and low cost.² Literature reported about the inulinase producer microorganisms such as fungi, yeast, and bacteria which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose). With the ever-increasing need for inulinase, it is necessary either to isolate different types of microorganisms capable of producing inulinase or to enhance the yield of inulinase-producing organisms³. Recently research groups around the world have reported the use of fungi as a source of inulinase enzyme; e.g., *Aspergillus* sp., *A. niger*, *A. ficuum*, *A. nidulans*, *Penicillium* sp. and *Fusarium oxysporum*. Inulinase production of *Aspergillus niger* and *Trichoderma harzianum* were studied, the highest inulinase activity from *A. alternata* was observed after 48h at optimum temperature 30°C, pH 5.0, and grown on Jerusalem artichoke powder as a sole carbon source. While the maximum inulinase activity of *T. harzianum* was recorded after 72h at 25°C⁴. Firstly, many experimental studies confirm that the content of the media affects the inulinase level. In 1979, Flemming and Grootwassink obtained high synthesis from *Kluyveromyces fragilis* via using a medium with low concentrations of glucose, fructose, and sucrose. In this context, High-level of the enzyme could be produced by *Aspergillus niger* when using corn steep liquor (CSL)-maltose medium in the absence of inulin. Secondly, other critical factors that influence inulinase production are aeration (air volume/broth volume minute) and agitation (revolution per minute). An effect of aeration was shown to be less significant in the production of inulinase (2,1- β -D-fructan fructanohydrolase, EC 3.2.1.7) by *Kluyveromyces marxianus* ATCC 16045, but the optimum condition was 50 to 550 rpm for agitation speed and 0.5 to 2.0 vvm for aeration rate⁵ while maximum inulinase activity *Kluyveromyces marxianus* var. *bulgaricus* was obtained at an aeration rate of 1 vvm. Thirdly, the temperature has also been reported to influence inulinase production and activity. The endoinulinase activity from fungi and bacteria was highest at 50–55°C⁶. demonstrated that 50°C was the optimal temperature of the purified inulinase from the marine yeast *C. aureus* G7a with stability up to 65°C. Although overproduction of inulinase by *P. guilliermondii* strain I is achieved at 60°C⁶. Fourthly, many researchers established that the purified inulinases are in the range of 4.5–6.0⁷. While optimal pH of the bacteria was higher than fungi and yeasts⁸. Many studies confirmed the use of inulin as carbon sources to produce high levels of inulinase⁹. The effects of carbon source on *Aspergillus niger* van Teighem on inulinase production was studied, the results showed that inulinase production was higher when the microorganism was inoculated in media formulated with inulin, and maximum productivity of inulinase (176 U ml) was achieved by using medium containing 5% (w/v) inulin and galactose¹⁰. High inulinase synthesis by *Aspergillus niger* result with employing corn steep liquor and (NH₄) H₂PO₄ or soybean meal as the nitrogen source which *Aspergillus niger* isolated from soil samples had a great capacity to produce extracellular inulinase with utilizing of casein as a source of nitrogen¹⁰. The effect of Metal ions and trace

elements in inulinase activity was approved in several experiments. Inulinase activity of *Streptococcus salivarius* KTA-19 may be stimulated by Mn²⁺(1mM). Although, at the same concentration Fe³⁺, Cu²⁺, Ag⁺, and Hg²⁺ act as inhibitors¹¹. There is a wide application of inulinase enzyme in both medical and industrial fields. Fructose, which is better for diabetic patients, since it is absorbed more slowly than glucose through the intestine. Fructose increases iron absorption as a result of the formation of the iron-fructose chelate complex which is better absorbed than inorganic iron. It has a high sweetening capacity so it can be used in the diet of obese persons. Fructose stimulates calcium absorption in postmenopausal women, stimulates the growth of *Bifidobacteria* in the large and small intestine, prevents colon cancer, and is used as dietary fibers because of its fat-like texture. Fructose is also widely used in many foods, pharmaceuticals, and beverages instead of sucrose. The best procedure involves the use of microbial inulinase which after one-step enzymatic hydrolysis of inulin yields 95% pure fructose⁹. Another industrial application of the inulinase enzyme is ethanol solvent that can be obtained by fermentation of acid hydrolyzed extracts, using classical *Saccharomyces cerevisiae* yeast, or without hydrolysis, using yeasts with inulinase activity such as *Kluyveromyces fragilis*. Recently, interest has been concentrated toward direct alcohol fermentation from inulin sources, Ethanol is the most employed liquid biofuel either as a fuel or as a gasoline enhancer¹², and they found Jerusalem artichoke tuber is one of the best raw materials for fuel ethanol production. However, Simultaneous production of ethanol and fructose enriched syrups was obtained from Jerusalem artichoke extract using a *Saccharomyces diastaticus*, this allowed the production of 42 g/L of ethanol and 70 g/L of inulin containing up to 92%fructose. In the nutrient field, the use of inulinase enzyme for the production of oligo-fructan production from inulin, Fructo-oligosaccharides constitute one of the most popular functional food components because of their bifidogenic and health-promoting properties. Inulin can be selectively hydrolyzed by the action of endoinulinase into IOSs¹³.

1.1 Research problem

The production of microbial enzymes has gained much attention in biotechnological studies to produce lipases, inulinases, proteases, etc. Inulinase is a potentially useful enzyme for the production of high fructose syrups (HFS) from inulin. Inulinases are considered as one of the most important classes of enzymes for the production of fructose and fructo-oligosaccharides, which are extensively used in the pharmaceutical and food industry due to their ability to produce 95% pure fructose². Inulinase catalyzes the removal of the terminal fructose residues from the non-reducing end of the inulin molecule known as exoinulinases, whilst the inulinase hydrolyzes the internal linkages in inulin to yield inulotriose, inulotetraose, and inulopentaose well-known as endoinulinase. In addition, during inulinase production, many factors affect the level and the activity of inulinase, some of these factors are Fermentation media, incubation temperature, percentage of moisture, pH of media, the concentration of inulin, carbon and nitrogen source, metal ions, and trace elements, etc. There is a wide application of inulinase enzyme in the medical field, as the synthesis of fructose syrup, which has beneficial effects which are emerging as a safe alternative sweetener than sucrose; In the

nutrient field, used of inulinase enzyme for production of oligo-fructan production from inulin¹³, and industrial field as the synthesis of ethanol solvent that can be obtained by fermentation of acid hydrolyzed extracts.

The present study aims to isolate, identify and grow t Fungi from different soil samples in different areas from Saudi Arabia kingdom, that produce the inulinase enzyme, as well as a comparison between these areas. In addition to this, the inulinase enzyme will be produced under specific conditions, so the present study aims to investigate and test this condition as well as assess the optimum conditions for inulinase enzyme production.

1.2 Research question

Recently, many of the industrial and medical fields depend on oligo-saccharides as the main source of sugar in their synthesis and application, so widely used inulin material as a source of the optimum and perfect sugar. Usually, chemical reaction to hydrolysis of inulin to low molecular weight oligosaccharides, but the recent research and studies recommend using microbial hydrolysis that produces an inulinase enzyme in secondary metabolites. Many of the microorganisms can release and synthesize this enzyme under specific conditions as bacterial and fungi. Based on the above mentions, this research aimed to assess and identify the fungi genus that can produce an inulinase enzyme that is present in the soils of the Saudi Kingdom. In the light of the above, the research problem can be solved in the following main question:

What is the fungi genus that is present in Saudi Arabia soils that can produce the inulinase enzyme?

From the main question, the following sub-questions are divided to:

- What is the DNA fingerprint of each fungi genus that is isolated from soils?
- What is the quantity of inulinase enzyme that is produced?
- What is the optimum condition for the incubation of fungi?
- What is the optimum condition to yield a high quantity of inulinase enzyme?

1.3 Research Objectives

The current research aims to:

- Identify the filamentous fungi indigenous to Saudi Arabian soils.
- occupant and grow the filamentous fungi extracted from soils to stimulate the inulinase enzyme.
- Assess the thermotolerant inulin-degrading fungal isolates.
- To investigate the high yield produced by local fungal stains of inulinase enzyme, and comparing with reference ones.
- Identify the growth parameters conditions that affect enzyme production of local fungal strains.

1.4 Research Significance

- By reviewing the relevance of previous studies, it was absent of article or research about the fungi that production of inulinase enzyme in soils of Saudi Arabia.
- Inulinases consider one of the most enzymes to constitute an important class of enzymes for the production of fructose and fructo-oligosaccharides. So, there are widely used inulin in the food industry and medical fields. This study suggests

alternative microbial hydrolysis of inulin instead of chemical hydrolysis.

- This research will allow further research into the efficacy of microbial hydrolysis of inulin. in addition to studying the critical factors that affect inulinase production level and activity.

2. MATERIALS & METHODS

2.1 Study Area

Fourteen soil samples were collected from the top layer (0-15 cm) of agricultural soils from different regions of the territory of the Kingdom of Saudi Arabia (by placing 100 g of the soil sample from each region), depending on the climatic differences. Soil samples' sites were Mecca, Al-Riyadh, AlQassim, AlBaha, AlGouf.

2.2 Isolation and identification of Fungi

Soil filamentous fungi for the acclimation study were conducted by placing 100 g of the soil sample from each region into a glass beaker, amended with 10 g of inulin, thoroughly mixed well with the soil. Then, all the beakers were incubated at 28-30°C for 10 days, considering the soil moisture through water addition. Then, ten grams of previously inulin-treated soil samples were suspended in 90 ml of sterilized distilled water. Subsequently, fungal cultures were isolated by serial dilution and plated onto Petri dishes Sabouraud glucose agar medium (contain per liter of distilled water, 10 g peptone, 40 g glucose, and 10 g agar supplemented after autoclaving with an antibacterial agent, 4 ml of chloramphenicol, 25 mg/ml). The plates were incubated at 28-30°C for 4 days. Individual colonies were picked by repetitively streaking through a series of plates to isolate the individual member. Single colonies were removed, streaked on slants of Potato Dextrose Agar, PDA (plus 4 ml per liter of chloramphenicol, 25 mg/ml, after autoclaving), and kept at 4°C until further use. All fungal isolates from inulin-enrichment soil samples were screened for their ability to grow on inulin, as the sole carbon source in basal mineral salts medium, and detection of inulinase enzyme by using plate method assay according to choose the super isolates that could grow¹⁴. For screening of inulin-degrading fungal isolates, the plate of mineral salts solid medium was separately inoculated from the spores' suspension of all fungal isolates by a loop and incubated for 3 to 7 days at 30°C. Pure culture of isolated fungi was identified using the key of the cultures were characterized to the genus level based on macroscopic characteristics (colonial morphology, color, and appearance of colony, shape, and diameter of the colony) and microscopic characterization (septation of mycelium, shape, diameter, and texture of conidia),^{15,16} Single spores were transferred to 2 % water agar medium. Then, the pure cultures were grown on potato dextrose agar (PDA) plates for one week at room temperatures (22-25°C) for also macroscopic characteristics (color, texture, appearance, and diameter of the colonies) and microscopic (microstructures) characterization. Cultures for DNA extraction were grown in 125-ml Erlenmeyer flasks containing 40 ml potato dextrose broth (PDA) and incubated on an orbital shaker (150 rpm) for 3-4 days at room temperature (22-25°C). Mycelia were harvested by filtering the liquid cultures through Whatman No. 2 filter paper discs, dried by blotting with paper towels, and kept in aluminum foil

at -20°C until use. Frozen mycelia were ground to a powder under liquid nitrogen with a mortar and pestle. The ground mycelia were transferred to 1.5 ml microcentrifuge tubes. Fungal DNA was extracted by using a CTAB method as modified by DNA concentrations were adjusted to 20 ng/ml

ITS 5 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ; ITS 4 5'-TCCTCCGCTTATTAGTATGC-3'¹⁹.

The PCR reaction contained 1 ng genomic DNA, 1× PCR buffer (Kapa Biosystems, Cape Town, South Africa), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.25 µM of each primer, and 1.0-unit Taq DNA polymerase (Kapa Biosystems) in a 30 µl reaction volume. The PCR program was: one cycle at 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and one cycle of 72°C for 5 min, and then held at 4°C. To check the PCR reproducibility, 2 µl from PCR products were run on 1.5% agarose gels stained with 0.5 µg/ml ethidium bromide in 0.5× Tris-borate EDTA (TBE) buffer. DNA was visualized, examined, and photographed under UV illumination¹⁷. PCR amplicons were then purified and sequenced by the Advanced Genetics Technology Center (AGTC, University of Kentucky, KY, USA). The DNA sequences were edited and aligned with Bio-Edit software (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>). Cleaned DNA sequences were searched against other 16S DNA sequences deposited in the GenBank database to identify the identity or the closest deposited sequences in the database. Fungal strain, *Aspergillus niger* van Tieghem NRRL 326 was used throughout as a reference strain for inulinase production, was obtained from NRRL (Agriculture Research Service Culture Collection). It was provided by the United States Department of Agriculture (USDA), New Orleans, Louisiana 70179. All the cultures of fungal strains were kept on potato dextrose agar (PDA) slants at 4°C until using¹⁸.

2.3 Inulinase enzyme produced

Petri dishes containing mineral salts medium supplemented with inulin as a sole carbon source were inoculated with spores' suspension of isolated fungus. From each isolate, one ml of spores' suspension (3.0×10⁶ spore/ml) was used as inoculums¹⁴. The plates were incubated at 30, 35, 40, 45, and 50°C for 6 days¹⁹. By using plate method assay according to the diameter of formed-colonies was measured. The fungal growth was expressed in terms of mm for the colony diameter¹⁴. Spores suspension was used for the inoculation of liquid cultures were prepared by subcultures on the potato dextrose agar (PDA) and incubated at 30°C for 6-10 days. Spores from the slants were suspended in sterile 0.85% saline containing 0.01% Tween 80, gently agitating the tube with a Tween 80 solution to remove the spores. The resulting spores' suspension was collected in a sterile tube, and the spores count was made using a Neubauer hemocytometer. Sufficient Tween 80 solution was added to the fungal spores' suspension to give a count within range 3.0×10⁶ - 3.0×10⁷ spores/ml. For screening of inulin-degrading fungal isolates, the plate of mineral salts solid medium was inoculated from the spores' suspension by a loop and incubated for 3 to 7 days at 30°C. For all the flask experiments, 0.5ml of this suspension was used. The medium used for inulinase production was prepared according to the method described by the Mineral salts medium contains: NH₄NO₃ 2.3 g, (NH₄)₂HPO₄ 3.7 g, KH₂PO₄ 1.0 g, MgSO₄ 0.5 g, yeast extract 1.5 g and inulin 10 g, the whole mixture was completed to 1 liter¹⁴. The pH was adjusted to 5.5 with

before use in the polymerase chain reactions (PCR)¹⁶. The internal transcribed spacer (ITS) region of the rDNA repeats was amplified from the fungal isolates recovered from the soil using the following forward and reverse primers :

hydrochloric acid (HCl). The fungal isolates were grown in liquid medium in 250 ml Erlenmeyer flasks containing 50 ml of medium per flask. One ml of spores' suspension was used for inoculating each flask. The flasks were incubated in a rotary shaker operating 200 rpm at 30°C for 7 days. The culture broth from each flask was filtered for mycelia separation. The clear culture filtrate was analyzed for pH, reducing sugar, total carbohydrate, and inulinase activity. Seven periods ranging from 1 to 7 days were tested. After 24 hours from zero time, the inulinase activity of the cell-free extract, final pH, fungal biomass, reducing sugar, and total carbohydrates was determined. These analyses were repeated with 2, 3, 4, 5, 6, and 7 days to determine which period was optimum to produce the highest productivity of inulinase for each tested fungal isolate while comparing the reference fungal strain.

2.4 Inulinase Enzyme Testing (physical factors & nutrients requirements)

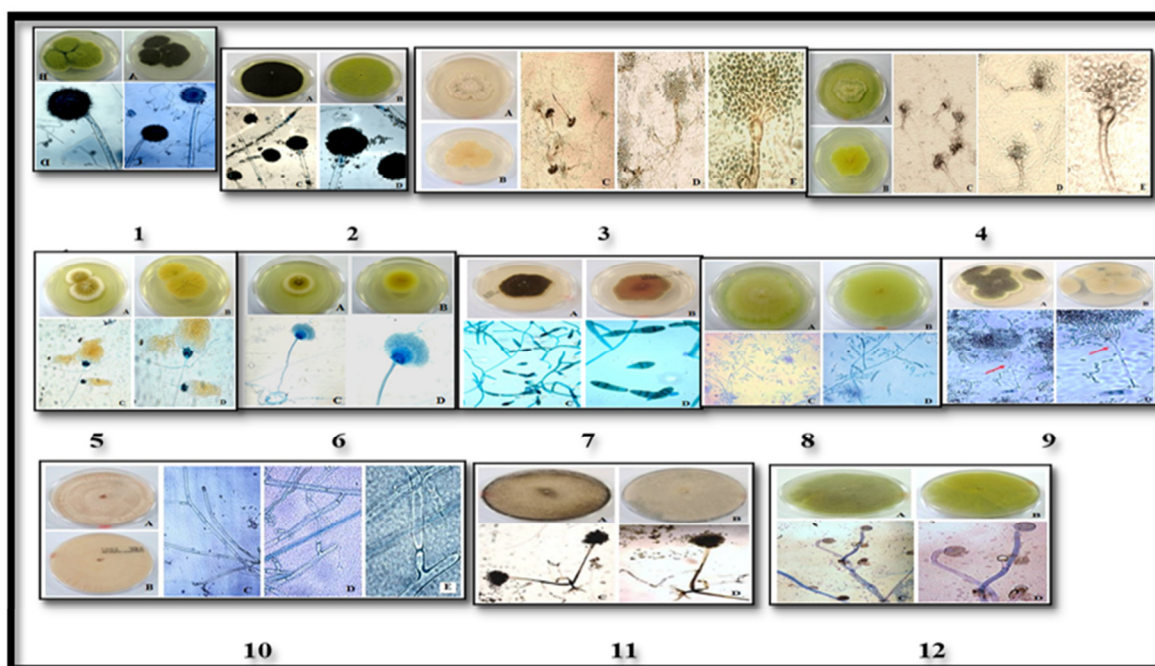
The effect of agitation, temperature, and pH on inulinase production was analyzed by adding the nutrients requirements of inulin concentration, carbon source, and nitrogen source to the basal medium. Optimization of these parameters was carried out with a "one at a time" strategy keeping all other variables constant except one. Agitation's effect on inulinase production was tested by analyzing the effect of shaking on the inulinase production with selected fungal isolates and the reference fungal strain; where two sets of flasks with the nutrient medium were prepared and inoculated with the prepared inocula. One set was incubated without shaking and the second set was kept continuously on a rotary shaker (200 rpm). Both groups were incubated at 30 °C for 72 hours. Analyzing the effect of incubation temperature on inulinase production by assays was conducted at different temperatures (10, 20, 30, 40, and 50°C). Flasks containing 50 ml of mineral salts medium were inoculated with isolated fungal, 1 ml of spore's suspension 8×10⁷ spore/ml used as inoculums. The flasks were incubated in a rotary shaker operating at 200 rpm at the different temperatures for 72 hours. After that, the mycelia separation by filtration to analyze the biomass, and the filtrate was analyzed for pH, free sugar, total carbohydrate, and inulinase activity. Effect of initial pH values on inulinase production via determining the impact of initial pH on enzyme production, the mineral salts medium was prepared with various pH values (3, 4, 5, 6, 7, 8 and 9) and the control pH 5.5, to find out the optimum pH of the inulinase production. These pH values were adjusted by using concentrated sodium hydroxide and hydrochloric acid. The flasks were inoculated with isolated fungal, 1 ml of spore's suspension 8×10⁷ spore/ml used as inoculums, then incubated in a rotary shaker operating at 200 rpm at 30 °C for 27 hours. Effect of different concentrations of inulin on inulinase production through prepared the mineral salts medium by containing different concentrations of inulin (zero, 0.25, 0.5, 1, 1.5, 2, 3, and 4 %) to determine the best concentration of inulin to the

production of the inulinase enzyme. The flasks were inoculated with isolated fungal, 1 ml of spore's suspension 8×10^7 spore/ml used as inoculums. The flasks were incubated in a rotary shaker operating at 200 rpm at 30 °C for 27 hours. Effect of different carbon sources on inulinase production by test the best carbon source for the production of the enzyme, the fungus was grown in the mineral salt's medium containing different carbon sources, including fructose, glucose, maltose, sucrose, lactose, galactose, and starch in addition to the inulin which was individually added instead of the initial value (2%, w/v) in the mineral salt's broth medium. The flasks were inoculated with isolated fungal, 1 ml of spore's suspension 8×10^7 spore/ml used as inoculums. The flasks were incubated in a rotary shaker operating at 200 rpm at 30 °C for 27 hours. Effect of different nitrogen source on inulinase production by evaluating the best nitrogen source for the enzyme production, the fungus was grown in the mineral salts medium containing various nitrogenous supplements, the organic sources were peptone, treptone, urea, yeast extract, and the inorganic source were ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$ & potassium nitrate, KNO_3 . We were added to the medium by replaced NH_4NO_3 and $(\text{NH}_4)_2\text{HPO}_4$ and added 6 g/l from the nitrogen source. The flasks were inoculated with isolated fungal, 1 ml of spore's suspension 8×10^7 spore/ml used as inoculums. The flasks were incubated in a rotary shaker operating at 200 rpm at 30°C for 27 hours. Assay of Inulinase activity in culture filtrate was estimated by determining the reducing sugars released from inulin according to the reaction mixture containing (in a test tube) 2 ml of 0.2% inulin solution and 2 ml of acetate buffer 0.1 M (pH 4.6)²⁰. To this mixture, 0.5 ml of culture filtrate was added and incubated at 50°C for 20 min. After incubation, the reaction was stopped by putting the test tubes in a boiling water bath for 10 min to inactivate the enzyme action. After the mixture cooling, reducing sugar was assayed by the DNS method as following, 3 ml of the above reaction added to 3 ml of 3,5-dinitrosalicylic acid (DNSA) reagent then 1 ml of 40% Rochelle salt solution and mixed well, then heated for 15 min in a boiling water bath and then cooled under running tap water. The absorbance of the solution was determined at 575 nm in a spectrophotometer (Ultrospec 2100 pro)²⁰. The amount of reducing sugar was estimated by comparison with a calibration curve which was made with fructose for inulinase activity. One unit of inulinase activity (U) was defined as the amount of enzyme in 1 ml of filtrate that liberates 1 μmol of fructose per minute under the assay conditions as described above. Measurement of Mycelial growth to determine the Fungal biomass by transferring the mycelium to dried and weighted. The mycelial pellets of each flask were harvested by filtration of the culture medium through pre-weighed filter paper (Whatman paper No.1) under suction. The biomass was determined after washing the mycelial mass with distilled water and dried at 105°C in an electric oven overnight until constant weight. The biomass dry weight was estimated from the difference between the mycelium plus filter paper and the filter paper itself. The biomass dry weight was expressed in terms of mg of the mycelium. Total sugar produced was

estimated by 3,5-dinitrosalicylic acid (DNSA) reagent according to a method of the modified reagent contained: 1% DNSA, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide, and 40% Rochelle salt²⁰. The modified reagent was prepared by placing all solid components in a container and dissolving them simultaneously by stirring with the required volume of sodium hydroxide solution and prepared the Rochelle salt solution independently. The color test tube was added to the mixture of reactants to the color's development. The color test was made with 3 ml of culture filtrate added to 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent then 1 ml of 40% Rochelle salt solution. The mixture was heated for 15 min in a boiling water bath then cooled under running tap water adjusted to ambient temperature. The color was measured using a spectrophotometer at 575 nm. Total carbohydrate produced was determined according to the method of using the phenol-sulfuric acid assay as follows: into thick test tubes, washed with distilled water, add 10 μl of culture filtrate, with 40 μl distilled water, then add 500 μl of 4% phenol (40 g phenol in 1-liter distilled water) followed by 2.5 ml 96% sulfuric acid²¹. The tubes were shaken and left to cool for minutes. A reagent blank containing 10 μl of water transferred the solutions from the test tubes to the cuvettes and measured the developed color in the spectrophotometer at 490 nm. A standard curve was prepared using (1 mg/ml) inulin in distilled water. Calibration sugar standards were prepared by transferring aliquots to 10 different 10-ml tubes in 5 μl increments from 5 to 50 μl with an accurate pipette.

3. RESULTS

Twelve soil samples were collected in Saudi Arabia soils. Most of the soil samples (7 samples) were collected from various sites in Riyadh city and the other five soil samples were collected from AlBaha, Makka, AlQassim, and AlGouf. All soil samples were enriched with inulin as previously described in the materials and methods section. After the incubation period of enriched soil and isolation procedure, mixed cultures were obtained. After purification, initially, thirty-seven individual fungal isolates were obtained. These fungal isolates showed a varied ability to grow on inulin as the sole carbon source. All (seven) fungal isolates of AlBaha, Makka, AlQassim, and AlGouf soil samples showed moderate growth on agar plates. However, the thirty fungal isolates of various Riyadh soil samples were different in their ability to grow on inulin as the sole carbon source. Whereas three fungal isolates of Riyadh (Riyadh A5, A6, and E6) displayed high colony growth, seven fungal isolates of Riyadh (Riyadh A4, B1, B3, C5, C6, E7, and G1) could not grow on inulin as the sole carbon source in mineral agar medium. Besides, fourteen fungal isolates of various Riyadh soil samples were moderately grown and the rest of the fungal isolates (six isolates, Riyadh A1, A3, B2, E4, E5, and F1) could grow poorly on inulin as the sole carbon source on agar plates. All fungal isolates (30 isolates), which displayed growth on inulin agar medium were identified and selected for further experiment.



(1); *Aspergillus niger* AlBaha B2 (2); *Aspergillus nidulans* Riyadh A2 (3); *Aspergillus nidulans* Riyadh A3 (4); *Aspergillus terreus* Riyadh A7 (5); *Aspergillus terreus* Riyadh E4 (6); *Alternaria* spp Riyadh B2 (7); *Fusarium solani* Riyadh D1 (8); *Penicillium* spp Riyadh C4 (9); *Rhizoctonia* spp Riyadh E6 (10); *Rhizopus* spp Riyadh E9 (11); and *Mucor* spp Riyadh E1 (12). Macromorphology on PDA agar plates for both surfaces using a digital camera : (A) Surface of the plate (B) Reverse surface of the plate. Micromorphology using optic microscope : (C) Conidiophores, viewed at 10x, (D) viewed at 20x.

Fig 1: Macro and micromorphology of *Aspergillus niger* NRRL 326

The impact of incubation temperature on the total of thirty inulin degrading-fungal isolates growth was ascertained by measuring the diameter of colonies on mineral salts agar medium containing inulin as the sole carbon source. Colonies

diameter was measured after incubation for 6 days at various temperature values (30, 35, 40, 45, and 50°C) as shown in Figure 2. The average diameters of the largest and smallest colony were recorded.

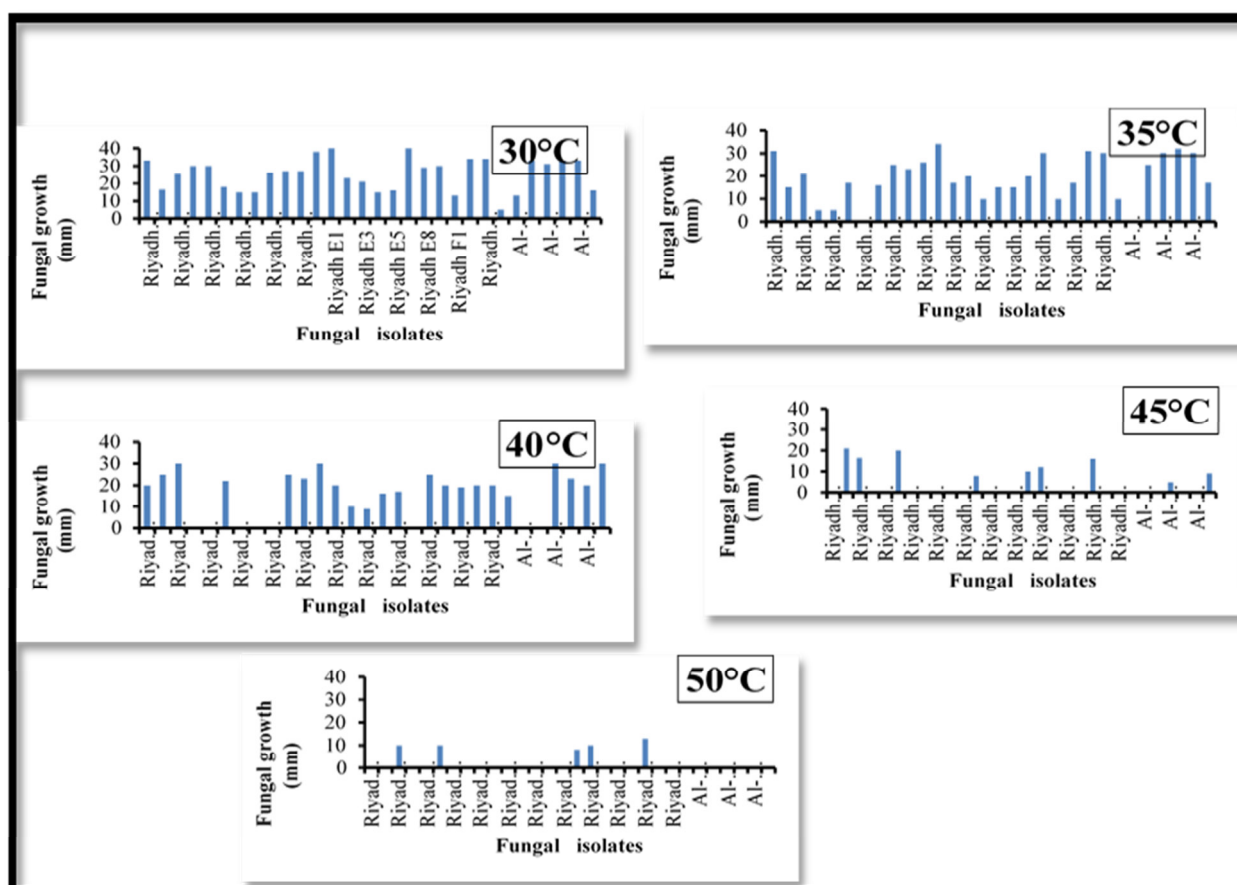


Fig 2. Growth of inulin degrading -fungi isolates (colony diameter in mm) at various incubation temperature

All the thirty-inulin degrading-fungal isolates were grown well at 30°C; as well most of them (28 fungal isolates) could also grow at 35°C. Moreover, at 40°C incubation temperature, twenty-two fungal isolates showed a clear growth. However, incubation temperature increment, not only, 9 fungal isolates could grow at 45°C, but five of them showed a slight growth at 50°C. From optimum temperature point of view for growth of all fungal isolates at tested various incubation temperature values found that growth of eight isolates (*Aspergillus* Riyadh A2, *Aspergillus* Riyadh A3, *Aspergillus* Riyadh A7, *Aspergillus* Riyadh E4, *Aspergillus* Riyadh E5, *Aspergillus* Riyadh F1, *Trichoderma* AlBaha B1, *Aspergillus* AlGouf) was improved by incubation temperature values increment, and their growth optimum temperature value was at 40°C. Moreover, during this survey, nine fungal isolates

(*Aspergillus* Riyadh A2, *Aspergillus* Riyadh A3, *Aspergillus* Riyadh A7, *Fusarium* Riyadh D1, *Aspergillus* Riyadh E4, *Aspergillus* Riyadh E5, *Aspergillus* Riyadh F1, *Alternaria* AlBaha B2, *Aspergillus* AlGouf) were showed that able to grow at 30-45°C could be considered as thermotolerant. The nine fungal isolates were identified, at the species level depending on both morphological and molecular characteristics (Table 1). Whereas, the cultures were grown in potato dextrose broth for DNA extraction. The PCR products of the rDNA-ITS region were sequenced in both directions using ITS4 and ITS5 primers. The DNA sequences were cleaned and edited by BioEdit software. Cleaned sequences were searched against the GenBank database to identify the identity or the closest known deposited sequences in the database.

Table 1: Identification of selected inulin degrading-fungal genera based on their morphological and molecular characterizations.

Fungal isolates	No.	Species ID	GenBank Accession number	Homology (identity %)
Riyadh A2	1a	<i>Emericella nidulans</i> (perfect stage or teleomorph)	GQ461904	509/509 (100%)
	1b	<i>Aspergillus nidulans</i> (anamorph stage)	HQ674656	508/509 (99%)
Riyadh A3	2a	<i>Emericella nidulans</i> (perfect stage or teleomorph)	GQ461904	509/509 (100%)
	2b	<i>Aspergillus nidulans</i> (anamorph stage)	HQ674656	508/509 (99%)
Riyadh A7	3	<i>Aspergillus terreus</i>	AB647191	549/549 (100%)
AlBAHA B2	4	<i>Aspergillus niger</i>	HQ379665	544/545 (99%)
Riyadh D1	5	<i>Fusarium solani</i>	HQ379665	523/524 (99%)
Riyadh E4	6	<i>Aspergillus terreus</i>	AB647191	550/550 (100%)
Riyadh F1	7	<i>Aspergillus terreus</i>	AB647191	549/549 (100%)
AlGouf	8	<i>Aspergillus niger</i>	HQ170509	545/545 (100%)

From Eight thermotolerant (that have the ability to grow at 30-45°C) inulin degrading-fungal isolates are *Aspergillus* nidulans Riyadh A2, *Aspergillus* nidulans Riyadh A3, *Aspergillus* terreus Riyadh A7, *Fusarium* solani Riyadh D1, *Aspergillus* terreus Riyadh E4, *Aspergillus* terreus Riyadh F1, *Aspergillus* niger AlBaha B2, and *Aspergillus* niger AlGouf, in addition to, *Aspergillus* niger van Tieghem NRRL 326. Also,

the effect of time course on inulinase productivity was investigated. Shaking flask cultures were incubated at 30°C. The extracellular inulinase production was determined within 1-7 days old cultures. The results obtained on the effect of the time course on inulinase production, total sugar, pH, and fungal biomass that are determined in shake flask cultures of the nine molds under investigation are mentioned in table 2.

Table 2. Time course of inulinase production in shake flask culture of *Aspergillus nidulans* Riyadh A2.

Time (days)	pH	Growth (DW mg/50 ml)	Enzyme Activity (U/ml)	Total sugar (%)
0	5.53	30	0.00	100
1	5.88	90	2.58	67.39
2	5.51	178	7.91	62.08
3	5.29	160	12.72	60.43
4	4.41	140	12.40	58.66
5	3.36	130	12.34	43.13
6	3.25	120	12.16	23.13
7	3.23	114	11.15	19.99

The medium contained basal parts (per liter, NH_4NO_3 2.3 g, $(\text{NH}_4)_2\text{HPO}_4$ 3.7g, KH_2PO_4 1.0 g, MgSO_4 0.5 g, yeast extract 1.5, and inulin 10 g. The initial pH was 5.5. The cultivation temperature was 30°C. Cultures were grown with shaking. Also, the Inulinase Enzyme activity was ranged between 11.76 to 39.16 U/ml which production of by various fungal isolates during 7 days in basal medium containing inulin (1%) under continuous shaking condition. Aeration by reciprocal shaking at a speed of 150 rpm resulted in greater growth and inulinase production for all tested strains. After 72 h of flask cultures, inulinase enzyme activity was within range 12.84-28.91 U/ml by *Aspergillus* niger AlGouf and *Aspergillus*

terreus Riyadh A7, respectively, in the case of static cultures. However, enzyme activity reached 19.96-39.88 U/ml in the shaken broth cultures of *Aspergillus* niger AlGouf and *Aspergillus* terreus Riyadh A7, respectively. The results also show that the type of culture had a pronounced effect on biomass dry matter. All strains possessed high growth in shaken than in static cultures. It was also found that in either static or shaken cultures the final pH values of the inulinase production medium lied in the acidic range (2.98-4.38). In any case, the type of culture had some effect on the final pH value of the enzyme production culture, which varied within a very limited range with the type of culture, Where, slightly

acid was produced in shaken cultures. As well as sugar utilization by *Aspergillus terreus* Riyadh A7, *Aspergillus niger* AlGouf, *Aspergillus niger* van Tieghem NRRL 326, *Fusarium solani* Riyadh DI show that the increment of inulinase production in shaken cultures by tested strains was an efficient process in consuming and conversion of sugar to biomass. Also, an increase in inulinase production with the increase of incubation temperature up to 30°C for the four tested fungal strains and thereafter, higher temperature resulted in a gradual lowering of inulinase formation. Besides, the results indicated that the pH greatly affected both growth and inulinase activity. The extracellular inulinase by tested strain was produced under both acidic and alkaline conditions and was found to be optimal at pH 6, where the enzyme activity was reached up to 48.14 U/ml. Outside the optimum pH value, the enzyme activity was slightly reduced. On other hand, the enzyme activity proportional produced by substrate concentration increment, and the enzyme production was decreased above 2% inulin concentration. Whereas, higher inulin concentrations lead to catabolic repression, consequently lowering the enzyme yields. The best concentration of inulin was 2% by which inulinase production reached up to 56.80 U/ml and consumption of inulin was more than 97% of total sugar. In addition, it was found that the production of inulinase is fungal growth dependent. Also, the fungal biomass was varied widely among the carbon sources utilized, ranging between 79 and 431 DW mg/50 ml obtained in the presence of galactose and maltose, respectively. Finally, treptone showed superiority over all organic and inorganic nitrogen sources, followed by yeast extract and NH₄NO₃, for the production of inulinase from *Aspergillus terreus* Riyadh A7.

4. DISCUSSION

Inulin is a linear fructose polymer that can be found as a reserve carbohydrate in plant tubers in which the fructans content is approximately 50% of the plant's dry weight. Inulin represents a source for the production of ultra-high-fructose syrups by enzymatic hydrolysis. Inulinase is a potentially useful enzyme for the production of high fructose syrups from inulin, where fructose shows several advantages in comparison to sucrose. Inulinase has also been employed for kidney disease diagnosis²². All the isolates identified as filamentous fungi belonging to the phyla Deuteromycota, Ascomycota, and Zygomycota. (Figures1), they were identified to the genus level as, *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*, *Rhizoctonia*, *Mucor*, *Alternaria*, and *Rhizopus*. *Aspergilli* groups are commonly found in soil and on decaying plant material. By converting resistant plant polysaccharides such as cellulose and pectin into small oligosaccharides and eventually into single sugar molecules, fungi such as *Aspergillus* spp. Play an important role in carbon cycling²³. Regarding the ability of these genera to inulin degradation, in 1989 Kim and Rhee used *Aspergillus ficuum* for the production of inulinase. Also, Reported that *Aspergillus awamori* was capable of inulinase synthesis, and the enzyme carried out complete hydrolysis of inulin to fructose²⁴. The present results show an *Aspergillus* spp. Are among the best-known producers of inulinase (table I), and the last study was informed that¹³. Moreover, *Rhizopus* sp²⁵ and *Alternaria alternata*²⁶ have also been studied for extracellularly inulinase production. The cultivation period for the maximum inulinase production was dependent on the tested fungal strains ranging between 2-6 days incubation period. Similarly, it was found by Nandagopal²⁷ and who

reported that the incubation period influenced inulinase production, and maximum inulinase activity was observed at 5 days of both *Penicillium purpurogenum* and *Aspergillus niveus* cultures. While both *Alternaria alternata* and *Aspergillus niger* reached maximum inulinase values on the 2nd day, however, *Trichoderma harzianum* at 3rd day of incubation period⁴. Moreover, total sugar was gradually reduced by fungal growth increasing and completely disappeared on the 7th day of incubation in the culture of the most active fungal strains. Also, found that the sugar was totally consumed in 72h old culture of *Aspergillus niveus* Blochwitz 4128URM²⁸. Among pH variation during the time course of enzyme production by all fungal strains, there was high acidification by total sugar decreasing and increment of both fungal growth and enzyme activity. The results are in accordance with those who reported that during the inulinase production by *A. niger* N402, the pH values decreased from 5.5 to 2.5 due to the formation of organic acids²⁹. The improvement strategies used in microbial enzyme productions were generally interested in classical optimization methods. There are many reports about classical optimization methods based on varying one-factor-at-a time³⁰. Generally, temperature influences the metabolic activity of cells. The obtained results for the growth of fungi at various incubation temperatures reveal that all tested fungal strains could tolerate and grow up to 50°C. But incubation temperature at 30°C was an optimum cultivation temperature for the growth. In this study, the finding of temperature parameters on fungal growth and enzyme production is close to earlier findings, such as Incubation temperature of 30°C was selected for further experiments using the best strain, *Aspergillus terreus* Riyadh A7 for inulinase production. Also, previous studies have used 30°C as fermentation temperature for the production of inulinase⁷. The pH of the culture medium showed a moderate effect on the fungal growth, which ranged from 212 to 330 DW mg/50 ml in initial pH values 3 and 6, respectively. Thus, from these data the optimum pH value 6 was chosen for further experiments. In general, the pH value of the culture medium mainly controls the course of enzyme production, the recent study also discussed the optimum pH is 5.0 for *Alternaria alternata* and *Trichoderma harzianum* and pH is 6.0 for *Aspergillus niger*⁴. Thus, the addition of inulin as a carbohydrate source usually is a prerequisite for inulinase enzyme formation. The culture was induced by cultivating it on different concentrations of inulin to get a high yield of inulinase enzyme observed higher inulinase activity of *A. niger* A42 in a medium in which carbon source was Jerusalem artichokes extract (10 g L⁻¹)¹⁴, but only after 192 hours of incubation reported that the best concentration was 4% of the chicory root extract in incubation media for elevating inulinase activity by thermophile *Thielavia terrestris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337³¹. Inulin is not only a significant carbon source but also an important inducer for inulinase production. A comparison among different C-sources in the cultivation of *Streptomyces* sp. Has also been performed; crude inulin was as good as pure inulin, except garlic extract, which showed a 60% inulinase yield enhancement concerning pure inulin²⁷. The present results demonstrated that treptone showed superiority over all organic and inorganic nitrogen sources, followed by yeast extract and NH₄NO₃, for the production of inulinase from *Aspergillus terreus* Riyadh A7. Also, utilized a medium composed of a combination of yeast extract and peptone that offered the best condition to inulinase production by *Kluyveromyces marxianus*³².

5. CONCLUSION

From twelve soil samples collected in Saudi Arabia soils from different locations, thirty fungal isolates displayed growth on inulin agar medium. They were identified to the genus level as, *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*, *Rhizoctonia*, *Mucor*, *Alternaria*, and *Rhizopus*. Eight fungal isolates (*Aspergillus* Riyadh A2, *Aspergillus* Riyadh A3, *Aspergillus* Riyadh A7, *Fusarium* Riyadh D1, *Aspergillus* Riyadh E4, *Aspergillus* Riyadh E5, *Aspergillus* Riyadh F1, *Alternaria* AlBaha B2, *Aspergillus* AlGouf) showed an ability to grow at 30-45°C and considered as thermotolerant, were chosen for inulinase production experiments and compared with reference strain *Aspergillus niger* van Tieghem NRRL 326. The effect of time course on inulinase productivity was investigated in shaking flask cultures, incubated at 30°C and the extracellular inulinase was determined within 1-7 days old cultures. Among all the tested fungal strains, inulinase production was varied. Enzyme activity was ranged between 11.76-39.16 U/ml, as well as the shaking at 150 rpm resulted in greater growth and inulinase production for all tested strains after 72 h compared with static cultures, in addition to, Incubation temperature at 30°C was an optimum

cultivation temperature for inulinase production and fungal growth; at pH 6 the enzyme production was reached up to 48.14 U/ml; the best concentration of inulin was 2% by which inulinase production reached up to 56.80 U/ml; and inulin served as the preferred carbon source for inulinase activity, 56.72 U/ml. It is clear that inulinase production by *Aspergillus terreus* Riyadh A7 is dependent on growth condition and especially on carbon source (incubation temperature 30°C; Initial pH 6.0; inulin 2% and trypton that resulted in enzymatic activity of 68.52 U/ml.).

Recommendation

After the optimization and considering the remarkable higher inulinase productivity obtained by local fungal strain in this study, will recommend the possibility to apply it at the commercial scale and use plant residues instead of inulin to reduce the cost of production.

6. CONFLICT OF INTEREST

Conflict of interest declared none.

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