



Optimization of inulinase production by a newly isolated strain *Aspergillus flavus* var. *flavus* by solid state fermentation of *Saccharum arundinaceum*

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ABSTRACT

The present study deals with the use of mangrove soil, a new source for the isolation of an inulinase producing strain followed by the enzyme production and optimization using hardy sugarcane, "*Saccharum arundinaceum*", a novel substrate. Four fungal isolates were obtained from the primary screening in inulin media. In the secondary screening, only *Aspergillus flavus* var. *flavus* strain ATCC 16883, a new strain, showed the zone of hydrolysis surrounding the fungal growth on the inulin medium plate. The action pattern of inulinase was determined by thin layer chromatography which confirmed it to be exoinulinase in nature. Solid state fermentation of *A. flavus* ATCC 16883 using *Saccharum arundinaceum* showed a maximum inulinase activity of 3.48U/gds after 96 h. The inulinase nature of the enzyme was confirmed by the resultant I/S ratio of 2.56. Central Composite Design (CCD) was used to optimize the media components and obtain a quadratic model using the five significant nutrients namely, inulin, K₂HPO₄, (NH₄)₂SO₄, KH₂PO₄ and NaCl. Using the optimum conditions of (g/gds) inulin- 0.1, K₂HPO₄- 0.05, (NH₄)₂SO₄- 0.002, KH₂PO₄- 0.005 and NaCl- 0.02, the inulinase activity was determined as 8.57U/gds which was 2.50-fold higher than the initial unoptimized conditions.

1. Introduction

Inulin is a polymer of fructose molecules composed primarily of linear β-2, 1-D fructofuranose links with a glucose moiety at the terminal end. This polysaccharide is non-edible in nature (Pandey et al., 1999). Inulin is found large amounts in plants like chicory root, Jerusalem artichoke, asparagus, fruits and vegetables such as onion, garlic, leek, banana, etc, thus ranks the second among the storage carbohydrates after starch (Mensink et al., 2015).

Inulinase enzyme hydrolyzes the (β-2, 1) inulin linkage to produce fructose, glucose, and inulo-oligosachharides (Neagu Bonciu and Bahrim, 2011). It is a single step process which results in the production of 95% fructose (Mohammadi et al., 2019). Inulinases are categorized into Exo and Endoinulinases based on their mode of action. Exoinulinase produces fructose as the main product by cleaving the terminal units of fructose from inulin whereas the inner linkages of inulin are hydrolyzed by endoinulinase into inulooligosaccharides (Chi et al., 2009). Inulinase is a very important enzyme mainly used for fructose production (Ricca et al., 2009), ultra- high fructose syrup (Maia et al., 2011) and ethanol (Ge and Zhang, 2005).

The potential sources for the production of inulinases are microbes

due to their easy culturing and high enzyme yields. A wide variety of yeast, fungi, bacteria, molds, and actinomycetes produces inulinases. The largest producers of inulinases as reported till date belongs to the fungal strain "*Aspergillus*" genus and the yeast strain "*Kluyveromyces*" genus (Das et al., 2019). In view of rising demand for the inulinases, it is vital to isolate strains which are highly productive and develop economically feasible processes.

The utilization of mangrove soil for the isolation of fungal species is based on their interesting adaptations and rich microbial diversity. The major component in the formation of the detritus pool in the mangrove environment is leaf litter. The detritus decomposition is triggered by filamentous fungi by the production of extracellular enzymes (Sridhar and Karamchand, 2010). Various enzymes like lipase, xylanase, pectate lyase, laccase, etc have been isolated and produced from mangrove-derived fungi (M.Prakash, 2013).

Usually, submerged fermentation is the mode of inulinase production, however, the use of solid state fermentation (SSF) has also been employed. Fungi and yeast belongs to the chief microbial class involved in the production of inulinases by SSF. Fungi are easy to cultivate on solid substrates as they grow between the particle spaces by penetrating their hyphae into it (Das et al., 2019). The most commonly used

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substrates for SSF are sugarcane bagasse, wheat bran, pressmud, rice bran, garlic, onion peels (Singh et al., 2016).

In the present research, a perennial grass, “*Saccharum arundinaceum*” also known as hardy sugarcane has been employed as a solid substrate for the production of inulinase by SSF, using *A. flavus* ATCC 16883. Till date, there are no literature reports on the utilization of *S. arundinaceum* as a substrate for the production of enzymes. They are monocotyledonous plants of the family of poaceae (Sharma and Borthakur, 2009). The stem has a solid pith with a very little amount of sucrose (Mukherjee, 1957). The cell wall of the cane is mainly composed of cellulose, hemicellulose, lignin, glucose, xylose, mannose, ash, etc (Hu et al., 2017). The culms of this cane are used to build thatched houses, making handles and screens of paint brushes (Bhat, 2014). Considering the necessity to develop a cost-effective substrate for the inulinase production by SSF, a small amount of inulin (as an inducer) can be mixed with *S. arundinaceum*.

In a fermentation process, the optimization of the media components and the operating conditions is a necessity in order to enhance the yield. One of the most relevant methods of optimization is a statistical method which includes response surface methodology (RSM) (Aydar, 2018). RSM analysis is carried out using multiple regression analysis and analysis of variance (ANOVA) (Speck et al., 2016). There are two types of response surface methodologies namely, Box-Behnken design (BBD) and central composite design (CCD) to determine the optimum process conditions in a fermentation (Sridevi et al., 2011). This is the first report on the inulinase production by SSF using *S. arundinaceum* as the solid substrate and *A. flavus* ATCC 16883 as the mangrove-derived fungal species.

2. Materials and methods

2.1. Isolation and screening of inulin- degrading fungal strains

Isolation was carried out using the topsoil of mangrove trees located in the Udyavara river estuary, Udupi Taluk (13°20'0"N, 74°43'13"E), India. The soil sample was collected in the month of June 2017. The soil sample was serially diluted. Czapek Dox Agar medium was used for fungal isolation consisting of (g/l): sucrose - 30, NaNO₃ - 2, K₂HPO₄ - 1, MgSO₄ - 0.50, KCl - 0.50, FeSO₄ - 0.01 and agar - 15. The media was maintained at a pH of 7.3 and autoclaved at 121 °C, 15–20 psi pressure for 45min. 0.1 ml of the serially diluted sample was plated on CDA plate and incubated at 30 °C for 3-4 days to obtain different fungal colonies (Guttikonda et al., 2017). In the primary screening, after getting different fungal colonies, individual colonies were picked up and aseptically plated on inulin medium consisting of (g/l): inulin - 6.00, NH₄Cl - 5, Na₂HPO₄ - 33.9, KH₂PO₄ - 15, NaCl - 2.50 and agar - 15. Inulin media was maintained at a pH of 6.7 and incubated at 30 °C for three days (Singh et al., 2018a,b). For the screening of extracellular inulinase producing microorganisms, Lugol's rapid plate assay was employed (Li et al., 2011).

2.2. Identification of the fungal strain

The fungal strain giving the maximum activity was identified using 18s rRNA gene sequencing (~571bp) at National Centre for Microbial Resource (NCMR) Pune, India. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Evolutionary analyses were conducted in MEGA6 based on best model fitting option (Frisvad et al., 2019). 1000 bootstrap was used for tree construction. The bootstrap support found for the parent node is 82. Viability of the strain was maintained by periodic subculturing and preserving at 4 °C (Chen et al., 2009).

2.3. Inoculum preparation

0.05% of NaCl solution was used to remove the fungal spores

completely from the agar plates followed by cell count using a haemocytometer (Improved Neubauer, Rohem, India). A suspension of 2×10^6 spores/mL was used for fermentation (Lafuente-Castaneda et al., 2012).

2.4. Pretreatment of solid substrate

S. arundinaceum plants were collected in heaps from the side of a local stream flowing by the Chitpady colony, Udupi, India. The leaves were discarded and the stems were thoroughly washed in cold water two to three times. The stems were cut into small cubes and sun-dried for seven days to remove moisture. Thereafter, the dried cubes were subsequently ground to a fine powder. The powder was then passed through a 1.18 mm sieve to remove larger size particles. After sieving, the resultant powder was used as a solid substrate for carrying out SSF.

2.5. Solid state fermentation

Fermentation was carried out in 250 ml Erlenmeyer flasks containing 2 g of solid substrate mixed with 5 ml of fermentative media. Moisture was adjusted to 77.8% on a dry basis. All the flasks were autoclaved at 121 °C, 15–20 psi pressure by covering them with hydrophobic cotton. After cooling, 2 ml of the previously prepared fungal inoculum was added to the flasks and carefully mixed to ensure uniform availability of nutrients. Incubation of the flasks was carried out for 5 day at 30 °C (Dilipkumar et al., 2011b). During the preliminary screening process, it was found that at the 96 h, the inulinase production was maximum. Thus, all the future experiments were carried out in three batches for 96 h.

2.6. Extraction of inulinase enzyme

After fermentation, 30 ml of sodium acetate buffer at a pH of 4.73 was added to the fermented matter and kept in a rotary shaker for 1 h for enzyme extraction. Subsequently the enzyme extract was filtered through a clean muslin cloth followed by centrifugation at 10,000 rpm for 10 min. The assay of the cell-free supernatant was carried out by the DNS method for the determination of reducing sugars (Miller, 1959).

2.7. Inulinase assay

For inulinase assay, substrate solution was prepared by adding 2 g inulin to 10 ml of 0.1 M sodium acetate buffer. Then the reaction mixture containing 0.1 ml of the supernatant and 0.9 ml of substrate solution was incubated for 10 min 1 ml of DNS reagent was added to the reaction mixture and boiled for 10 min. Blank consisted of preheating the reaction mixture for enzyme deactivation followed by DNS method. The quantity of enzyme that produces 1 μmol of fructose per minute under the above conditions is defined as one unit of inulinase activity (Garuba et al., 2018).

Invertase assay was determined similar to inulinase assay, except that the reaction mixture consisted of sucrose and invertase activity is defined with respect to sucrose activity (Dinarvand et al., 2013).

2.8. Inulinase to sucrose activity (I/S ratio)

Generally, I/S ratio helps to distinguish between inulinase and invertase activities. I/S ratio plays a crucial role in inulinase characterization because inulinase enzyme also has some activity on sucrose. Higher inulinase production in culture media is determined by an I/S ratio of greater than 10^{-2} , whereas higher invertase production is determined by an I/S ratio of less than 10^{-4} (Neagu Bonciu and Bahrim, 2011).

2.9. Optimization of inulinase production

Response surface methodology (RSM) consisting of a group of

empirical methods is used to determine the relationship between a group of controlled experimental factors and calculated response. To obtain an accurate model, prior knowledge and understanding of the related bioprocesses are essential (Behera and Ray, 2016). The experimental data were analyzed by the statistical software package “Minitab 16.2.2.0”. A quadratic model was obtained using the central composite design (CCD) which consists of star points and factorial trials. CCD helps in determining the quadratic effects and central points determine the variability of the pure process with the production of inulinase as the response (Olawoye, 2016). The five significant nutrients which enhance the production of inulinase was optimized using RSM. The five independent factors were studied at five different levels with the central coded value as zero, and sets of 32 experiments were carried out. The response (Y) is the maximum inulinase activity after the completion of the 32 experiments. The data analysis using multiple regression was carried out by submitting the results obtained from CCD to ANOVA (analysis of variance) (Raja and Murty, 2013). Finally, an empirical model was obtained that shows a relationship between the measured response and the independent factors (Dilipkumar et al., 2013).

2.10. Analysis of end products

Thin layer chromatography of the optimized broth was performed to determine the action pattern of inulinase enzyme on inulin. For hydrolysis reaction, a mixture of 2.0 ml of a cell-free crude extract and 0.02 g of inulin was prepared. Incubation was carried out for 6 h at 56 °C followed by terminating the hydrolysis reaction mixture by boiling for 10 min. The TLC plate was spotted with inactivated inulinase (I), glucose (G), sucrose (S), and fructose (F) as standards along with the test sample (T). Then, it was dried in air and dipped in a developer consisting of butanol: acetone: water (4:5:1). Again the plate was dried in air and sprayed with diphenylamine reagent (1% diphenylamine, 1% aniline, and 10% phosphoric acid). Diphenylamine reagent reacts with sugars and develops colours. The plate was then dried at 90 °C for 10 min in a hot air oven (Singh and Chauhan, 2017).

2.11. Seliwanoff's test

Seliwanoff's test is a colour test to distinguish between ketose and aldose sugar. Ketose sugar, like fructose dehydrate more rapidly to give furfural derivatives on reaction with concentrated acid. On condensation with resorcinol, it gives a cherry red or burgundy red colour (Sánchez-Viesca and Gómez, 2018). This test was performed with the cell-free supernatant containing inulinase and inulin along with three standard controls namely glucose, fructose, and inactivated inulinase with inulin. To one ml of seliwanoff's reagent (0.5 g resorcinol per liter of 10% HCl), half ml of cell-free supernatant containing inulinase and inulin was added, mixed and heated for 2 min in a water bath. One ml of standard glucose, one ml of standard fructose and one ml of pre-heated cell-free supernatant containing inactivated inulinase and inulin were also added to one ml of seliwanoff's reagent and heated for 2 min. Prolonged heating is avoided as aldoses like glucose will also produce a red colour (V Devmurari et al., 2018).

2.12. Osazone formation test

Osazone test is a simple test for the identification and qualitative determination of sugar. Sugar reacts with phenylhydrazine and forms characteristic osazone crystals, for example, monosaccharides like fructose and glucose gives the needle and broom-shaped osazone crystals. This test was performed with the standard fructose and the cell-free supernatant. Two ml of the cell-free supernatant containing inulinase and inulin and two ml of standard fructose was added to phenylhydrazine hydrochloride solution and thoroughly mixed. Both the test and control samples were heated in a boiling water bath for 30 min. The solutions were cooled and observed for crystals formation (Hassid and

McCready, 1942). The images were observed at 40x magnification under a light microscope (Euromex iscope, Holland).

3. Results and discussion

3.1. Microorganism

In the preliminary screening, four fungal isolates which grew on inulin media were selected for Lugol's rapid plate assay. Fig. 1a and Fig. 1b indicate the fungal strain DD MSF 2 which has grown on inulin medium simultaneously forming a clear halo in Lugol's iodine solution. The isolate was highly branched with lime colored woolly mycelia and globose conidia as shown in Fig. 1a and Fig 1c. Similar characteristics have been reported for *Aspergillus flavus*-INU2 (Garuba et al., 2015) and for *Aspergillus flavus* (Hedayati et al., 2007). Furthermore, the fungal strain DD MSF 2 was sequenced by NCMR Pune, India and found to have 100% similarity with *Aspergillus flavus* var. *flavus* strain ATCC 16883 as shown in Fig. 2. This is the first report on the isolation of *A. flavus* ATCC 16883 from mangrove soil. The ribosomal RNA gene sequence of the newly isolated strain DD MSF2 have been deposited in the GenBank (Accession number [KU729026.1](https://www.ncbi.nlm.nih.gov/nuclot/KU729026.1)). The genus *Aspergillus* falls under one of the most commonly used fungal inulinase producers. Among the reported literature, species such as *A. niger* NK-126 (Kango, 2008), *A. fumigatus* MTCC 3009 (Gill et al., 2006), *A. terreus*, *A. versicolor*, *A. parasiticus* (El et al., 2014), *A. tubingensis* CR16 (Trivedi et al., 2012), have been used for inulinase production.

3.2. Fermentation and optimization of the significant nutrients

The fungal isolate *A. flavus* ATCC 16883 gave a maximum inulinase activity of 3.48 U/gds at 96 h of fermentation. After 96 h, the activity decreased with increase in incubation time probably due to catabolic repression, depletion of carbon source in the medium or due to protein denaturation caused by the secretion of proteolytic proteins (Dinarvand et al., 2013). The duration observed was in agreement with the time for maximum activity (96 h) reported on *A. terreus* and inulinase activity for the species was 5.680 ± 0.183 U/gds (El et al., 2014). The I/S ratio of fungal isolate *A. flavus* ATCC 16883 obtained was 2.56 which confirmed the specificity of inulinase enzyme. The results are in relation with other inulinase producers like *P. oxalicum* BGPUP-4 gave an I/S ratio of 3.38 (Singh and Chauhan, 2017), *Rhizopus microspores* 13aIV gave an I/S ratio of 0.57–2.49 (Flores-Gallegos et al., 2015), *Penicillium* sp. NFCC 2768 gave I/S ratios in the range of 0.53–0.66 (Rawat et al., 2015a,b). In contrast, *Mucor circinelloides* BGPUP-9 gave a very high I/S ratio of 72.86 (R. Singh et al., 2018a,b).

Five significant nutrient factors, namely, inulin, K_2HPO_4 , $(NH_4)_2SO_4$,

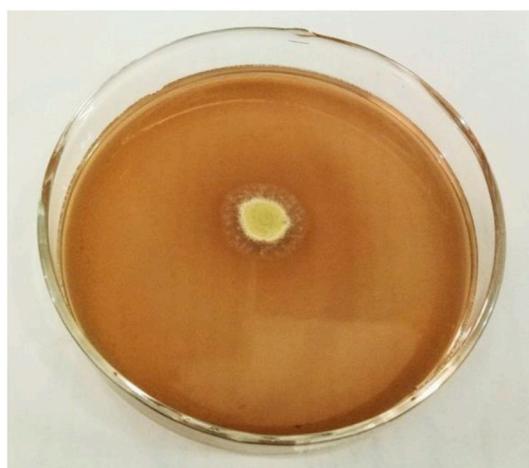


Fig. 1a. Growth of DD MSF 2 on inulin media.



Fig. 1b. Formation of clear halo around DD MSF 2 after flooding with Lugol's iodine.



Fig. 1c. Microscopic feature of DD MSF 2 showing globose conidia (10x).

KH_2PO_4 and NaCl were screened during the preliminary fermentation process. Results obtained by the current study are compared with the reported literature. Xiong et al. (2007) screened inulin, corn steep liquor and $(\text{NH}_4)_2\text{SO}_4$ as the significant factors for inulinase production and optimization by *Kluyveromyces* S120 in SSF. Dilipkumar et al. (2014) screened K_2HPO_4 , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and soya bean cake as the significant factors for inulinase optimization by *P. rugulosum* MTCC-3487 in SSF. CCD was used to determine the optimal values of the quadratic and the interaction effects between the significant factors. The highest value obtained for inulinase activity was observed in run 6 (5.82 U/gds) and the lowest was in run 31 (3.27 U/gds) as shown in Table 1. There is low variation between the central points which indicates that the experimental data is significantly reproducible. The efficiency and fitness of the model were calculated by using ANOVA for the experimental design used as shown in Table 2. The model F value is 7.38 as shown in Table 2, which shows that the model is significant. Similarly, the model fits the data in a better way since the F value of the model (7.38) is higher than the F value of the lack of fit (0.87). The insignificant lack of fit value (0.573) also signifies that the design space can be navigated using the model. In the current model, at a confidence level of 95% and a p-value ($p < 0.05$), the linear model terms X_1 , X_2 , X_3 , and X_4 are significant. Basically, a smaller p value has a greater influence and only factors having p values < 0.05 are considered to be significant (Vinayaka B Shet et al., 2018). Among the square interactions, X_2^2 is significant. Among two way interactions, $X_1 * X_2$, $X_1 * X_3$, $X_1 * X_5$, $X_2 * X_3$, $X_3 * X_5$, and $X_4 * X_5$ are

significant. The determination coefficient (R^2) measures the strength of the linear relationship between the predicted and experimental values (Vinayaka Babu Shet et al., 2018). The determination coefficient (R^2) was calculated as 0.9306 for the inulinase activity which is close to 1 and responsible for 93.06% variability of the response. These results were in comparison with R^2 value of 0.9279 obtained from RSM using CCD to optimize inulinase production from *Streptomyces* sp. in SSF using copra waste (Dilipkumar et al., 2013), 0.9354 using pressmud (Dilipkumar et al., 2011a) and 0.8931 using garlic (Dilipkumar et al., 2011b). An R^2 value of 0.95 was reported from RSM using CCD to optimize inulinase production from *Cryptococcus aureus* G7a in SSF using wheat bran and rice husk (Sheng et al., 2009). Similarly, an R^2 value of 0.92 was reported from RSM using CCRD to optimize inulinase production from *Kluyveromyces marxianus* NRRL Y-7571 using sugarcane bagasse, corn steep liquor (CSL), pre-treated cane molasses, and soybean bran (Mazutti et al., 2010).

3.2.1. Regression equation for the model in coded units

$$\text{Inulinase activity (U/gds)} = 2.25 + 3.3 X_1 + 153.0 X_2 - 320 X_3 - 72 X_4 - 19 X_5 + 28.5 X_1^2 - 1092 X_2^2 + 3984 X_3^2 + 2537 X_4^2 - 1448 X_5^2 - 529 X_1 * X_2 - 1802 X_1 * X_3 + 1348 X_1 * X_4 + 2605 X_1 * X_5 + 2222 X_2 * X_3 + 613 X_2 * X_4 - 956 X_2 * X_5 + 2667 X_3 * X_4 + 8889 X_3 * X_5 - 8963 X_4 * X_5$$

The 3-D surface plot in Fig. 3 shows the dependency of inulinase on inulin and $(\text{NH}_4)_2\text{SO}_4$. The inulinase activity reaches up to 5.74U/gds when the concentration of inulin and $(\text{NH}_4)_2\text{SO}_4$ increases up and decreases to 4.61U/gds when the concentration of inulin and $(\text{NH}_4)_2\text{SO}_4$ is further increased. The 3-D surface plot in Fig. 4 shows the dependency of inulinase on NaCl and KH_2PO_4 . The inulinase activity reaches up to 5.58U/gds when the concentration of KH_2PO_4 and NaCl increases but decreases to 3.27U/gds when the concentration of KH_2PO_4 and NaCl is further increased. Due to insufficient nutrients at a lower concentration, the activity of inulinase was found to be low. Decrease in the inulinase activity observed at higher concentrations could be due to inhibitory effects (Dilipkumar et al., 2013). Carbon sources provide skeletal support and energy to the microbial cells. Inulin is a significant carbon source that acts an inducer in the production of inulinase from *A. flavus* ATCC 16883. However, catabolite repression (Gill et al., 2003) may occur in the presence of a higher amount of inulin leading to a reduction in the enzyme activity which is evident from Fig. 3. Ammonium sulphate is considered as an important source of providing nitrogen for the growth of *A. flavus* ATCC 16883. However, it decreases the enzyme activity in higher concentration which is evident in Fig. 3. This could be due to excess release of ammonium ions which causes an inhibitory effect on the inulinase production (Singh et al., 2006). KH_2PO_4 supports the basic growth of a cell and acts as a buffering agent. NaCl maintains the osmotic balance in the growth media. As the concentration of NaCl increases, activity decreases as shown in Fig. 4. This could be due to salinity stress which creates a low water activity in the fungal cells thereby slowing down the transportation rate in and outside the fungal cells (Seesuriyachan et al., 2012). With an increase in KH_2PO_4 the activity decreases as shown in Fig. 4. This could be due to the excess release of the phosphate ions which cause an inhibitory effect on the enzyme activity (Singh and Singh, 2014). Inulin and $(\text{NH}_4)_2\text{SO}_4$ have been reported as two of the most significant factors for inulinase production by *Kluyveromyces* S120 (Xiong et al., 2007), K_2HPO_4 has been reported as one of the important factors for inulinase production by *Streptomyces* sp. MTCC-3119 (Dilipkumar et al., 2011b), and *P. rugulosum* MTCC-3487 (Dilipkumar et al., 2014). KH_2PO_4 has been reported as one of the important factors for inulinase production by *Mucor circinelloides* BGPUP-9 (Singh et al., 2018a,b). As the concentration of inulin, K_2HPO_4 , NaCl , KH_2PO_4 and $(\text{NH}_4)_2\text{SO}_4$ increases above optimum level, the inulinase activity is repressed, although the cell growth continues slowly. On the other hand, both the microbial growth and the inulinase activity would rapidly reduce if there is a decrease in

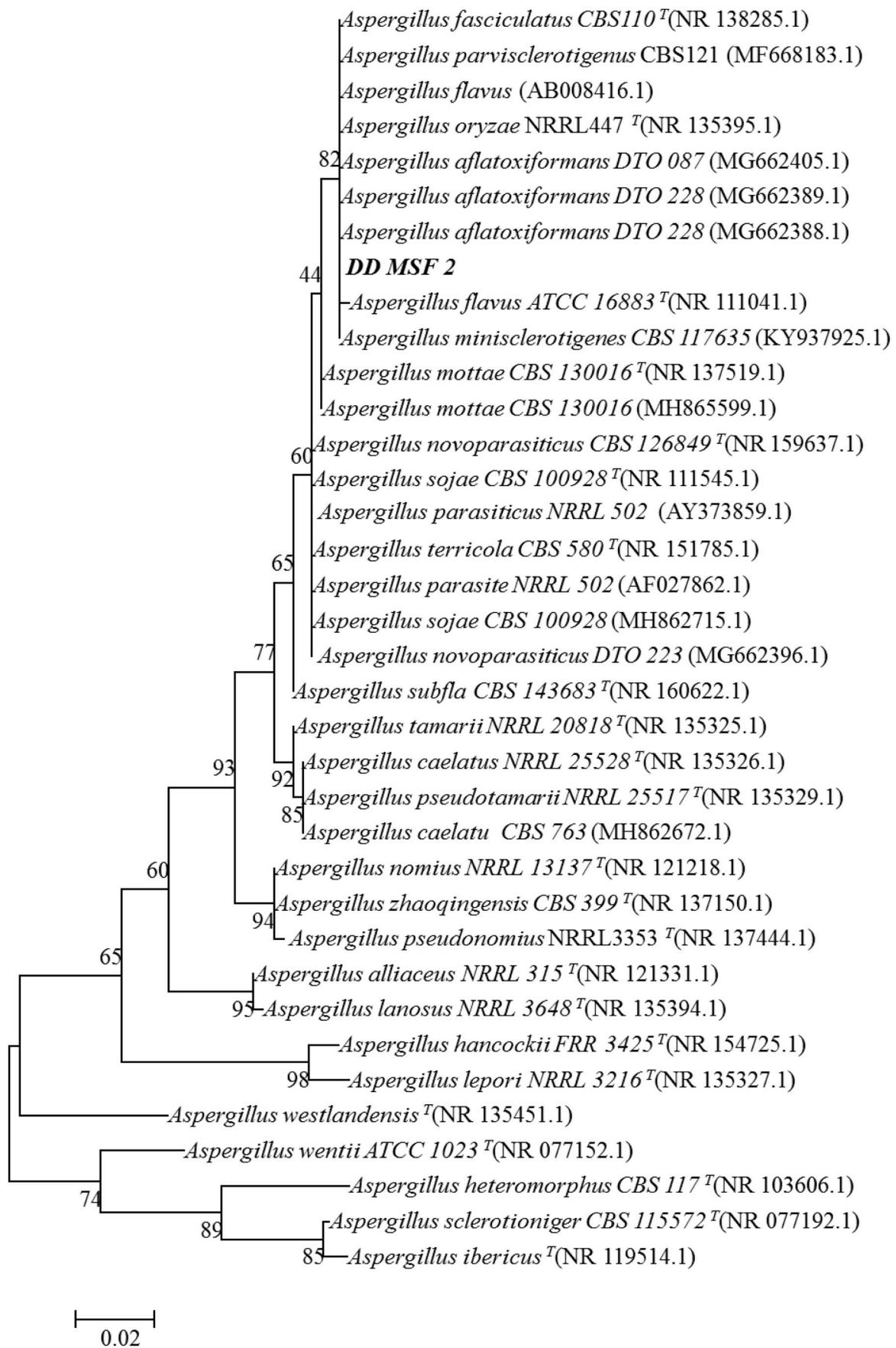


Fig. 2. Phylogenetic tree of 18S rRNA of the isolate *Aspergillus flavus* ATCC 16883 shown as DD MSF 2.

Table 1

Matrix for the central composite design to determine the optimum values of the five nutrients for the production of inulinase.

| Std Order | Run Order | Inulin (X ₁) | K ₂ HPO ₄ (X ₂) | (NH ₄) ₂ SO ₄ (X ₃) | KH ₂ PO ₄ (X ₄) | NaCl (X ₅) | Experimental | Predicted |
|-----------|-----------|-------------------------------------|---|---|---|------------------------|----------------------------|-----------|
| Runs | | Nutrient factors with codes (g/gds) | | | | | Inulinase activity (U/gds) | |
| 24 | 1 | 0.055 | 0.075 | 0.011 | 0.02 | 0.011 | 5.60 | 5.64 |
| 27 | 2 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.011 | 4.74 | 4.96 |
| 9 | 3 | 0.0325 | 0.0625 | 0.0065 | 0.01625 | 0.0065 | 5.74 | 5.74 |
| 20 | 4 | 0.055 | 0.1 | 0.011 | 0.0125 | 0.011 | 3.91 | 3.97 |
| 18 | 5 | 0.1 | 0.075 | 0.011 | 0.0125 | 0.011 | 4.61 | 4.50 |
| 10 | 6 | 0.0775 | 0.0625 | 0.0065 | 0.01625 | 0.0155 | 5.82 | 5.86 |
| 2 | 7 | 0.0775 | 0.0625 | 0.0065 | 0.00875 | 0.0065 | 5.18 | 5.25 |
| 22 | 8 | 0.055 | 0.075 | 0.02 | 0.0125 | 0.011 | 5.27 | 4.96 |
| 23 | 9 | 0.055 | 0.075 | 0.011 | 0.005 | 0.011 | 4.80 | 5.12 |
| 21 | 10 | 0.055 | 0.075 | 0.002 | 0.0125 | 0.011 | 5.49 | 5.62 |
| 19 | 11 | 0.055 | 0.05 | 0.011 | 0.0125 | 0.011 | 4.84 | 4.94 |
| 15 | 12 | 0.0325 | 0.0875 | 0.0155 | 0.01625 | 0.0065 | 5.45 | 5.48 |
| 17 | 13 | 0.01 | 0.075 | 0.011 | 0.0125 | 0.011 | 5.62 | 5.31 |
| 26 | 14 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.02 | 5.11 | 4.72 |
| 7 | 15 | 0.0325 | 0.0875 | 0.0155 | 0.00875 | 0.0155 | 4.98 | 5.23 |
| 28 | 16 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.011 | 5.14 | 4.96 |
| 11 | 17 | 0.0325 | 0.0875 | 0.0065 | 0.01625 | 0.0155 | 4.21 | 4.16 |
| 31 | 18 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.011 | 4.75 | 4.96 |
| 5 | 19 | 0.0325 | 0.0625 | 0.0155 | 0.00875 | 0.0065 | 4.80 | 5.09 |
| 4 | 20 | 0.0775 | 0.0875 | 0.0065 | 0.00875 | 0.0155 | 4.60 | 4.63 |
| 29 | 21 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.011 | 4.82 | 4.96 |
| 13 | 22 | 0.0325 | 0.0625 | 0.0155 | 0.01625 | 0.0155 | 4.83 | 5.09 |
| 14 | 23 | 0.0775 | 0.0625 | 0.0155 | 0.01625 | 0.0065 | 4.56 | 4.68 |
| 16 | 24 | 0.0775 | 0.0875 | 0.0155 | 0.01625 | 0.0155 | 4.71 | 4.78 |
| 6 | 25 | 0.0775 | 0.0625 | 0.0155 | 0.00875 | 0.0155 | 5.02 | 4.55 |
| 3 | 26 | 0.0325 | 0.0875 | 0.0065 | 0.00875 | 0.0065 | 5.18 | 5.17 |
| 1 | 27 | 0.0325 | 0.0625 | 0.0065 | 0.00875 | 0.0155 | 5.06 | 5.27 |
| 25 | 28 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.002 | 4.77 | 4.74 |
| 30 | 29 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.011 | 5.14 | 4.96 |
| 32 | 30 | 0.055 | 0.075 | 0.011 | 0.0125 | 0.011 | 5.29 | 4.96 |
| 8 | 31 | 0.0775 | 0.0875 | 0.0155 | 0.00875 | 0.0065 | 3.27 | 3.38 |
| 12 | 32 | 0.0775 | 0.0875 | 0.0065 | 0.01625 | 0.0065 | 5.01 | 4.83 |

Table 2

Analysis of variance for the quadratic regression model obtained from central composite design for optimal inulinase activity(U/gds).

| Source | Effect | Coefficient | F-value | P- value |
|--------------------------------|---------|-------------|---------|----------|
| Model | | | 7.38 | 0.001* |
| Linear | | | 11.88 | 0.000* |
| Constant | | 4.9993 | | |
| X ₁ | -0.3417 | -0.1708 | 13.31 | 0.004* |
| X ₂ | -0.4550 | -0.2275 | 23.61 | 0.001* |
| X ₃ | -0.3017 | -0.1508 | 10.38 | 0.008* |
| X ₄ | 0.3200 | 0.1600 | 11.68 | 0.006* |
| X ₅ | 0.0600 | 0.0300 | 0.41 | 0.535 |
| Square | | | 4.58 | 0.017* |
| X ₁ ² | 0.0289 | 0.0144 | 0.12 | 0.740 |
| X ₂ ² | -0.3411 | -0.1706 | 16.22 | 0.002* |
| X ₃ ² | 0.1614 | 0.0807 | 3.63 | 0.083 |
| X ₄ ² | 0.0714 | 0.0357 | 0.71 | 0.417 |
| X ₅ ² | -0.0586 | -0.0293 | 0.48 | 0.503 |
| 2-Way Interaction | | | 6.53 | 0.002* |
| X ₁ *X ₂ | -0.2975 | -0.1488 | 6.73 | 0.025* |
| X ₁ *X ₃ | -0.3650 | -0.1825 | 10.13 | 0.009* |
| X ₁ *X ₄ | 0.2275 | 0.1137 | 3.94 | 0.073 |
| X ₁ *X ₅ | 0.5275 | 0.2638 | 21.16 | 0.001* |
| X ₂ *X ₃ | 0.2500 | 0.1250 | 4.75 | 0.052* |
| X ₂ *X ₄ | 0.0575 | 0.0287 | 0.25 | 0.626 |
| X ₂ *X ₅ | -0.1075 | -0.0537 | 0.88 | 0.369 |
| X ₃ *X ₄ | 0.0900 | 0.0450 | 0.62 | 0.449 |
| X ₃ *X ₅ | 0.3600 | 0.1800 | 9.85 | 0.009* |
| X ₄ *X ₅ | -0.3025 | -0.1512 | 6.96 | 0.023* |
| Lack of Fit | | | 0.87 | 0.573 |

the medium components.

3.2.2. Validation of the experimental model

For validation of the model, further studies were carried out using the following optimized values of the factors for inulinase production:

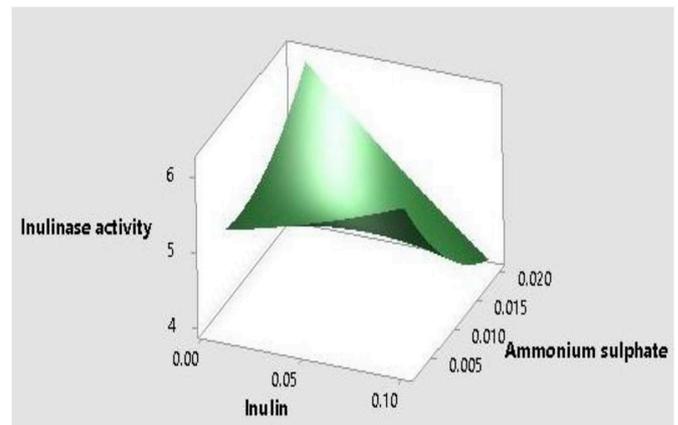


Fig. 3. 3D surface plot showing the effect of Inulin and (NH₄)₂SO₄ on the inulinase activity.

Inulin-0.1, K₂HPO₄- 0.05, (NH₄)₂SO₄- 0.002, KH₂PO₄- 0.005 and NaCl-0.02 (g/gds). Using the optimum values, the experimental activity obtained was 8.57U/gds. The activity obtained corresponds well with the predicted activity of 7.69U/gds as given by the software. In Table 3, inulinase activities of the present studies are compared with the inulinase activity reported by some of the other fungal species using different solid substrates. It can be concluded from Table 3 that the inulinase yield obtained in the present work is higher than the reported literature.

The literature on *A. flavus* reported till date is on submerged fermentation from *A. flavus* MTCC 277 (Gupta et al., 1994), *A. flavus* INU2 (Garuba et al., 2015) and *A. flavus* NFCCI 2364 (Rawat et al., 2015a,b). In conclusion, the study on the inulinase production from the novel fungal isolate *A. flavus* ATCC 16883 on *S. arundinaceum* as a novel

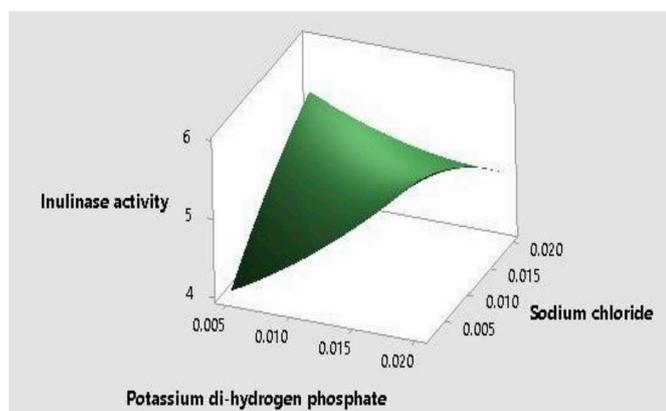


Fig. 4. 3D surface plot showing the effect of NaCl and KH_2PO_4 on the inulinase activity.

Table 3

Inulinase activity in comparison to the inulinase activity obtained by some of the other fungal species.

| Microorganism | Substrate | Inulinase yield | References |
|--------------------------------------|------------------------------------|-------------------------------|------------------|
| <i>Aspergillus terreus</i> | Garlic wastes | $0.022 \pm 0.03\text{U/gds}$ | El et al. (2014) |
| <i>Aspergillus niger</i> AUMC 9375 | Lettuce roots | 0.0879U/gds | Housseiny (2014) |
| <i>Aspergillus parasiticus</i> | Artichoke leaves | $0.177 \pm 0.125\text{U/gds}$ | El et al. (2014) |
| <i>Aspergillus niger</i> AUMC 9375 | Sunflower tubers and lettuce roots | 0.232U/gds | Housseiny (2014) |
| <i>Aspergillus versicolor</i> | Orange rinds | $1.917 \pm 0.016\text{U/gds}$ | El et al. (2014) |
| <i>Aspergillus flavus</i> ATCC 16883 | <i>Saccharum arundinaceum</i> | 8.57U/gds | Present work |

substrate has shown the feasibility of SSF to produce inulinase using this strain on a laboratory scale for the first time. Generally, the literature survey reports that the exoinulinase yield from wild type strains have been low and not feasible enough to carry out production at the industrial level (Wang et al., 2011). The yield of inulinase production from *A. flavus* ATCC 16883 can be increased using recombinant DNA technology. Various microbial inulinases like *Aspergillus ficuum* JNSP5-06 was overexpressed in *Escherichia coli* (Chen et al., 2013), *Pichia guilliermondii* strain 1 was expressed in *Pichia pastoris* X-33 (Zhang et al., 2009), *Kluyveromyces marxianus* CBS 6556 was expressed in *Pichia pastoris* (Zhang et al., 2012), etc. It would also be of interest in future to explore the various other factors like the application of moistening agents like corn steep liquor, utilization of different types of solvents for inulinase extraction, use of process parameters and different set of nutrient factors for optimization, increase in substrate concentration, to enhance the production of inulinases by *A. flavus* ATCC 16883.

3.3. Analysis of hydrolysis products

Reddish brown colored spots were seen in the developed TLC plate spotted with the standards (I, F, G, S) and test sample (T) as shown in Fig. 5. The spots for the test sample (T) resembled that of fructose and glucose standards. This shows that the hydrolysate contains only fructose and a small amount of glucose. The TLC plate did not show any presence of fructooligosachharides. It can be concluded that the fungal isolate *A. flavus* ATCC 16883 is exoinulolytic in nature. Exoinulinase removes the terminal fructose units from inulin and produces fructose as the main product. Many exoinulolytic fungal species like *Mucor circinelloides* BGPUP-9 (Singh et al., 2018a,b), *Penicillium subrubescens* (Mansouri et al., 2013), *Aspergillus fumigatus* (Gill et al., 2006), *Fusarium solani* JALPK (Kamble et al., 2019) have been reported for fructose

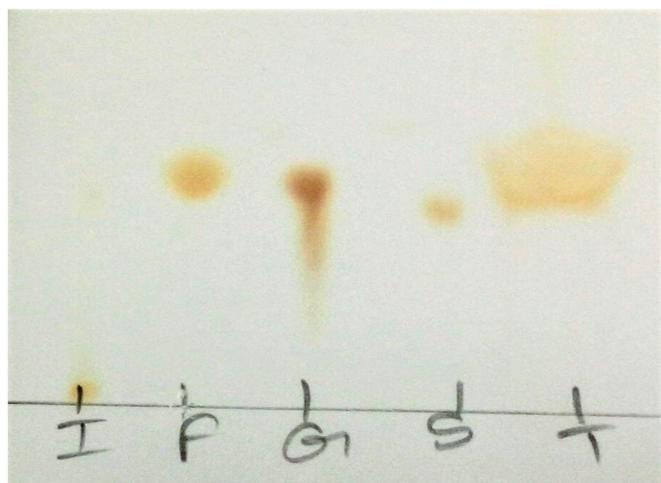


Fig. 5. Thin layer chromatogram of inulin hydrolysate formed by crude inulinase from fungal isolate, DD MSF 2, I- control (inactivated inulinase + inulin), F- fructose standard, G- glucose standard, S- sucrose standard, T – products of hydrolysis.

liberation as the main product.

3.4. Seliwanoff's test

Seliwanoff's test rapidly gave a distinctive red colour on heating the sample fructose (B) and the test sample (D) as shown in Fig. 6 which proves the presence of ketose sugar in the fermented broth as reported by (Elzagheid, 2018) for the determination of ketohexoses. Test sample (C) containing the inactivated enzyme showed no change in colour. Being an aldose sugar, the standard glucose (A) gave no colour.

3.5. Osazone formation test

The free carbonyl group in the sugars reacted with phenylhydrazine forming needle and broom-shaped crystals. Fig. 7 represents light microscope image (40x) where the distinctive needle-shaped osazones formed by both the fermented broth and the fructose standard can be observed. Distinctive needle-shaped osazone crystals were reported by (Kamble et al., 2019) and (Hassid and McCready, 1942).

4. Conclusion

In recent years, it has been found out that the diversity of inulinase



Fig. 6. The distinctive colours are given by the seliwanoff's test, A- standard glucose, B- standard fructose, C- inactivated inulinase + inulin, D- inulinase + inulin.

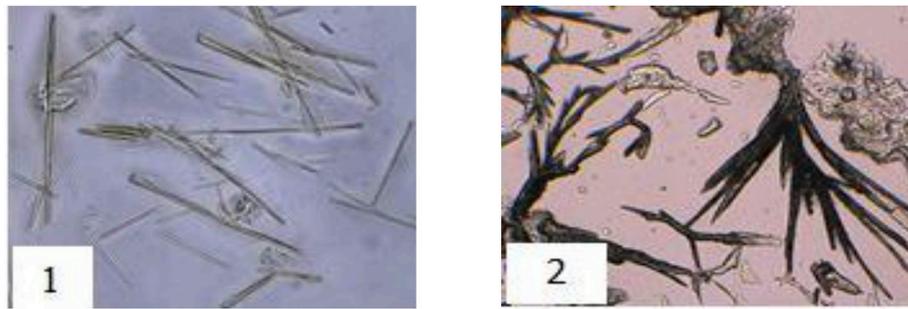


Fig. 7. The distinctive needle-shaped osazones from standard fructose on the left and fermented broth on the right.

producing microbes is very rich. However, to our knowledge, microbes from the mangrove soil environment is still an untouched bioresource for extracellular inulinase enzyme production. It is very interesting to observe from the results obtained in this study that fungal species producing inulinase occurred in mangrove environments. The study showed that *Aspergillus flavus* ATCC 16883 proved to be a potential inulinase producing organism in SSF. *Saccharum arundinaceum* constituted as a good source of a cheap and readily available solid substrate. The study using statistical experimental design for inulinase production resulted in a 2.50 fold increase under SSF. The importance of medium components on the production of inulinase was studied by CCD. The four factors (Inulin, K_2HPO_4 , KH_2PO_4 and $(NH_4)_2SO_4$) were found to be significant. Literature review reveals that there is no report on inulinase production from *A. flavus* ATCC 16883 by SSF using *S. arundinaceum* as the solid substrate. Therefore, *A. flavus* 16883 proved to be an attractive source for fructose production.

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Research involving human participants and/or animals

There was no involvement of human and animal participation in the research.

Informed consent

All the authors have gone through the manuscript and well informed about the research.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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