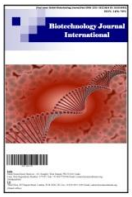

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# Exo-Inulinase Production by a Catabolite Repression-Resistant Mutant Thermophilic *Aspergillus tamarii*-U4 in Solid State Fermentation

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author EOG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AAO supervised the study. Both authors read and approved the final manuscript.*

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## ABSTRACT

In this study, spores of inulinase-producing thermophilic *Aspergillus tamarii* were subjected to UV mutagenesis, and colonies obtained were screened for inulinase production on inulin-glucose agar. The thermal stability of the inulinase was also investigated. A mutant strain U4 was found to produce 2.8 times inulinase titre (62.1U/mL) as against the wild strain (22.2U/mL). Inulinase production by this U4 strain was also found not to be significantly (P≤0.05) affected by the presence of glucose. The inulinase produced retained 64% of its activity after incubation at 65ºC for three hours. Solid-state fermentation for inulinase production by the strain U4 showed that wheat bran supported the highest inulinase titre 218.3U/gds while banana peels supported the lowest inulinase production titre of 80.5U/gds. Further optimization of cultural parameters revealed that incubation time of 5 days, 60% initial moisture content of the substrate, 2% inoculum density 2%, temperature 55ºC and pH 4.5 were optimal for inulinase production. Under optimized conditions, inulinase titre of 426.6 U/gds was observed. The pattern of inulin hydrolysis by the inulinase revealed the presence of monosaccharide as the main product of hydrolysis. Inulinase

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production at elevated temperatures by the mutant *Aspergillus tamarii*-U4 and its catabolite resistant properties showed that the organism is a potential industrial candidate for the production of exo-acting inulinases.

*Keywords: Aspergillus tamarii; exo-inulinases; thermostability; catabolite repression.*

## 1. INTRODUCTION

Exo-inulinases (E.C. 3.2.1.80; β-fructanohydrolase) which cleave inulin (a polymer of fructose linked by β-(2,1) bonds and terminated by a sucrose residue) and other fructan polymers have been reported in various plants and microorganisms [1]. Although these enzymes are reported to be involved in carbohydrate metabolisms and also play important roles in the regulation of the process of cell differentiation and developments [2], they have received so much attention because of their potential application in the production of fructose via inulin hydrolysis thereby acting as alternative to three (3) glycosidase enzymes on starch [3]. Additionally, exo-inulinases have also been reported to found application in a single step fermentation of inulin to yield ethanol which is the most employed liquid biofuel [4]. Furthermore, inulinases have been reported to be useful in the production of inulooligosaccharides with functional properties similar to that of the fructooligosaccharides whose beneficial effect on humans has been well documented [5].

Although several authors have investigated inulinase production from various microorganisms and the properties of these inulinases characterized [6,7,3,8,9,10,11] and the production of these inulinases is most commonly repressed by the presence of glucose in the production medium (a phenomenon known as catabolite repression). Furthermore, the reported inulinases have not been successfully utilized in the hydrolysis of inulin on an industrial scale, primarily due to the limited solubility of inulin at ambient temperature which necessitates its complete hydrolysis at elevated temperature (60°C and above). At this temperature however, most of the reported inulinases rapidly loose there activity hence the need for constant replenishment [12]. This has made inulinase with high temperature optimal and thermal stability of great importance in the industrial production of fructose from inulin. In this paper we report the production of inulinase production by a mutant strain *Aspergillus tamarii*-U4 which was generated from wild type *Aspergillus tamarii*-INU4 by treatment with ultraviolet (UV)-light.

## 2. MATERIALS AND METHODS

### 2.1 Microorganism and Culture Conditions

The thermophilic strain of *Aspergillus tamarii*-INU4 (Accession: KJ423064.1) used in this study was obtained from the Culture Collection of the Department of Microbiology, University of Ibadan, Nigeria. It was previously isolated from a waste dumpsite located within the Ibadan metropolis and maintained on Yeast Extract-Inulin- Peptone (YIP) agar slants [12] at 4°C.

### 2.2 Mutagenesis

The spores of A*spergillus tamarii*-INU4 that was used for mutagenic studies were prepared by flooding the surface of a seven (7) days old agar slant of the organisms with sterile physiological saline followed by agitation mechanically. The concentration of suspension obtained was adjusted by appropriate dilution using sterile physiological saline to obtained suspension of $10^2$ spores/ml and this was used for mutation. An aliquot of 1mL spore suspension (containing approximately 102 spores/mL) was dispensed into each of six Petri plates which thereafter were exposed to UV radiation from a 30W UV-bulb (at a distance of 10cm) for variable time periods (5, 10, 15, 20, 25 30, 35, 40, 45, 50, 55 and 60 min). After exposure, the plates were kept in the dark for half an hour to prevent photo reactivation [13] following which they were transferred to the surface of the inulin-agar of Kim [14] as described by Garuba et al. [12] and incubated at 50ºC for 5 days. In each case, the experiment was replicated three times. A control experiment consisting of 1 mL of the untreated spore suspension was also kept in the dark for half an hour and then plated on inulin-agar followed by incubation at 50ºC for 5 days. Percentage survival of the organism was calculated as follows

Percentage survival rate (%) = a/b ×100

Where,

a and b indicate the average number of colonies on the inulin agar medium of the treated and

untreated spores respectively. Organisms growing from treatment which represent approximately 10% survival rate were selected for further studies.

## 2.3 Screening of Mutants

Spores of organisms obtained from treatments corresponding to approximately 10% survival rate were prepared as earlier described and 1 mL spore suspension (containing $2.0\times10^4$ spores/mL) was used to inoculate 100 mL of inulin broth of Kim [14] supplemented with 5% glucose. Control experiments were also set up without glucose supplementation and the cultures incubated at 50ºC for 5 days. After 5 days of incubation, the fermented broth was centrifuged at 6000 rpm for 20 minutes and the supernatant was taken as the crude enzyme. Inulinase activity and thermal stability of the inulinase produced was determined as previously described by Garuba et al. [l12]. The mutant with the highest inulinase production in the presence of a 5% glucose coupled with high thermal stability was selected for inulinase production in solid-state.

## 2.4 Solid-state Fermentation

### 2.4.1 Substrate screening

Screening of different substrates as an appropriate medium for inulinase production in solid-state fermentation was carried out as earlier described by Onilude et al. [10] using wheat bran; peels of yam, cassava and banana; and sugar cane bagasse. Five grams of each of the dried pre-treated substrate was mixed with 1mL of acidified mineral solution containing 3.0mg of $MnSO_4•H_2O$, 9.0 mg $FeSO_4$. $6H_2O$, 2.5mg $ZnSO_4.7H_2O$, and 3.5 mg $CaCl_2$ (for 100 g of the substrates) as described by Xiong et al. [15] and the pH adjusted to 5.0 in 100ml Erlenmeyer's flasks and the moisture content of the substrate adjusted to 65% by the addition of an appropriate quantity of sterile distilled water. The Erlenmeyer's flasks were plugged with non-absorbent cotton wool and sterilized in an autoclaved at 121°C for 20 min after which they were allowed to cool to ambient temperature. The cooled substrates were then inoculated with 1 mL spore suspension (containing $2.0\times10^6$) spore suspension of the mutant strain of the fungus and incubated at 50ºC for 5 days in a static or stationary mode.

### 2.4.2 Inulinase extraction and inulinase activity

Inulinase extraction was carried out according to the method of Mazzutti et al. [7] by the addition of sodium acetate buffer at ten times (v/w) of the fermented matter (0.1M at pH 4.8) and incubated at 50°C with agitation at 150 rpm for 30min. This whole content was then filtered using Whatman filter paper No. 1 and the supernatant was used as the crude enzyme preparation. Inulinase activity was measured as previously described by Garuba et al. [12] and one unit of inulinase activity was defined as the amount of inulinase enzyme that produced lμ mole of fructose per minute under defined assay conditions.

## 2.5 Time Course on Inulinase Production

The time course of inulinase production was investigated by inoculating 5g of sterile wheat bran medium with 1 mL spore suspension (containing $2\times10^8$ spores) and incubated at 50°C for 10 days. Inulinase production was monitored at 24-h interval by assaying for inulinase produced during the fermentation period.

## 2.6 Effect of %Moisture Content on Inulinase Production

To determine the effect of the initial moisture content of the substrates on the inulinase production, five different (50, 55, 60, 65 and 70%) moisture content levels were established in the solid substrate before sterilization. After sterilization, inoculation was done stated above and the set up was incubated for 6 days at 50°C.

## 2.7 Effect of Inoculum Density on Inulinase Production

Optimization of the size of inoculum on inulinase production by the organism was studied using various levels of inocula (1, 2 4, 6, 8 %w/v). The sterile substrate with 65% was inoculated with the various inoculum levels and the set up was incubated at 50°C for 6 days.

## 2.8 Effect of Temperature on Inulinase Production

Optimum temperature for inulinase production by this organism was investigated as follows; the sterile substrate was initially inoculated with 4% inoculum and its moisture content adjusted to 60%. The cultures were incubated at different

temperature regimes viz, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C for 6 days, after which inulinase produced quantified.

### 2.9 Effect of the Initial pH of the Fermentation Medium on Inulinase Production

The effect of different pH levels on the production of the inulinase was done according to the method of Gill et al. [16]. A buffer of varying pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) was used for the adjustment of the moisture content. Thereafter, sterilization was done and the substrate inoculated with a 4% inoculum level. The setup was incubated for 9 days at 50°C.

### 2.10 Pattern of Inulin Hydrolysis

Pattern of hydrolysis of inulin (whether Exo- or Endo-acting) by the crude inulinase extract was determined by spotting the reaction product (after incubating inulin and the crude inulinase for 2 hours at 50ºC) on a pre-coated TLC plate (Merck Germany). The plate was developed with a solvent system of chloroform: acetic acid: water (30:35:5 v/v/v) at room temperature and sugars were visualized by heating the plate at 120ºC for 10 min after which it was sprayed with 1% α-naphthol (containing 10% phosphoric acid) acid as described by Azhari et al. [17].

## 3. RESULTS AND DISCUSSION

### 3.1 Survival and Screening of Strains

Fig. 1 shows the survival pattern of spores of *Aspergillus tamarii*-INU4 after exposure to UV-light. As shown, initial exposure time of 10 minutes produced about 90 colonies which further decreased as the time of exposure increased, with no colony growing after 55 minutes of exposure. From the treatments (including the various replicates), twenty-eight different colonies were isolated from treatment corresponding to 10% survival rate (90% lethal dose) and screened for enhanced inulinase production in inulin medium with and without 5% glucose.

The results of inulinase production by the putative mutants as presented in Table 1 showed that mutant strain U4 produced highest inulinase titre of 62.1U/mL which is 2.8 times inulinase titre of the wild strain (22.2 U/mL). The growth of this strain U4 and that of the wild type on inulin-agar medium is shown in Plate 1. Enhanced inulinase production after UV-light treatment has been well documented in the literature. *A. niger* mutant UV11 gave 3 times the inulinase yield of the parent strain [18]. Similarly, Yu et al. [19] reported a 2.6 times increase in inulinase production when the ascospores of *Pichia guilliermondii* were subjected to UV and LiCl treatment. The same mutant produced 455.9 U/gds of inulinase activity under optimized conditions [9]. High inulinase production is particularly important from the commercial point of view. In this study, enhanced inulinase production by the mutant in inulin medium with and without glucose supplementation was not statistically significant ($p \leq 0.05$) (Table 1). Various concentration of glucose was also investigated and data obtained (not shown) indicated that inulinase production is not statistically significant ($p \leq 0.05$).

Plate 1 shows the growth of this mutant and the wild strain on inulin agar medium supplemented with 5% glucose. The resistance of mutant *Aspergillus tamarii*-U4 to catabolite repression could be as a result of an alteration in the promoter, terminator, or regulatory sequence of inulinase synthesis. This resistance to catabolite repression observed in this report could provide excellent material for studying the synthesis, regulation, and secretion of inulinase in filamentous fungi. Additionally, this resistance to catabolite repression could be advantageous when considering the use of a substrate with a high concentration of monosaccharides for inulinase production. As a result, this mutant strain U4 was selected for further studies.

Of the solid substrates investigated in this study, Wheat bran supported the highest inulinase production with an inulinase titre of 218.3±0.55 U/gds (Table 2). This observation is similar to the report by Trivedi et al. [20] who reported the highest inulinase production in wheat bran substrate by a strain of *Aspergillus tubingensis* CR16. Apart from agro-residues acting as carbon sources, complex media also contain substances such as salts, which can inhibit and stimulate cellular growth and microbial enzyme production [10]. Wheat bran supporting the highest inulinase titre could be as a result of the fact that it acts as a complete nutritious feed for microorganisms, having all the ingredients and remains loose even under moist conditions providing a large surface area. Moreover it contains various soluble sugars that help the initiation and growth of microorganisms.

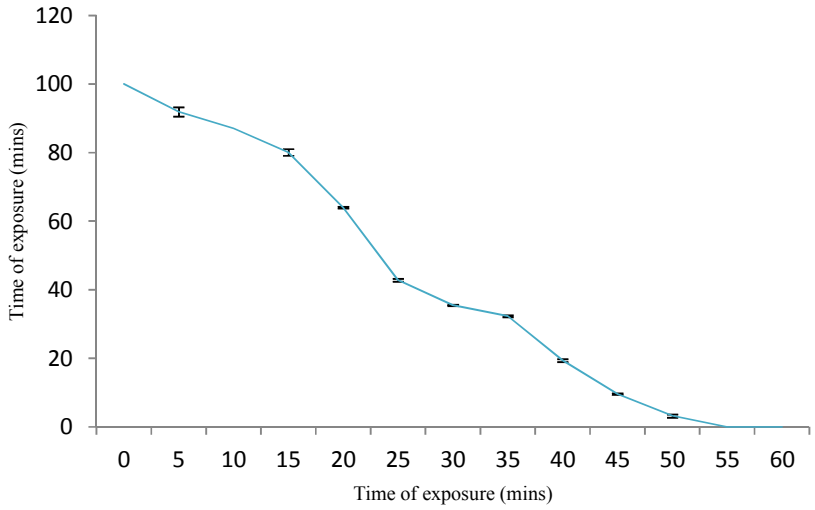


**Fig. 1. Survival rate of spores of *Aspergillus tamarii*-INU4 on exposure to 30 wattage of UV-light at 10 cm**

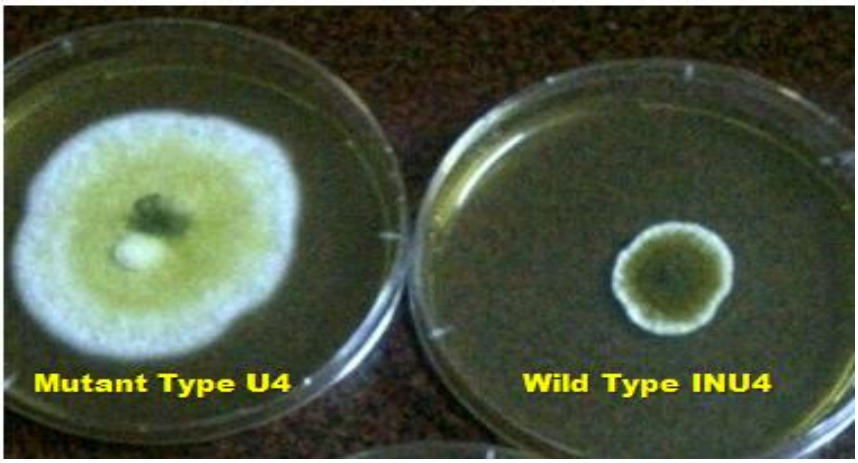


**Plate 1. Three-day old culture of wild and mutant strains of *Aspergillus tamarii* on inulin-agar medium**

Results of the time course of inulinase production as presented in Fig. 2 revealed that the accumulation of extracellular inulinase increased with an increase in fermentation time with a peak inulinase production of 312.6±1.4U/gds at the 6$^{th}$ day of fermentation. Optimum inulinase production after 5 days of incubation by *Aspergillus ficuum* JNSP5-06 has also been reported by Jing et al. [21]. Similarly, Housseiny [11] reported seven days for maximum accumulation of inulinase by *Aspergillus niger* AUMC 9375. This optimum incubation period in this study is however lower than that reported by Gupta et al. [22] who observed highest inulinase production by *Aspergillus aureus*, *A. fisheri*, *A. flavus* and *A. nidulans* on the 9$^{th}$ day of fermentation. Available literature shows that there exists great variation in optimum inulinase production time and this considered variation in incubation periods for optimum inulinase production could be due to differences in strain types, medium composition, and culture conditions [23]. The decline in inulinase titer after the optimum incubation period could be as a result of the exhaustion of the carbon or the accumulation of autotoxic products of the organism in the medium.

**Table 1. Inulinase production by wild type *Aspergillus tamarii*-INU4 and putative mutants**

| Strain | Inulinase activity (U/mL) in medium without glucose | Inulinase activity (U/mL) in medium with 5% glucose | Relative activity after heating at 65ºC for 3hrs |
|---|---|---|---|
| INU4 | 22.21±0.2 | 9.12±0.32 | 62 |
| U11 | 18.23±0.3 | 5.0±0.48 | 50 |
| U22 | 12.46±1.2 | 1.8±0.81 | 57 |
| U33 | 5.24±0.5 | 1.57±0.45 | 60 |
| U44 | 16.48±0.6 | 7.2±0.46 | 18 |
| U55 | 12.56±0.2 | 3.9±0.4 | 44 |
| U66 | 25±0.01 | 19.0±0.41 | 53 |
| U77 | 28±0.2 | 17.2±0.86 | 60 |
| U88 | 30.56±0.4 | 15.4±0.71 | 11 |
| U99 | 22.49±0.3 | 19.9±0.81 | 60 |
| U1 | 32.46±0.57 | 21.4±0.62 | 41 |
| U2 | 14.81±0.1 | 12.1±0.6 | 54 |
| U3 | 29.13±0.28 | 21.1±0.62 | 62 |
| U4 | 62.18±0.01 | 61.2±0.12 | 64 |
| U5 | 51.28±0.56 | 38.6±0.21 | 60 |
| U6 | 28.18±0.18 | 18.9±0.43 | 30 |
| U7 | 29.12±0.11 | 19.0±0.36 | 50 |
| U8 | 43.61±0.34 | 35.1±0.87 | 42 |
| 1U1 | 49.72±0.56 | 32.8±0.21 | 20 |
| 1U2 | 39.16±0.08 | 30.5±0.2 | 36 |
| 1U3 | 24.18±0.42 | 15.2±0.41 | 49 |
| 1U4 | 42.8±0.48 | 29.3±0.40 | 52 |
| 1U5 | 45.14±0.62 | 32.4±0.20 | 10 |
| 1U6 | 7.81±0.38 | 3.0±42 | 30 |
| 1U7 | 34.21±0.56 | 24.6±0.08 | 46 |
| 1U8 | 32.19±0.31 | 20.6±0.54 | 40 |
| 1U9 | 24.27±0.72 | 19.2±0.34 | 52 |

**Data are means of three replicates.**Means with different letters within each column differ significantly (p≤0.05) using Duncan Multiple Range Test*

**Table 2. Inulinase production by *Aspergillus tamarii*-U4 in solid-state fermentation**

| Substrate | Inulinase activity(U/gds)* |
|---|---|
| Banana peel | $80.5\pm1.4^{e}$** |
| Yam peel | $115.4\pm0.5^{c}$ |
| Sugar cane bagasse | $161.5\pm0.8^{b}$ |
| Cassava peel | $91.0\pm1.2^{d}$ |
| Wheat bran | $218.3\pm1.4^{a}$ |

*Data are means of three replicates. *U/gds-Units per grams of dry substrate **Means with different letters within each column differ significantly (p≤0.05) using Duncan Multiple Range Test*

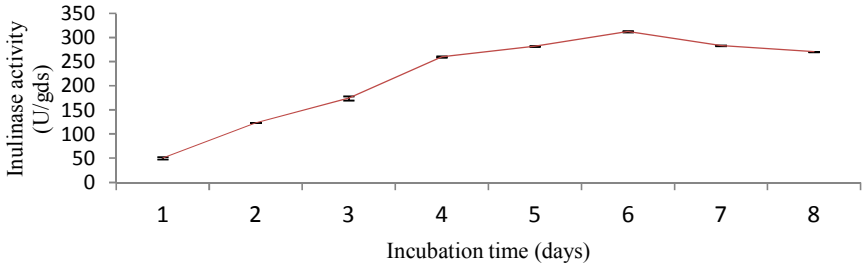


**Fig. 2. Time course of inulinase production by mutant *Aspergillus tamarii*-U4 in solid-state fermentation**

The results of the initial % moisture content of the substrate (Fig. 3) showed that inulinase titre rapidly increased from 210.3±1.2 U/gds to 289.8±0.8 U/gds when the % moisture content increased from 50% to 55% in wheat bran reaching a peak inulinase titre of 398.4±1.7 U/gds at 60% moisture content followed by a gradual decrease in inulinase accumulation. This optimum moisture level is in the range of 30-75% reported for SSF systems[10]. Substrate moisture has been reported as a critical factor in SSF as it has a great influence on growth and biosynthesis including the secretion of different metabolites [7]. Low enzyme titre below the optimum moisture content has been attributed to due to a reduction in the nutrient solubility, low degree of swelling and high water tension [10] while a decline above the optimum moisture levels might be due to allosteric hindrance of the growth of the organisms by the reduction in porosity (interparticle spaces) of the solid matrix, thus interfering with oxygen transfer [24]. The maximum inulinase production at low moisture level by this *Aspergillus tamarii*-U4 is advantageous as the chance of contamination of the fermentation medium by other microorganisms having high water activity will be greatly reduced.

Investigation of the effect of inoculum density on inulinase production as shown in Fig. 4 revealed maximum accumulation of inulinase titre of 409.1±0.7 U/gds at 2% below and above which low inulinase titre were observed. The 2% optimum inoculum density observed in this study is similar to the 2% that reported for A. *niger* were reported by de-Souza-Motta et al. [6]. This value is however lower compared to 5% inoculum density reported for a strain of *Aspergillus tamarii* AR-IN9 by Saber and El-Naggar [25]. It is necessary to provide an optimal inoculum size in fermentation as lower inoculums density may give insufficient biomass and permits the growth of undesirable organisms whereas a higher inoculums density usually produce too much biomass that can rapidly deplete the available substrate nutrient before the organisms are physiologically ready to start enzyme formation [10].

The results effect of incubation temperature on inulinase production (Fig. 5) revealed that inulinase production increased from 356.1±1.3 U/gds at 45°C to 390.2±0.4U/gds at 50°C reaching a peak titer of 410.5±1.8U/gds at 55°C and thereafter, exhibits a gradual decrease to 378.2±0.4U/gds at 60°C and finally to 301.1±0.2U/gds at 70°C (Fig. 5). Optimum inulinase production at 55ºC by this *Aspergillus tamarii*-U4 is advantageous as it allows the fermentation to be carried out at elevated temperatures thereby preventing contamination by mesophilic organisms [3,8]. Low inulinase activity observed at low temperature in this study may be as a result of the fact that the organisms were not metabolically active even though they were in the exponential phase of growth while low inulinase titer at temperatures beyond the optimum could be due to the reduction of oxygen solubility in the medium [26]. Furthermore, temperature has been reported to affect the secretion of extracellular enzymes, possibly by changing the physical properties of the cell membrane [27].

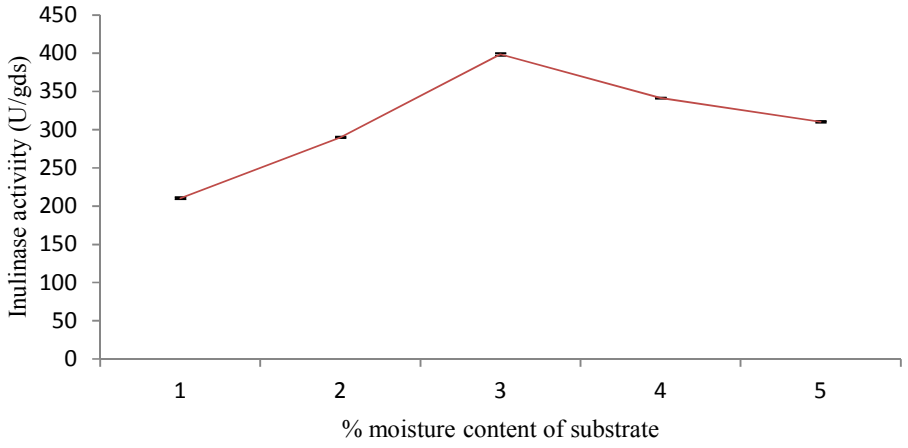


**Fig. 3. Effect of different percentage moisture content of substrate on inulinase production by Mutant *Aspergillus tamarii*-U4**

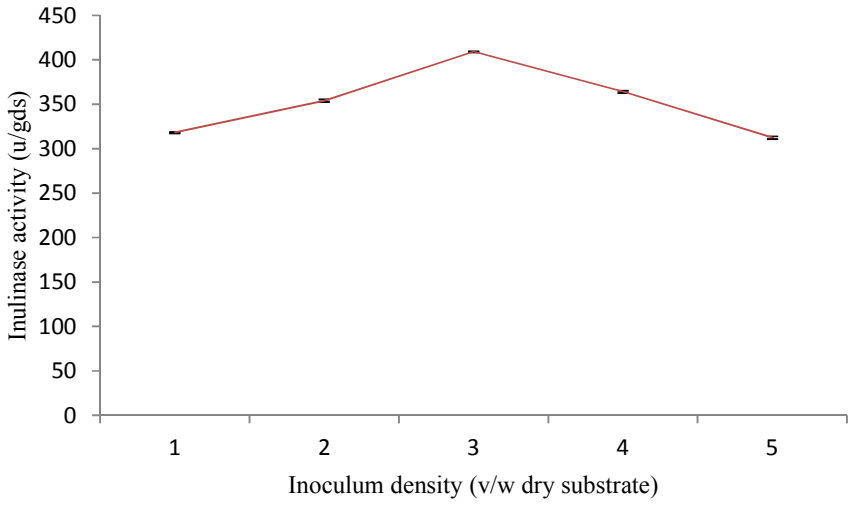


**Fig. 4. Effect of inoculum density on inulinase production by mutant *Aspergillus tamarii*-U4**

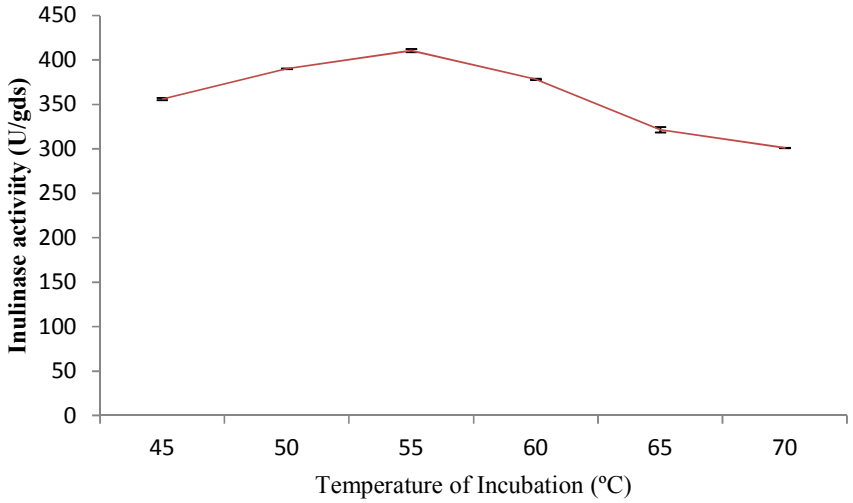


**Fig. 5. Effect of different incubation temperatures on inulinase production by mutant *Aspergillus tamarii*-U4**

In this study, optimum pH for inulinase production using wheat bran as substrate was found to be 4.5 (426.6±0.4U/gds). The initial pH of the solid substrate is considered an important factor in the SSF cultivation of microorganisms and enzyme production as it affects the metabolic activities of microorganisms. The optimum pH reported in this study is among the limits described for inulinase production by fungi [10]. In general, it is noted that the pH varies among the different microbial species and according to the type of substrate [23].

The thin layer chromatographic analysis of the product of the hydrolysis of inulin (50 C, during two hours) by the inulinase produced revealed fructose as the main product of hydrolysis (Fig. 7). Fructose as the main product of hydrolysis indicates that the inulinase splits off the terminal fructose unit from the non-reducing end of inulin producing about 95% fructose [3]. Exo-acting inulinase reported in this study is similar to the report of Singh and Singh[5] who documented that inulinases from fungi are generally exo-acting.

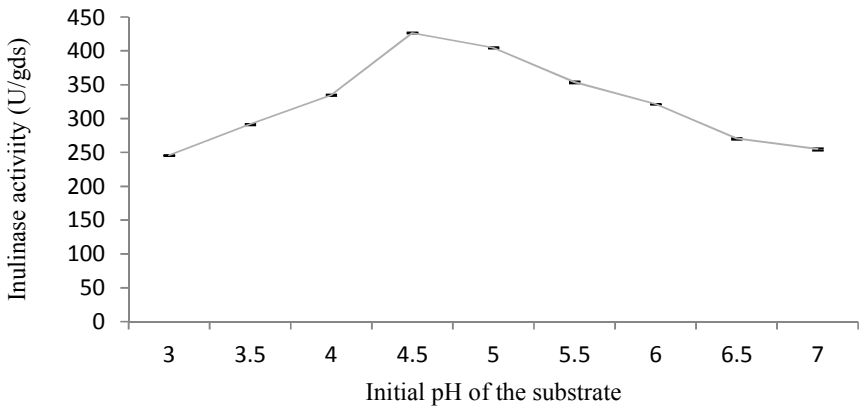


**Fig. 6. Effect of initial pH of the substrate on inulinase production by mutant *Aspergillus tamarii*-U4**

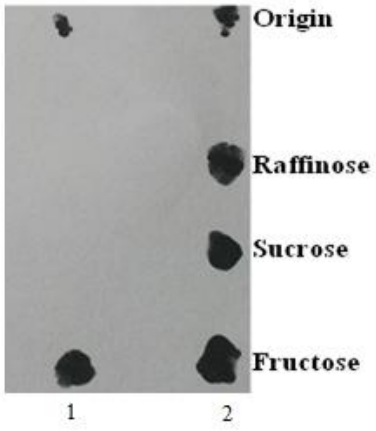


**Fig. 7. Thin layer chromatogram of the pattern of inulin hydrolysis by the inulinase produced by mutant *Aspergillus tamarii*-U4 (Lane 1); sugar standards (Lane 2)**

## 4. CONCLUSION

In conclusion, the optimum temperature of 55 ºC for inulinase production reported in this study together with the high thermal stability of the produced inulinase at elevated temperatures makes the mutant strain *Aspergillus tamarii*-U4 a promising organism for the production of exo-acting inulinase in SSF. Furthermore, the fact that exo-inulinase production wasn't affected by the presence of glucose in the inulin-agar medium further gives credit to the potential industrial importance of this organism for exo-inulinase production especially when considering the choice of glucose-containing substrate. Nevertheless, further work is required to characterize the exo-inulinase produced by this organism to determine its suitability for industrial application.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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