

# Response surface methodology to optimize inulinase production by a newly isolated *Penicillium amphipolaria* strain by solid-state fermentation of *Saccharum arundinaceum*

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## Research

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# Abstract

In the present investigation, a new fungal inulinase producer *Penicillium amphipolaria* KAS 2555 has been isolated from the soil of dead mangroves litter area, followed by the inulinase production and optimization by solid-state fermentation using a low-cost substrate – hardy sugarcane (*Saccharum arundinaceum*). While screening, only *Penicillium amphipolaria* KAS 2555 showed the hydrolysis zone on the plate containing inulin media. The exoinulolytic nature of inulinase and its form of action was confirmed by thin-layer chromatography (TLC). After 96 h of the fermentation period, an activity of 2.45 U/gds was obtained. The I/S ratio of 0.59 proved that the enzyme is inulolytic in nature. Media optimization was performed to obtain a regression model using Central Composite Design (CCD). For optimization, five significant media components viz., inulin,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$  and, NaCl were used. A 3.10-fold increase in activity of inulinase (7.59 U/gds) was obtained under the optimal settings of (g/gds) inulin- 0.1,  $(\text{NH}_4)_2\text{SO}_4$ - 0.002,  $\text{K}_2\text{HPO}_4$ - 0.1,  $\text{KH}_2\text{PO}_4$ - 0.02 and NaCl- 0.02.

## 1. Introduction

A variety of hydrolysing enzymes of industrial significance from various sources of microbes have been identified. Inulinases belong to one of these significant class of enzymes that acts on  $\beta$ , 2 – 1 linkages of inulin to yield different industrial products [1]. The  $\beta$ , 2 – 1 inulin linkages are affected by inulinases differently. They are therefore categorized as: exoinulinase and endoinulinase according to their pattern of action on inulin. Exoinulinase breaks inulin sequentially, while endoinulinase breaks the polymer internal links randomly to create varying chain length fructooligosaccharides [2].

The two main uses of inulinases are the processing of high fructose syrup and fructooligosaccharides [3]. Other inulinase applications are production of bioethanol [4], lactic acid [5], single-cell oil [6], and citric acid [7].

Being a low calorie sweetener, fructose is beneficial over traditional sucrose and fructooligosaccharides are effective prebiotics [8]. Usually, production of fructose from starch involves the use of three enzymes namely,  $\alpha$ -amylase, amyloglucosidase and glucose isomerase which leads to only 45% yield. In contrast to this, inulinases are used in a single enzymatic inulin hydrolysis step which yields 95% of fructose [9].

Inulinase producing microbes have been widely isolated from sources like marine soil [10], rotten wood [11], inulin rich plants [12], etc. Mangrove soil is also proved to be an efficient source of inulinase producing fungi [13]. A mangrove habitat consists of two parts, production-based where living mangrove trees are found and dead-based where dead mangroves are found. Dead mangroves litter is made up of leaves, barks, seeds, branch, wood, etc. A wide range of microbes inhabits the different parts of the dead-based mangrove environment like soil, leaf, wood, algae, and sand. In the present study, topsoil from the rotten wood of mangrove trees has been used for the isolation of inulinase producing microbes [14]. Mangrove soil is a rich source of microbes that produces enzymes like chitinase, cellulase, protease, and amylase [15]. To date, there is no report on the production of inulinase or any other enzymes from

*Penicillium amphipolaria* species. This is the first report on the solid state production of inulinase enzyme using this new fungal species isolated from the mangrove soil in the dead mangroves area.

Microbial sources of inulinases are bacteria, yeast and filamentous fungi. Nevertheless, there has been a considerable increase in fungal sources because of thermal and pH stability and growth on low cost substrates to produce important industrial products. Some active fungal inulinase producers are *Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp., etc [16]. In the present study a new fungal species "*Penicillium amphipolaria*" has been isolated. Till date there is no report on the application of this fungus in any field of research.

In general, inulinase is produced by submerged fermentation, nonetheless, nowadays, solid-state fermentation (SSF) is gaining a lot of attention because of the low cost process. Several cheap substrates such as sugarcane bagasse, peels of onion, pressmud, wheat bran and rice bran have been used for the production of inulinase enzyme [17]. Very recently, our research group have documented the use of a cost-effective solid substrate – "*Saccharum arundinaceum*", usually called as hardy sugarcane, for the inulinase production by employing *Aspergillus flavus* var. *flavus* strain ATCC 16883 [13].

In the process of fermentation, media optimization is necessary to achieve optimal growth of microbes and enhance the synthesis of metabolites. The relevant statistical method of optimization i.e., response surface methodology (RSM) uses analysis of variance (ANOVA) and multiple regression analysis to analyse the optimum model [18]. RSM determines the optimum process conditions using Central composite design (CCD) and Box-Behnken design (BBD) [19]. Therefore, this method was used to optimize the media components of inulinase production by SSF from the rotten wood mangrove soil isolated fungus, *P. amphipolaria*. This is the first report on the production of inulinase from *P. amphipolaria* using dead-based mangrove soil as the chief source of fungal isolation.

## 2. Materials And Methods

### 2.1 Isolation and screening of inulin- degrading fungal strains

The method of isolation was performed using the surface soil of the rotten wood of the mangrove detritus situated in the estuary of Halady river, on the Herikudru island, Kundapura Taluk (13°38'28"N, 74°42'01"E), India. Here, the mangrove area is mostly comprised of mangrove trees that have died due to urbanization and deforestation. The soil collection was done in July 2017. Firstly, a serial dilution of the soil sample was carried out followed by plating 0.1ml of the sample on the Czapek Dox Agar medium (CDA). CDA medium consists of (g/l): sucrose - 30, K<sub>2</sub>HPO<sub>4</sub> - 1, NaNO<sub>3</sub> - 2, KCl - 0.50, MgSO<sub>4</sub> - 0.50, FeSO<sub>4</sub> - 0.01 and agar - 15. It is used for the isolation of fungal species. The pH of the media was kept at 7.4 and autoclaved for 45min at 121°C and 15-20 psi. The plates were incubated for 3-4 days at 30°C to get diverse colonies of fungal species [13]. In the process of primary screening, individual colonies from different fungal colonies were selected and plated aseptically on inulin media. The inulin media consists of (g/l): inulin - 6.00, Na<sub>2</sub>HPO<sub>4</sub> - 33.9, NH<sub>4</sub>Cl - 5, NaCl - 2.50, KH<sub>2</sub>PO<sub>4</sub> - 15, and agar - 15. The plates were

incubated for three days at 30°C. The pH of the media was kept at 6.7 [21]. Lugol's plate assay was performed to screen the microorganisms producing extracellular inulinases [22].

## 2.2 Identification of the fungal strain

The fungal strain which gave the highest activity was recognized using 18s rRNA gene sequencing (571bp) at National Centre for Microbial Resource (NCMR) Pune, India. Using the Tamura-Nei model, the evolutionary history was inferred by using the Maximum Likelihood method. The tree was constructed using 1000 bootstrap. The bootstrap support is 95 for the parent node. Tree construction method was selected based on the best model fitting option in MEGA6 [41].

## 2.3 Inoculum preparation

The fungal spores were totally removed from the agar plates using 0.05% of sodium chloride solution. A haemocytometer (Improved Neubauer, Rohem, India) was used to count the number of cells. For SSF,  $2 \times 10^6$  spores/ mL of spore suspension was used [23].

## 2.4 Pretreatment of solid substrate

Stems of *S. arundinaceum* collected from Chitpady colony, Udupi, India were cleaned properly couple of times in running water. Small cubes of the stems were prepared and sun-dried for a couple of days to remove moisture. Subsequently, fine powder was made by grinding the dried cubes in a mixer grinder and passed through a 1.18mm sieve. After sieving, the particles with larger sizes were removed and the fine powder was used in SSF as a solid substrate.

## 2.5 Solid-State Fermentation

SSF was carried out by mixing 2g of *S. arundinaceum* powder and 5ml of inulin media in 250ml conical flasks. Moisture on a dry basis was maintained at 77.8%. All the flasks were tightly covered with hydrophobic cotton and autoclaved. The flasks were allowed to cool followed by the addition of 2ml of the spore suspension prepared earlier. Proper mixing of the solid substrate, fermentation media and the spore suspension was done to ensure the availability of nutrients uniformly. The flasks were incubated at 30°C for five days [42]. During the process of preliminary screening, it was found that the maximum production of inulinase occurred at 96<sup>th</sup> h. All the subsequent experiments were thus carried out for 96 h.

## 2.6 Extraction of Inulinase enzyme

For the extraction of enzyme after the completion of fermentation, the fermented contents were mixed with 30ml of sodium acetate buffer (0.1molar) having a pH of 4.75. The flasks were then kept for 1 h on a rotary shaker (180rpm) to extract the enzyme. Subsequently, a clean muslin cloth was used to filter out the enzyme extract followed by centrifugation at 844 G-force (10,000 rpm in rotor 9, Tabletop Superspin Centrifuge –V/F<sub>M</sub>, Plasto Crafts., Mumbai) at 4°C for 10 min. The determination of reducing sugars was done by the dinitrosalicylic acid (DNS) method using the cell-free supernatant [25].

## 2.7 Inulinase assay

For the assay of inulinase enzyme, 2g inulin was added to 10ml of 0.1M sodium acetate buffer to make a substrate solution. Then the reaction mixture which consisted of 0.9 ml of substrate solution and 0.1 ml of the cell-free supernatant was incubated for 10 min, followed by the addition of 1ml of DNS reagent. The mixture was heated for 10 min in a water bath. For the blank, the enzyme was deactivated by preheating the reaction mixture. The quantity of enzyme which produces 1 $\mu$ mol of fructose per min is defined as one unit of inulinase activity.

Similar to inulinase assay, the invertase assay was determined using sucrose in the reaction mixture and the activity of invertase was determined with respect to the sucrose activity [21].

## 2.8 Inulinase to Sucrose activity (I/S ratio)

Usually, the inulinase activity is characterized from the activity of invertase using the I/S ratio. An I/S ratio higher than  $10^{-2}$  proves to have a high production of inulinase whereas an I/S ratio lower than  $10^{-4}$  proves to have a high production of invertase enzyme in the culture media [2].

## 2.9 Optimization of Inulinase Production

Proper understanding and former knowledge of bioprocess are necessary to obtain a model with accuracy. The data obtained from the experiments were studied using "Minitab 16.2.2.0", a design software. Using Central composite design, a quadratic model was obtained. A fractional CCD 2(5-1) was used here and the significance level was kept at 95%. CCD consists of factorial trials and star points which assist the defining of quadratic effects. The centre points assess the pure variance with inulinase production as the output variable [26]. The optimization of the five significant media constituents which increased the inulinase production was done using RSM. A set of thirty-two experiments were performed by studying the five components at 5 different levels, keeping zero as the central coded value. The experimental results of all the 32 experiments – the inulinase activity (Y) were recorded and submitted to ANOVA to carry out the analysis of data using multiple regression [27]. Lastly, an empirical model showing the relationship between the independent components and the measured response was achieved.

## 2.10 Analysis of end products

The pattern in which inulinase enzyme works on inulin was figured out by thin-layer chromatography (TLC) analysis. To perform the reaction of hydrolysis, 1.0 ml of the cell-free supernatant was mixed with 0.01g of inulin. The reaction mixture was incubated at 56°C for six h. Then, the reaction was stopped by boiling the reaction mixture for 10 min. Along with the sample test (T), four standards namely, deactivated inulinase (I), fructose (F), glucose (G), sucrose (S), were spotted on the TLC plates. It was then air-dried and dipped in a developer solution composed of butanol:ethanol:water (50:30:20). The plate was again air-dried and sprayed with diphenylamine reagent (1% diphenylamine, 10% phosphoric acid and 1%

aniline). This reagent develops colors on reaction with sugars. Finally, the plate was kept in a hot air oven for 10 min at 120°C for drying [21].

### 2.11 Seliwanoff's test

To distinguish between aldoses and ketoses, Seliwanoff's color test is carried out. Ketose sugar can be differentiated from aldose sugar by the formation of a burgundy or cherry red color with resorcinol on condensation, whereas aldose sugar doesn't give any color [28]. Heating for a long time is avoided as aldose sugars will also give a red color [29]. To perform this test, three controls namely fructose, glucose and deactivated inulinase with inulin was used and a test sample containing the supernatant along with inulinase and inulin was used. 0.5 ml of the test sample was mixed with 1ml of Seliwanoff's reagent and heated for 2 min. To 1ml of Seliwanoff's reagent, 1ml each of the following, namely, standard fructose, standard glucose, and a pre-heated mixture of deactivated inulinase and inulin were also mixed and heated for 2 min.

### 2.12 Osazone formation test

Osazone test is performed to determine and identify the sugars qualitatively. It is a simple test in which sugars form distinctive crystals of osazone on reaction with phenylhydrazine. Distinctive broom and needle-shaped osazones are formed by monosachharides like glucose and fructose. The following test was carried out using the optimized cell-free liquid of inulinase and inulin mixture along with the fructose control. 2 ml of the control fructose and 2 ml of the supernatant were mixed with phenylhydrazine hydrochloride solution followed by heating for 30 min in a water bath [30]. After cooling down of the solutions, the distinctive images of the osazone crystals were visualized by a light microscope (Euromex iscope, Holland) at 40x magnification.

## 3. Results And Discussions

### 3.1 Microorganism

The fungal isolate that has grown on the inulin media were chosen for Lugol's plate assay. The fungal strain which had formed a clear halo in the plate assay has been indicated as DD MSF 1 in Fig. 1a and Fig. 1b. Figure 1a shows that the isolate has moderately deep colonies, white mycelia, and floccose texture. Visagie et al., (2016) reported the same features for *Penicillium amphipolaria* KAS 2555. They isolated this species from the quarter main mountains of the dry valleys of Antarctica [31]. Till date, there is no work reported in any field on this fungal species. Figure 2 shows DD MSF 1 represented in the phylogenetic tree as sequenced by NCMR Pune, India. DD MSF 1 was found to have a 99% similarity with *Penicillium amphipolaria* strain KAS 2555. This study reports the isolation of this fungal species from detritus mangrove soil for the first time. In the GenBank under the (Accession number KT887872.1), the gene sequence of the ribosomal RNA of DD MSF 1 has been deposited. Among the fungal producers of inulinases, the *Penicillium* genus is one of the most common. According to the literature survey, species

such as *P. brevicompactum* [11], *P. rugulosum* [32], *P. citrinum* [23], have been used for the production of inulinase enzyme.

## 3.2 Fermentation and optimization of the significant nutrients

After the completion of 96 h of fermentation, DD MSF 1 yielded a maximal activity of 2.45 U/gds. The activity gradually decreased after 96 h with the increase in the incubation period possibly due to the carbon source exhaustion in the fermentation media, catabolic repression, or due to the secretion of proteolytic proteins causing the denaturation of proteins. The experimental time of fermentation is consistent with the reported values. A maximal inulinase activity of 3.48 U/gds was obtained from *A. flavus* ATCC 16883 [13], *A. terreus* gave a maximum activity of  $5.680 \pm 0.183$  U/gds [11], a maximal activity of 319 U/ml was obtained from *A. niger* ATCC 20611 in shake flask [33]. *Penicillium amphipolaria* KAS 2555 gave an I/S ratio of 0.59, hence, proved the specificity of the enzyme inulinase. The result obtained in the present study had similarities with other microbial producers of inulinases as reported in the literature. An I/S ratio of 2.56 was obtained from *A. flavus* ATCC 16883 [13], a ratio of 3.38 was obtained from *P. oxalicum* BGPUP-4 [21], a ratio in the range of 0.53–0.66 was obtained from *Penicillium* sp. NFCC 2768 [34], a ratio of 0.57–2.49 was obtained from *Rhizopus microspores* 13aIV [35].

During the preliminary process of traditional screening, five nutrient components viz., inulin,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ , and NaCl were screened out to be significant and their levels were determined for CCD as shown below in Table 1a. Thirty two experiments were performed according to the combinations given by the software. The number of runs were kept randomized. The process parameters were kept constant as follows: temperature (30°C), moisture (77.77%), inoculum size (2 ml of  $2 \times 10^6$  fungal spores), amount of solid substrate (2 g), fermentation media (5 ml), pH (7). The results are compared with the published reports. Four significant nutrients namely  $\text{NH}_4\text{NO}_3$ , soya bean cake,  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , and  $\text{K}_2\text{HPO}_4$  were screened by Dilipkumar et al., (2011) for the of inulinase production by *Streptomyces* sp. in SSF [24]. Three significant nutrients viz.  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , yeast extract, and  $\text{NH}_4\text{NO}_3$  were screened by Dilipkumar et al., (2011) for inulinase optimization in SSF by *Streptomyces* sp [36]. Five significant factors namely, inoculum size, moisture, the ratio of the quantity of wheat bran to rice husk, pH and temperature were screened by Sheng et al., (2009) for the optimization of inulinase enzyme using *Cryptococcus aureus* G7a in SSF [37].

Among the significant term, the optimum values were determined by CCD. In run 10, the highest activity of 7.38 U/gds and in run 22, the lowest activity of 5.74 U/gds were obtained as presented in Table 1b. The data obtained from the experiments are reproducible significantly as there is a small difference between the central points. ANOVA was used for the design of the experiment to determine the model efficiency and fitness as shown in Table 2. F value of the model is shown as 10.24 which proves that it is significant.

At a 95% level of confidence and a (p-value < 0.05),  $X_1$  is significant among the linear model factors,  $X_2^2$  and  $X_3^2$  are significant among the square interactions and  $X_1 * X_2$ ,  $X_2 * X_4$  are significant among the two way interactions. The coefficient of determination ( $R^2$ ) for the activity of the inulinase enzyme was calculated as 0.9490 which is responsible for causing a 94.90% variation in the response.

Table 1

a- Levels of the independent variables chosen for Central Composite Design.

Nutrient factors (g/gds)	Levels				
	(-2)	(-1)	(0)	(+1)	(+2)
Inulin	0.0100	0.0325	0.0550	0.0775	0.1000
$K_2HPO_4$	0.0500	0.0625	0.0750	0.0875	0.1000
$(NH_4)_2SO_4$	0.0020	0.0065	0.0110	0.0155	0.0200
$KH_2PO_4$	0.0050	0.00875	0.01250	0.01625	0.0200
NaCl	0.0020	0.0065	0.0110	0.0155	0.0200

Table 1

**b-** Central composite design matrix to determine the optimum values of the five nutrient factors for the production of inulinase.

<b>Runs</b>	<b>Nutrient factors with codes (g/gds)</b>					<b>Inulinase activity (U/gds)</b>	
Run Order	Inulin (X <sub>1</sub> )	K <sub>2</sub> HPO <sub>4</sub> (X <sub>2</sub> )	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (X <sub>3</sub> )	KH <sub>2</sub> PO <sub>4</sub> (X <sub>4</sub> )	NaCl (X <sub>5</sub> )	Inulinase activity	Predicted activity
1	0.055	0.075	0.011	0.02	0.011	6.90	6.81
2	0.0775	0.0625	0.0065	0.00875	0.0065	5.92	5.74
3	0.055	0.075	0.002	0.0125	0.011	6.70	6.88
4	0.055	0.075	0.02	0.0125	0.011	7.16	6.99
5	0.055	0.075	0.011	0.0125	0.011	6.49	6.53
6	0.0775	0.0625	0.0155	0.01625	0.0065	6.17	6.12
7	0.055	0.075	0.011	0.0125	0.011	6.51	6.53
8	0.055	0.075	0.011	0.005	0.011	6.59	6.69
9	0.055	0.075	0.011	0.0125	0.002	6.67	6.82
10	0.0325	0.0625	0.0065	0.01625	0.0065	7.38	7.30
11	0.0325	0.0875	0.0065	0.01625	0.0155	5.78	5.85
12	0.0325	0.0875	0.0065	0.00875	0.0065	6.58	6.53
13	0.0325	0.0625	0.0155	0.00875	0.0065	7.01	6.97
14	0.055	0.1	0.011	0.0125	0.011	6.25	6.10
15	0.055	0.075	0.011	0.0125	0.011	6.50	6.53
16	0.055	0.075	0.011	0.0125	0.011	6.56	6.53
17	0.0325	0.0875	0.0155	0.01625	0.0065	6.31	6.40
18	0.0325	0.0625	0.0155	0.01625	0.0155	7.24	7.32
19	0.0775	0.0875	0.0065	0.00875	0.0155	7.05	7.02
20	0.0775	0.0875	0.0155	0.00875	0.0065	7.20	7.19
21	0.0325	0.0875	0.0155	0.00875	0.0155	6.15	6.26
22	0.0775	0.0625	0.0155	0.00875	0.0155	5.74	5.72
23	0.0325	0.0625	0.0065	0.00875	0.0155	7.01	6.96
24	0.055	0.075	0.011	0.0125	0.02	6.88	6.74

<b>Runs</b>	<b>Nutrient factors with codes (g/gds)</b>					<b>Inulinase activity (U/gds)</b>	
25	0.055	0.075	0.011	0.0125	0.011	6.57	6.53
26	0.055	0.075	0.011	0.0125	0.011	6.60	6.53
27	0.0775	0.0875	0.0065	0.01625	0.0065	6.60	6.55
28	0.055	0.05	0.011	0.0125	0.011	5.91	6.07
29	0.0775	0.0625	0.0065	0.01625	0.0155	6.53	6.47
30	0.1	0.075	0.011	0.0125	0.011	5.96	6.07
31	0.0775	0.0875	0.0155	0.01625	0.0155	6.79	6.90
32	0.01	0.075	0.011	0.0125	0.011	6.64	6.54

Table 2  
 Analysis of variance for the quadratic regression model obtained  
 from central composite design for optimal inulinase activity

Source	Effect	Coefficient	F-value	P-value
<b>Model</b>			10.24	0.000
Linear			2.85	0.069
Constant				
(X <sub>1</sub> ) Inulin	-0.2350	-0.1175	12.29	0.005
(X <sub>2</sub> ) K <sub>2</sub> HPO <sub>4</sub>	0.0117	0.0058	0.03	0.865
(X <sub>3</sub> ) (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.0567	0.0283	0.71	0.416
(X <sub>4</sub> ) KH <sub>2</sub> PO <sub>4</sub>	0.0633	0.0317	0.89	0.365
(X <sub>5</sub> ) NaCl	-0.0383	-0.0192	0.33	0.579
<b>Square</b>				
X <sub>1</sub> <sup>2</sup>	-0.1125	-0.0563	3.44	0.091
X <sub>2</sub> <sup>2</sup>	-0.2225	-0.1112	13.46	0.004
X <sub>3</sub> <sup>2</sup>	0.2025	0.1013	11.15	0.007
X <sub>4</sub> <sup>2</sup>	0.1100	0.0550	3.29	0.097
X <sub>5</sub> <sup>2</sup>	0.1250	0.0625	4.25	0.064
<b>2-Way Interaction</b>				
X <sub>1</sub> *X <sub>2</sub>	0.8875	0.4437	116.84	0.000
X <sub>1</sub> *X <sub>3</sub>	-0.0200	-0.0100	0.06	0.812
X <sub>1</sub> *X <sub>4</sub>	0.0275	0.0137	0.11	0.744
X <sub>1</sub> *X <sub>5</sub>	0.1650	0.0825	4.04	0.070
X <sub>2</sub> *X <sub>3</sub>	0.1400	0.0700	2.91	0.116
X <sub>2</sub> *X <sub>4</sub>	-0.3925	-0.1962	22.85	0.001
X <sub>2</sub> *X <sub>5</sub>	-0.1200	-0.0600	2.14	0.172
X <sub>3</sub> *X <sub>4</sub>	0.0850	0.0425	1.07	0.323

Source	Effect	Coefficient	F-value	P-value
$X_3 * X_5$	-0.0825	-0.0412	1.01	0.337
$X_4 * X_5$	0.0800	0.0400	0.95	0.351

### Regression model equation with uncoded values

$$\text{Inulinase activity (U/gds)} = 7.61 - 121.2 X_1 + 70.9 X_2 - 200.7 X_3 + 161.9 X_4 - 44.2 X_5 - 111.1 X_1^2 - 712 X_2^2 + 5000 X_3^2 + 3911 X_4^2 + 3086 X_5^2 + 1578 X_1 * X_2 - 99 X_1 * X_3 + 163 X_1 * X_4 + 815 X_1 * X_5 + 1244 X_2 * X_3 - 4187 X_2 * X_4 - 1067 X_2 * X_5 + 2519 X_3 * X_4 - 2037 X_3 * X_5 + 2370 X_4 * X_5$$

The main source of energy and skeletal support to the cells of microbes are provided by the carbon sources. Thus, inulin came out to be the most significant among the other variables. *Penicillium amphipolaria* KAS 2555 produces inulinase using inulin as the only carbon substrate which induces the production process. Yet a large amount of inulin leads to a decrease in the inulinase activity as shown in Fig. 3 due to catabolite repression.  $K_2HPO_4$  and  $KH_2PO_4$  act as a buffer and helps in basic cell growth. The inulinase activity decreases with an increase in  $KH_2PO_4$  as presented in Fig. 4. This inhibitory effect on the inulinase activity could be the result of phosphate ions released in excess. Ammonium sulphate is an important source which provides nitrogen in the growth of *P. amphipolaria* KAS 2555. However, the inulinase activity decreases in higher concentration possibly due to excess release of ammonium ions [43]. Another important factor, NaCl helps to maintain the osmotic balance in the growth media. The inulinase activity decreases as the concentration of NaCl increases possibly due to salinity stress which slows down the water transportation rate in and outside the fungal cells [44].

Singh et al., (2018) reported  $KH_2PO_4$  as one of the significant factors in the inulinase production from *Mucor circinelloides* BGPUP-9 [38]. Dilipkumar et al., (2011) reported  $K_2HPO_4$  as one of the significant factors in the production of inulinase from *Streptomyces sp.* MTCC-3119 [24]. Xiong et al., (2007) reported inulin as one of the significant factors in the production of inulinase from *Kluyveromyces* S120 [39]. Abd El Aty et al., (2014) reported  $KH_2PO_4$  as one of the significant factors in the production of inulinase from *Aspergillus terreus* [11]. As the concentration of the five media components is increased above the peak level, the activity of the inulinase enzyme is suppressed although there is continued slow growth of the cells. On the other hand, if the concentration of the media components decreases, the growth of the microbes and the activity of the inulinase enzyme will rapidly decrease [13].

## 3.3 Regression model validation

The model validation was performed by the respective optimized values of the significant terms for the production of inulinase: Inulin-0.1,  $K_2HPO_4$ - 0.1,  $(NH_4)_2SO_4$ - 0.002,  $KH_2PO_4$ - 0.02 and NaCl- 0.02 (g/gds). The experimental inulinase activity using these values was found out to be 7.59 U/gds which was in agreement with the predicted inulinase activity of 7.53 U/gds. The inulinase activity obtained in the

current study is 3.1 times higher than the un-optimized value. A comparison of the inulinase activity with the existing literature for few fungal species is given in Table 3.

Table 3  
Comparison of inulinase activity with few fungal species

Microorganism	Substrate	Inulinase yield (U/gds)	References
<i>Aspergillus versicolor</i>	Orange rinds	1.917	[11]
<i>Aspergillus terreus</i>	Artichoke leaves	4.433	
<i>Aspergillus flavus</i> ATCC 16883	<i>Saccharum arundinaceum</i>	8.57	[13]
<i>Penicillium amphipolaria</i> KAS 2555	<i>Saccharum arundinaceum</i>	2.45 (un-optimized)	Present work
<i>Penicillium amphipolaria</i> KAS 2555	<i>Saccharum arundinaceum</i>	7.59 (optimized)	Present work

There is no literature to date, on the production of inulinase from *Penicillium amphipolaria* KAS 2555 both by submerged and solid-state fermentation. For the first time, using hardy sugarcane, this new fungal strain has shown the viability of producing the enzyme inulinase on a laboratory scale by SSF. Optimized inulinase yield (7.59U/gds) as obtained from the present study is comparable with the optimized inulinase yield (8.57U/gds) from our previous study. Only a yield difference of 0.11U/gds is observed between them. This study proved the efficiency of the mangrove soil containing dead mangroves litter also has good inulinases producing microbes like the soil from living mangroves litter.

### 3.4 Analysis of hydrolysis products

The TLC plate showed spots of fructose standard (F), glucose standard (G), sucrose standard (S) and the test sample (T) containing the products of hydrolysis. No spot was observed for the control (I) containing inactivated inulinase and inulin. A single-colored spot obtained for the test sample proved that only fructose and a minute quantity of glucose is present in the hydrosylate. No fructooligosachharides were present which concludes the exoinulolytic nature of *Penicillium amphipolaria* KAS 2555. Some of the reported fungal microbes like *A. fumigatus* [40], *A. flavus* ATCC 16883[13], *P. oxalicum* BGPUP-4 [21], etc are exoinulolytic in nature.

### 3.5 Seliwanoff's test

In the Seliwanoff's test, as depicted in Fig. 6, the test solution (4) and the fructose control (1) quickly formed a red color while heating, whereas there was no change in color in the test sample (3) containing the heat-inactivated inulinase enzyme and the glucose control (2). This color visibility proves that the fermented culture contains ketose sugar.

## 3.6 Osazone formation test

Phenylhydrazine contains a free carbonyl moiety which reacts with the sugars to form broom and needle-shaped crystals. Similar shaped osazones were visualized under (40x) magnification in a light microscope for both the standard fructose and the test broth. Similar shaped osazones were also reported by Hassid and McCready (1942)[30], Kamble et al., (2019) [12], Das et al., (2019)[13].

## 4. Conclusion

In the present study, a potential new inulinase producing fungus *Penicillium amphipolaria* KAS 2555 was isolated from the detritus mangrove environment. According to our previous study using *Aspergillus flavus* var. *flavus* ATCC16883, living mangrove environment proved to be a rich source of organisms capable of exhibiting inulinase activity. In the present study, dead mangrove environment also proved to be a rich source of microbes producing inulinase. This fungal species gave a good yield of extracellular inulinase in SSF using hardy sugarcane. An increase of 3.10 fold was obtained in SSF using the statistical design of experiment. The study by CCD helped in determining the significance of the nutrients present in the media. Among the five factors, inulin was found to be significant. As per the cited literature, no work has been done on *Penicillium amphipolaria* KAS 2555 for the production of inulinase or any other enzymes. Thus, the newly isolated fungal species, *Penicillium amphipolaria* KAS 2555 proved to be an efficient producer of inulinase enzyme, leading to the production of fructose in a single step. Further, scale-up studies can be done to enhance the enzyme production.

## Abbreviations

### RSM

Response Surface Methodology

### CCD

Central Composite Design

### ANOVA

Analysis of Variance

### SSF

Solid Substrate Fermentation

### CDA

Czapex Dox Agar medium

### DNS

dinitrosalicylic acid

### TLC

Thin Layer Chromatography

## Declarations

## **6.1 Funding**

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## **6.3 Author contributions:**

The original idea was suggested by MRB and RS. The experimental work was carried out by DD with guidance from MRB and RS. DD drafted the article. All the authors involved in the critical revision of the article for important intellectual content. All the authors read and approved the final manuscript.

## **6.4 Competing interests:**

The authors have declared no competing interests

## **6.5 Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## **6.6 Ethics approval and consent to participate**

Not applicable.

## **6.7 Consent for publication**

Not applicable.

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## Figures

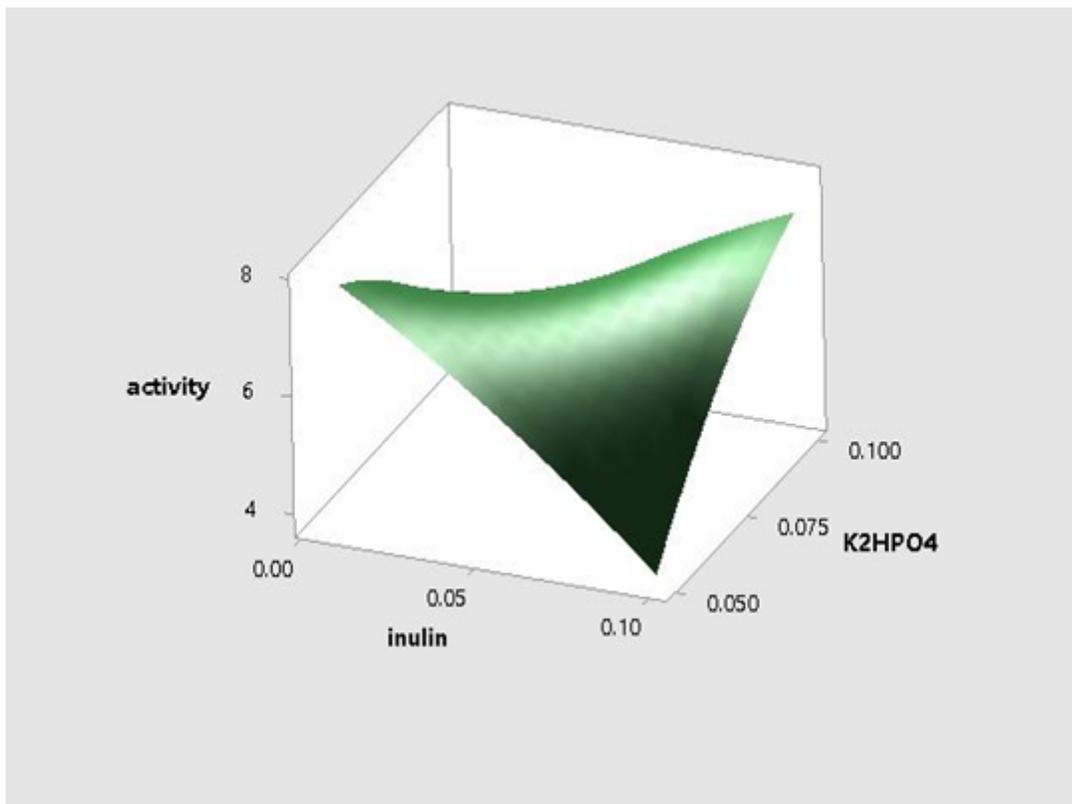


Figure 1

3D surface plot showing the effect of Inulin and K<sub>2</sub>HPO<sub>4</sub> on the activity of inulinase enzyme.

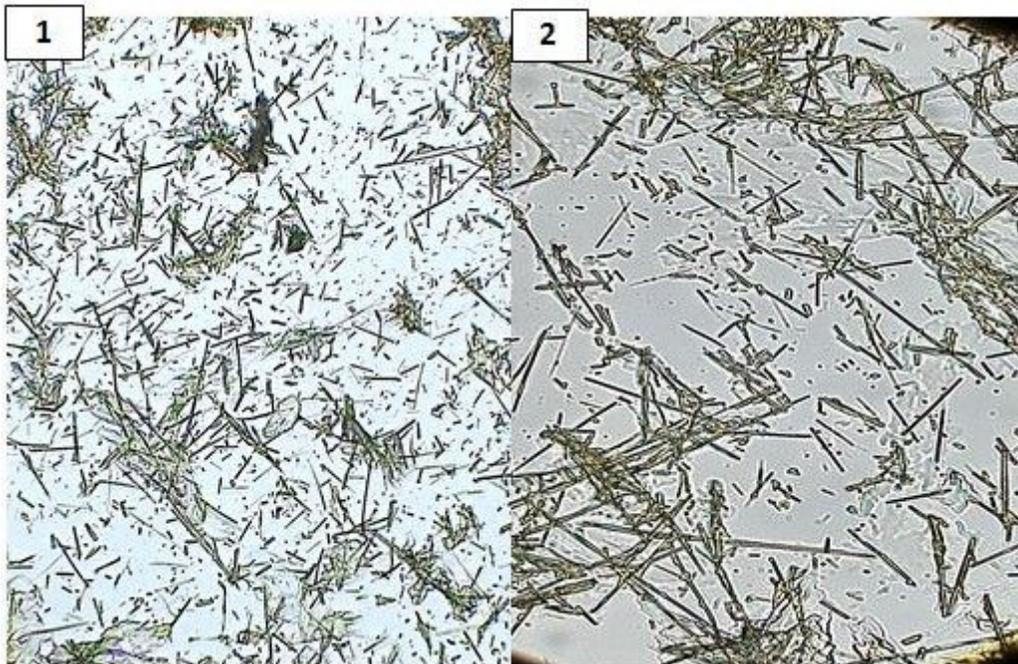


Figure 2



Figure 3

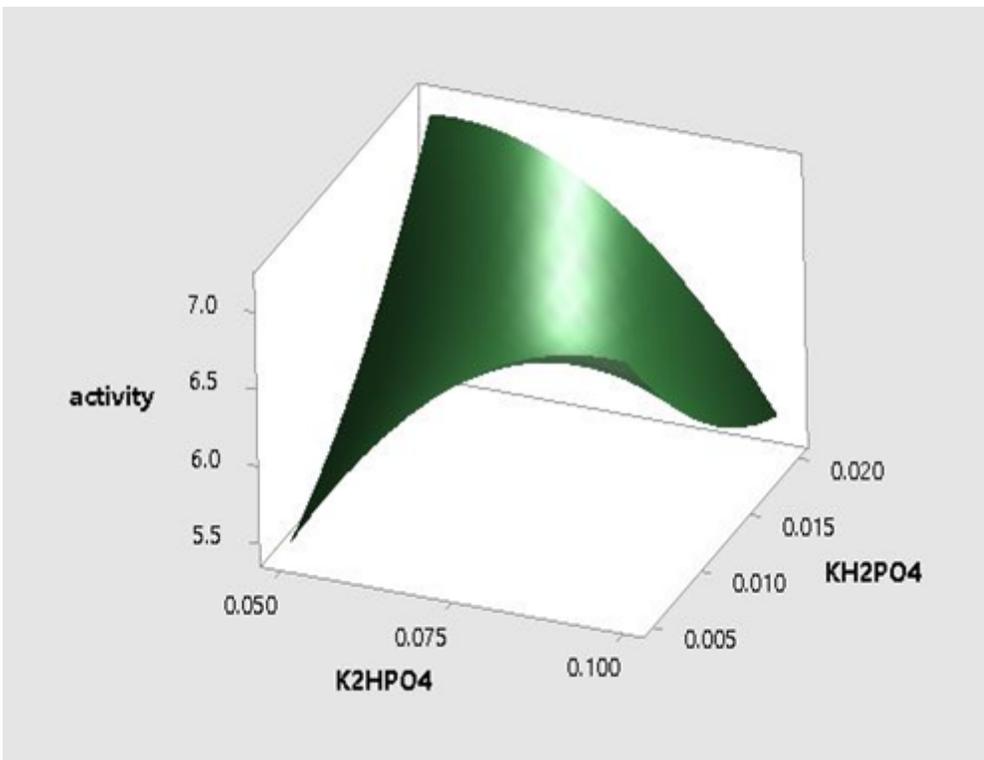
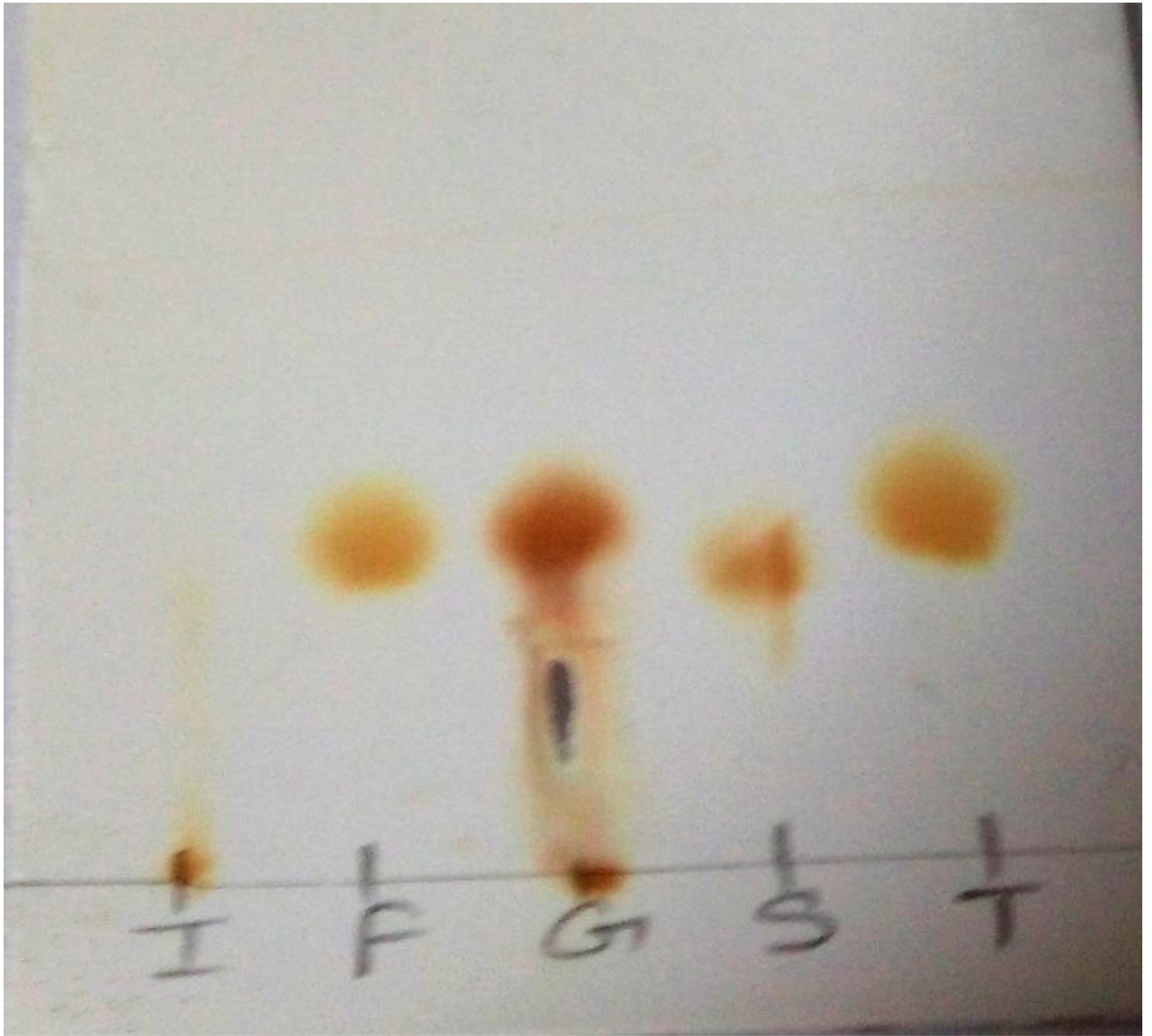


Figure 4

3D surface plot showing the effect of K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> on the activity of inulinase enzyme.



**Figure 5**

Thin layer chromatogram of inulin hydrolysate formed by crude inulinase from fungal isolate, DD MSF 1, I- control (inactivated inulinase + inulin), F- fructose standard, G- glucose standard, S- sucrose standard, T – products of hydrolysis.

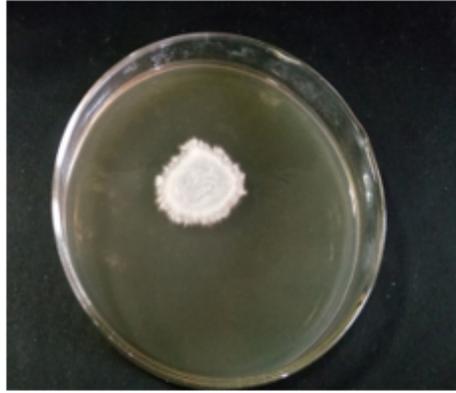


Fig. 1a- Growth of DD MSF 1 on inulin media

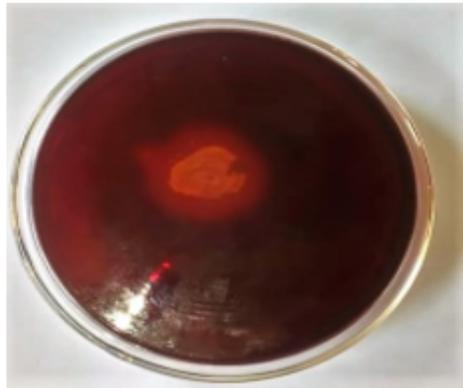


Fig. 1b- Formation of clear halo around DD MSF 1 after flooding with Lugol's iodine

## Figure 6

1a- Growth of DD MSF 1 on inulin media. 1b- Formation of clear halo around DD MSF 1 after flooding with Lugol's iodine.

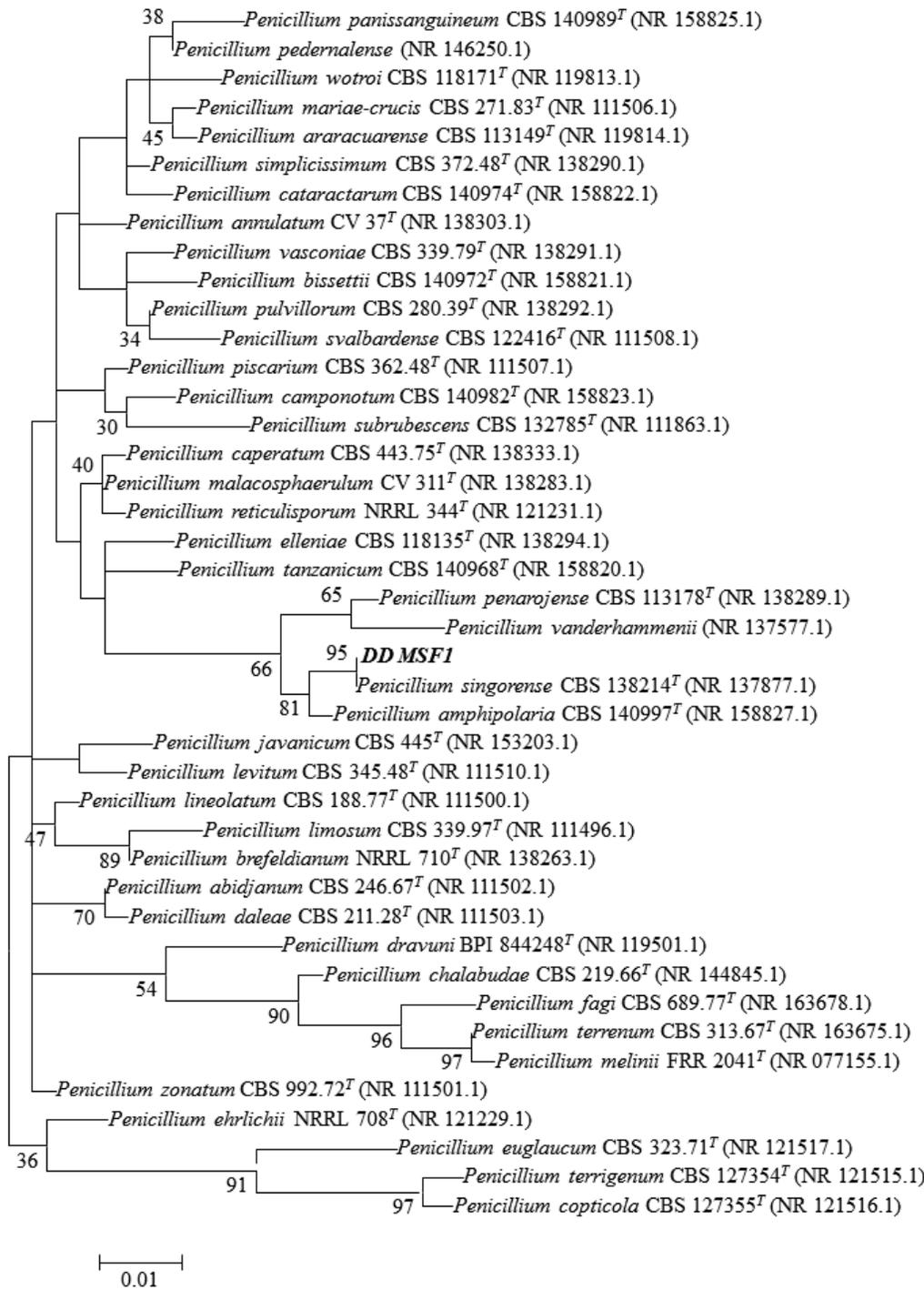


Figure 7

Phylogenetic tree of 18s rRNA of the isolate *Penicillium amphipolaria* strain KAS 2555 shown as DD MSF 1.

## Supplementary Files

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