

Optimization of process parameters for inulinase production from endophytic fungi *Fusarium solani* LBKURCC67, *Humicola fuscoatra* LBKURCC68 and *Fusarium oxysporum* LBKURCC69

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Abstract

Endophytic fungi isolates of *Fusarium solani* LBKURCC67, *Humicola fuscoatra* LBKURCC68 and *Fusarium oxysporum* LBKURCC69 are local strains that are isolated from dahlia tubers (*Dahlia variabilis*). Inulinase is an enzyme that hydrolyses the β (2-1) D-fructofuranoside bond of inulin into fructose or fructooligosaccharide. This study aimed to determine the optimum pH of inulinase production (3,5; 4,0; 4,5; 5,0; and 5,5), the optimum temperature (30, 35, 37 and 40°C) and the optimum agitation speeds (50, 100, 150 and 200 rpm) for inulinase production from these three isolates.

In this research, the activity of inulinase was determined with diffusion method and dinitrosalicylic (DNS) method. The result showed that *F. solani* LBKURCC67 did not produce inulinase while *H. fuscoatra* LBKURCC68 and *F. oxysporum* LBKURCC69 were able to produce inulinase with the highest inulinase activity found in *H. fuscoatra* LBKURCC68. The optimization of process parameters was obtained at pH 5,0 temperature of 35°C and agitation speeds of 100 rpm with crude inulinase extract activity of 188.89×10^{-3} U/mL, protein content 0.91 mg/mL and the specific enzyme activity of 211.96×10^{-3} U/mg protein.

Keywords: Dahlia, endophytic fungi, *Humicola fuscoatra*, inulinase, optimization.

Introduction

Inulinase is an enzyme that is capable of hydrolyzing inulin to produce molecules of fructose or fructooligosaccharide. Inulin is hydrolyzed by two types of inulinase; exoinulinase (β -D-fructanfructohydrolase, EC 3.2.1.80) which produces fructose and endoinulinase (2,1- β -D-fructanfructano hydrolase, EC 3.2.1.7) which produces fructooligosaccharide. Inulin is a polymer formed from polifruktose, through β (2-1) D-fructofuranoside bond with the beginning of the chain containing a molecule of glucose. Inulin can be found in some plants such as Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium endivia*) and dahlia (*Dahlia* sp.).^{1,2}

Inulinase is commercially produced by microorganisms including *Aspergillus* spp., *Penicillium* spp., *Bacillus* spp., *Pseudomonas* spp., *Xanthomonas* spp., *Kluyveromyces* spp., *Cryptococcus* spp., *Pichia* spp., *Sporotrichum* spp. and *Candida* spp.^{3,4} The apparent applications of inulinase can be seen in the production of high fructose syrup, inulooligosaccharide, fructooligosaccharide, sorbitol, pullulan, ethanol, acetone and butanol.⁵ Conventional production of fructose from starch molecules requires three enzymatic reactions using α -amylase, amiloglucosidase and invertase with a yield of fructose produced at about 45% whilst the production of fructose directly from inulin by inulinase requires an enzymatic reaction with a yield of fructose produced at about 90 %.^{6,7}

Inulinase can be produced by the endophytic microbes including fungi. Endophytic fungi live inside plant tissues but not detrimental to the host plant.^{3,8,9} In a previous study, Lestari¹⁰ had carried out the isolation of various dahlia tubers from the area of Padang Panjang, Sumatera Barat and gained 3 isolates of *Fusarium solani* LBKURCC67 from yellow-flowered dahlia tubers, *Humicola fuscoatra* LBKURCC68 from red-flowered dahlia tubers and *Fusarium oxysporum* LBKURCC69 from pink-flowered dahlia tubers which had previously been identified.¹¹

A series of qualitative tests were conducted to endophytic fungi that could produce extracellular enzymes such as catalase and inulinase but did not produce amylase. It has been observed that there is currently lack of information about enzymatic fermentation conditions for endophytic fungi isolated from dahlia tubers, so it is necessary to find the optimum conditions for endophytic fungi to produce inulinase.

Material and Methods

As many as three endophytic fungal isolates were obtained from dahlia tubers from the area of Padang Panjang, Sumatera Barat i.e. *Fusarium solani* LBKURCC67, *Humicola fuscoatra* LBKURCC68 and *Fusarium oxysporum* LBKURCC69 which were subcultured in potato dextrose agar (PDA) medium. These isolates were collections of Research Laboratories of Enzyme, Fermentation and Biomolecular Engineering, Universitas Riau.

Preparation of Inulin from Dahlia Tubers: A total of 200 g dahlia tubers were peeled, washed and sliced, heated in 500 ml of boiling water for 15 minutes and filtered with chiffon or cotton cloth. The precipitate was re-heated to 100 mL of boiling water for 15 minutes and filtered again with a cloth. The filtrates were mixed, added with alcohol 70% with the same volume as the filtrate and stored at a temperature of 5°C for one night. The precipitate formed was separated by a vacuum pump, washed 2 times with 50% ethanol and dried in an oven at 60°C.

Semiquantitative Analysis of Inulinase Activity with Diffusion Method: A test of inulinase activity of endophytic fungi used medium with inulin 1% as the sole carbon source in a Petri dish with the composition of 10 g inulin, 1,5 g NaNO₃, 2g (NH₄)₂SO₄, 1g KH₂PO₄, 0,5g MgSO₄.7H₂O, 0,1g FeSO₄.7H₂O and 18 g agar. All components were dissolved in 1000 mL of demineralized water and heated. Media solution was sterilized by autoclaving at a pressure of 15 psi, at 121°C for 15 minutes. Each endophytic fungi *F. solani* LBKURCC67, *H. fuscoatra* LBKURCC68 and *F. oxysporum* LBKURCC69 subcultured on PDA were inoculated on 1% inulin agar medium and incubated for 2-3 days at room temperature.

Determination of inulinase activity was carried out by placing the culture in a refrigerator at 0°C for 5 days and 7 days. After that, a few drops of iodine were dropped over the surface of fungal colonies on day 5 and 7 and then leveled by shaking the Petri dish evenly throughout the media for 15 minutes to allow iodine to be absorbed. Positive test was indicated by the formation of a clear zone around the growth of fungal colonies as a result of hydrolyzed inulin. The colony area (K) and clear zone (Z) were measured using a caliper. Inulinase activities were grouped into three categories, namely low activity with Z/K ratio <1, moderate with Z/K 1-2 and high with Z/K>2.⁹ The isolate which produced the highest Z/K ratio was used as a next step to determine optimum process parameters for inulinase production.

Inulinase production: A fungal isolate with the highest inulinase activity was used for inulinase production optimization. A total of 1-2 dose of the isolate was inoculated into 100 ml of potato dextrose broth in a 250 mL Erlenmeyer flask and incubated at 30°C on an incubator shaker with the agitation speed of 100 rpm for 72 hours. Measurement of optical density (OD) was performed using a spectrophotometer at a wavelength of 600 nm to determine the approximate number of cells in the inoculum for the production of inulinase in which OD_{600nm} was equivalent to 0.1 × 10⁷ CFU/ml.¹²

The suspension of fungi growing on this medium was used as a starter inoculum. Each inoculum was inserted with the same relative number of cells in media production with the composition of 10 g inulin, 0,5 g MgSO₄.7H₂O, 0,16 g FeSO₄, 2 g NaCl, 0,5 g KCl and 10 g yeast extract. All of the

materials were dissolved in 1000 mL of 0.05 M acetate buffer with a pH of 5.0. The liquid medium was sterilized and incubated for 24 hours.

Optimization of pH for inulinase production: As much as 10% of inoculum was inoculated into 100 mL liquid medium with a series of different pH (3,5; 4,0; 4,5; 5,0; and 5,5) respectively in Erlenmeyer flask. The mixture was incubated at 37°C with the agitation speed of 100 rpm for 5 days.⁹ Furthermore, the liquid medium containing the crude enzyme extract was cooled in a refrigerator for ± 1 hour, then centrifuged at 9500 rpm for 10 minutes to precipitate cell biomass. Crude enzyme extract was filtered with glass fiber filter. Each crude extract's inulinase activity was determined by measuring the levels of reducing sugars with dinitrosalicylate method (DNS).¹³ The crude extract was added with NaN₃ 0.02% (w/v) and stored at 4°C if it was not directly used. The best pH which generated the highest inulinase activity was used to optimize the temperature.

Optimization of temperature for inulinase production at the optimum pH conditions: Determination of optimum temperature was carried out at the optimum pH obtained. The mixture was incubated at a series of temperatures (30, 35, 37 and 40°C). All the procedures followed the previous steps to obtain the inulinase activity.

Optimization of agitation speed for inulinase production at optimum pH and temperature: Determination of optimum agitation speed was carried out at the optimum pH and temperature obtained. The mixture was incubated with the variation of agitation speeds (50,100, 150 and 200 rpm) for 5 days. All the procedures followed the previous steps to obtain the inulinase activity.

Determination of specific enzyme activity: A total of 0.5 mL of inulinase obtained from the optimum pH, temperature and agitation speed was incorporated into each Eppendorf and added with 1 mL of 80% cold acetone. The solution was homogenized and allowed to stand for one night at -20°C. After that, each Eppendorf was cold centrifuged at 13.000 rpm for 10 minutes. The filtrate was separated from the precipitated protein and removed by decantation. The precipitated protein was dissolved in 200 ml of 0.05 M acetate buffer pH 5.0. A specific activity of inulinase was indicated by protein content measured using method of Lowry et al¹⁴.

As much as 1 mL of each protein solution was pipetted into a test tube and added with the reagent A [9.8 ml Na₂CO₃ 2% in 0.1 N NaOH] and reagent B [0.1 mL potassium sodium tartrate 2.7% and 0.1 mL of 1% CuSO₄], homogenized and allowed to stand for 10 minutes. After that, 1 mL of Folin-Ciocalteau reagent was added and allowed to stand for 30 minutes at room temperature. As a blank, 0.05 M acetate buffer pH 5.0 was used while for protein, Bovine Serum Albumin was used prepared with various concentrations (100-700 ppm). The specific activity was measured with the

absorbance of UV-Vis spectrophotometry at a wavelength of 700 nm.

$$\text{Specific enzyme activity} = \frac{\text{enzyme activity}}{\text{mg protein}}$$

$$= \frac{Y \text{ } \mu\text{mole of reducing sugar/mL.min.mg protein}}{\text{mg protein}}$$

$$= Y \text{ unit / mg protein}$$

Data analysis: Data were determined by analysis of variance (ANOVA) and expressed as means and standard errors of 3 replicates.

Results and Discussion

Inulinase semi quantitative analysis with diffusion method:

Inulin is a polymer with a high molecular weight with a range of 3500-5500, so it cannot be transported into the cells of microorganisms.² Inulinase was retained on the surface of the cell wall or released out of the cell. Inulinase secreted in growing media isolates will diffuse into the surface of the agar medium.¹⁵ The presence of inulinase was indicated by the formation of a clear zone around the growth of fungal colonies as a result of hydrolyzed inulin. The wider the clear zone was formed, the higher was the inulinase activity generated.⁷

Analysis of inulinase-producing ability was carried out on *F. solani* LBKURCC67, *H. fuscoatra* LBKURCC68 and *F. oxysporum* spores LBKURCC69 by observing clear zone produced around the colony. Images of inulinase activity determination of *F. solani* LBKURCC67, *H. fuscoatra* LBKURCC68 and *F. oxysporum* LBKURCC69 isolates on day 5 and day 7 are shown in figure 1, figure 2 and figure 3 respectively. In this study, it was observed that inulinase activity is equivalent to the value of Z/K, in which the result could be seen in table 1. The results indicated that isolates of *H. fuscoatra* LBKURCC68 and *F. oxysporum* LBKURCC69 produced clear zone around the colony. The values of Z/K shown by the *F. oxysporum* LBKURCC69 on day 5 and day 7 were 0.28 and 0.21 respectively which are lower than those yielded from *H. fuscoatra* LBKURCC68 with a value Z/K of 1.72 and 1.44 on day 5 and day 7.

Saryono et al⁷ obtained three fungal isolates with the highest inulinase activity produced by *Geotrichum* sp. with the value of Z/K at 2.3 which was categorized as high activity, whereas *Humicola grisea* and *Aspergillus niger* yielded 1,4 and 1,9, respectively which were included into the category of moderate activity. Inulinase activity of *H. fuscoatra* LBKURCC68 fell into moderate activity and *F. oxysporum* LBKURCC69 belonged to the category of low activity. Therefore, the isolate of *H. fuscoatra* LBKURCC68 was subsequently used for optimization of pH, temperature and agitation speeds for inulinase production.

Optimization of pH for inulinase production: Enzyme production from microorganism is strongly influenced by pH, temperature and agitation speeds of fermentation as

external factors and genes as an internal factor. Fungi requires an optimum pH for their best growth; the effect of pH on the growth of the fungi is associated with enzyme activity. If pH in the environment is not suitable, it would interfere with the work of these enzymes and eventually disrupts the growth of the fungi.¹⁶

In line with that, the best inulinase production from *H. fuscoatra* LBKURCC68 required the optimum pH. Inulinase production from *H. fuscoatra* LBKURCC68 with variations of pH at 37°C and 100 rpm for 5 days with a constant factor of fermentation conditions is shown in figure 4.

The highest inulinase activity (120.211×10^{-3} U/mL) of *H. fuscoatra* LBKURCC68 was obtained at pH 5.0. In general, fungi need an optimum pH, ranging from 5.0-7.0 and could grow in pH ranging from 3.0 to 8.5.¹⁷ The buffer that was used in this study is an acetate buffer 0.05 M which served to maintain the pH of the production environment so that the enzyme produced is not denatured. Another study³ stated that for inulinase production, endophytic fungus *Aspergillus clavatus* (BB5) had to be in an optimum pH of 5.0 to produce reducing sugars of 2.940 mg/mL, in which the inulinase activity is equivalent to 1.089×10^{-3} U/mL. The optimum pH for some enzymes production including inulinase from several strains varied and was generally obtained at low pH.

Low pH value of inulinase production was beneficial to the application of inulinase in the manufacture of fructose syrup because the low pH reduced the color of the results of this enzymatic reaction and the possibility of contamination by bacteria.^{3,18} Marlida et al¹⁹ stated that another enzyme, phytase, produced by both *Rhizoctonia* sp. and *F. verticillioides* had pH optimum at 4.0 and 5.0 respectively. Based on the data in figure 4, pH 5.0 was subsequently chosen to optimize temperature for inulinase production.

Temperature Optimization for Production of Inulinase at the Optimum pH Conditions:

Each microorganism has an optimum temperature to grow and even to produce enzymes. Increasing temperature, until a certain point, would lead to the increment of enzyme activity. After passing the optimum temperature, the enzyme activity was getting lower. This occurred due to slow cell growth and cell components became inactive.²⁰ Figure 5 shows the inulinase production from *H. fuscoatra* LBKURCC68 at pH 5.0 and agitation speeds of 100 rpm for 5 days with varying temperatures.

The highest inulinase activity of *H. fuscoatra* LBKURCC68 (188.889×10^{-3} U/mL) was obtained at 35°C. In a previous study, the production of inulinase as conducted by Saryono et al³ from endophytic fungi *Aspergillus clavatus* Gmn 11.3 had the optimum temperature of 37°C to produce reducing sugars of 2.500 mg/mL and the inulinase activity was equivalent to 944×10^{-3} U/mL. On the other hand, Marlida et al¹⁹ reported that *Acremonium* sp., an endophytic fungus isolated from forest trees, reached their optimum at

incubation temperature of 30°C in producing raw sago starch degrading enzyme.

Based on figure 5, the highest inulinase activity of *H. fuscoatra* LBKURCC68 was found at 35°C which was 188.889×10^{-3} U/ml and significantly different ($p < 0.05$) from the other temperatures. Further analysis to optimize the agitation speed for inulinase production was done by using pH of 5.0 and temperature of 35°C with the same media.

Optimization of Agitation Speeds for Production of Inulinase at Optimum pH and Temperature: The agitation speeds aimed to equalize nutrients and dissolve oxygen in the growth media. It helps to speed up nutrients absorption for growth and meet the needs of oxygen for microbial growth.²² Production of inulinase from *H. fuscoatra* LBKURCC68 with variation of agitation speeds for 5 fermentation days with a constant factor of fermentation conditions is shown in figure 6.

The highest inulinase activity of *H. fuscoatra* LBKURCC68 was obtained at agitation speeds of 100 rpm (188.889×10^{-3} U/mL) and was significantly different ($p < 0.05$) from the other agitation speeds. This was presumably because the nutrients in the media were distributed properly. However, high agitation speeds might lead cell metabolism to be disrupted and the production of enzymes secreted also reduced.²³ Enzyme production was also affected by the supply of nutrients for energy and cell growth. According to Bibek²⁰, these nutrients could affect the growth of microbial cells and eventually could lead to death.

The consequence of improper distribution was shown in low enzyme activity.²⁴ The production of inulinase as conducted by Park and Yun²⁵ from endophytic fungi *Xanthomonas* sp. demonstrated that the conditions of production medium were at pH 7.0, temperature of 37°C and agitation speeds of 100 rpm with inulinase activity of 18.700×10^{-3} U/mL.

The protein content produced by *H. fuscoatra* LBKURCC68 at the agitation speeds of 100 rpm, optimum pH (5.0) and optimum temperature (35°C) as shown in table 2 was not significantly different ($p \geq 0,05$) from those yielded at other agitation speeds. On the other hand, the highest specific inulinase activity (211.961×10^{-3} U/mg protein) was obtained at the agitation speed of 100 rpm. The specific activity of the enzyme could be used to measure of the magnitude of the isolated enzyme purity.

The higher is the specific activity of the enzyme, the higher is the purity level of the enzyme. This is due to non-enzyme protein loss at some stage of separation passed in the enzyme purification. The specific activity also indicated that the protein produced by fungi in growing medium was certain protein target.²⁶ The results of the same study for inulinase production by Saryono et al³ obtained inulinase from the isolate of *Aspergillus clavatus* Gmn 11.3 at the optimum fermentation conditions of pH 4.5, temperature of 37°C and agitation speed of 100 rpm yielded a specific enzyme activity of 0.157 U/mg protein.

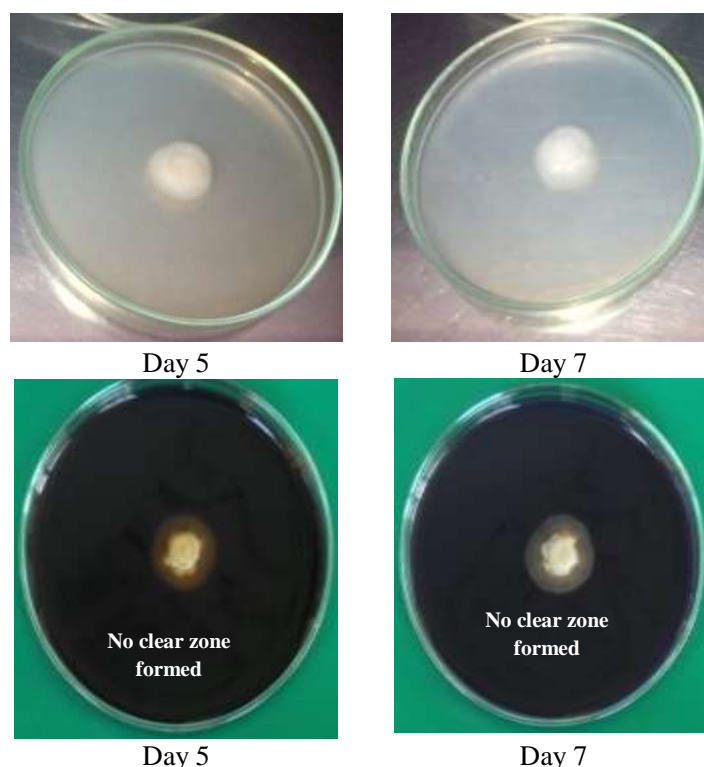


Figure 1: Inulinase activity of *F. solani* LBKURCC67 on day 5 and day 7 (top: before having dripped iodine solution; bottom: after having dripped iodine solution).

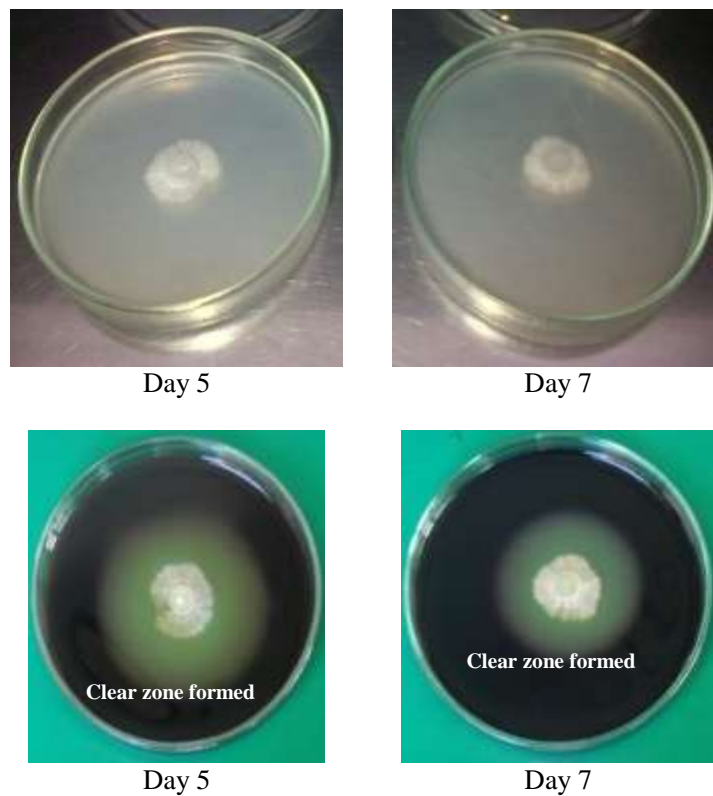


Figure 2: Inulinase activity *H. fuscoatra* LBKURCC68 on day 5 and day 7 (top: before having dripped iodine solution; bottom: after having dripped iodine solution).

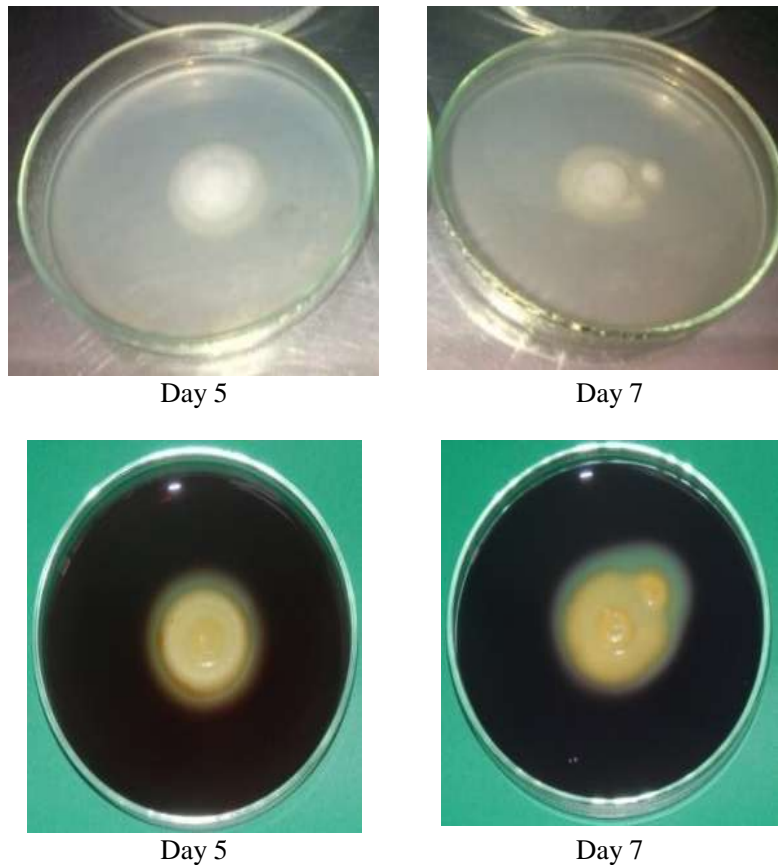


Figure 3: Inulinase activity of *F. solani* LBKURCC69 on day 5 and day 7 (top: before having dripped iodine solution; bottom: having dripped iodine solution).

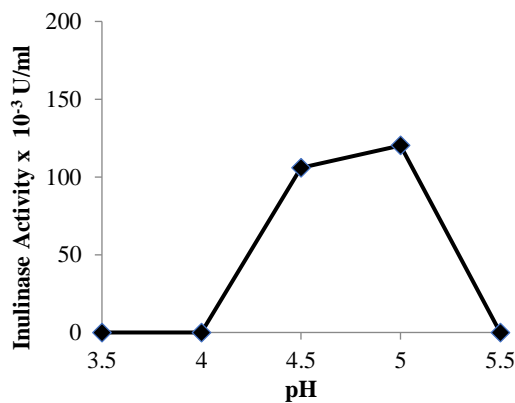


Figure 4: Inulinase activity of *H. fuscoatra* LBKURCC68 with variation of pH

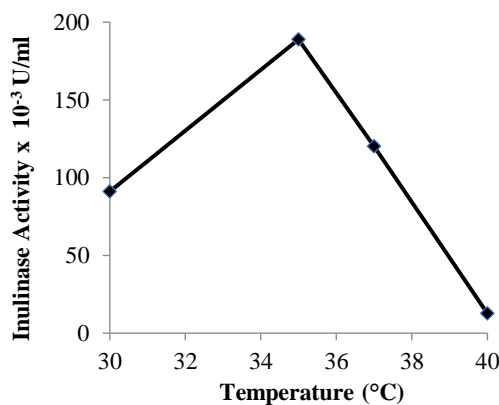


Figure 5: Inulinase activity of *H. fuscoatra* LBKURCC68 with varying temperatures

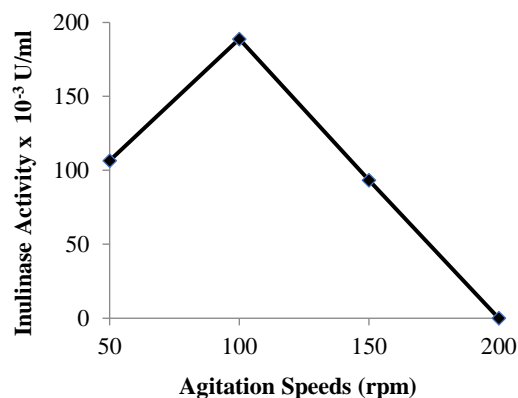


Figure 6: Inulinase activity of *H. fuscoatra* LBKURCC68 with variation of agitation speeds

Table 1
Inulinase semiquantitative analysis of 3 endophytic fungal isolates

S. N.	Endophytic fungi	Day 5			Day 7		
		Colony Diameter (cm)	Clear Zone Diameter (cm)	Z/K	Colony Diameter (cm)	Clear Zone Diameter (cm)	Z/K
1	<i>F. solani</i> LBKURCC67	-	-	-	-	-	-
2	<i>H. fuscoatra</i> LBKURCC68	2,17 ± 0,091	3,73 ± 0,092	1,72	1,97 ± 0,030	2,84 ± 0,030	1,44
3	<i>F. oxysporum</i> LBKURCC69	2,89 ± 0,000	0,80 ± 0,000	0,28	3,43 ± 0,007	0,71 ± 0,007	0,21

Table 2
Inulinase activity, protein content and specific activity of *H. fuscoatra* LBKURCC68 at pH 5,0 and temperature of 35°C with variation of agitation speeds

Agitation Speeds (rpm)	Enzyme Activity $\times 10^{-3}$ (U/ml)	Protein Content (mg/ml)	Enzyme specific Activity $\times 10^{-3}$ (U/mg protein)
50	106,67 \pm 0,028 ^b	0,76 \pm 0,119 ^a	143,38 \pm 0,021 ^b
100	188,89 \pm 0,013 ^a	0,91 \pm 0,149 ^a	211,96 \pm 0,033 ^a
150	93,33 \pm 0,007 ^b	0,86 \pm 0,042 ^a	109,31 \pm 0,006 ^c

Note: a, b, c) The average value of the enzyme activity of five repetitions. The values with the same letter in the same column are not significantly different ($p \leq 0,05$).

Conclusion

Based on semiquantitative analysis using diffusion method for the endophytic fungal isolates of fungi *F. solani* LBKURCC67, *H. fuscoatra* LBKURCC68 and *F. oxysporum* LBKURCC69, the study shows that the highest inulinase activity was produced by *H. fuscoatra* LBKURCC68. The optimum condition for inulinase production from *H. fuscoatra* LBKURCC68 was obtained at pH 5.0, temperature of 35°C and agitation speed of 100 rpm with inulinase activity reaching 188.889×10^{-3} U/mL, protein content of 0,909 mg/mL and specific activity of 211.961×10^{-3} U/mg protein.

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