Production of Inulinase by *Fusarium* sp. and its Application for Fructooligosaccharide Production for use as Prebiotics

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ABSTRACT

Fructooligosaccharides (FOS) are useful due to their applications in food and pharmaceutical industry. *Fusarium* spp., isolated from dahlia rhizosphere, produced endoinulinases in a medium containing inulin or sucrose as carbon substrate. Characterisation of exo-inulinase and production of FOS were investigated. Temperature and pH optimum of the enzyme was found to be 60 °C and pH 6.0, respectively. The optimisation of fermentation conditions for inulinase production was carried out using one at a time approach. The optimised medium with sucrose as a carbon source and yeast extract as a nitrogen source were found to be optimal for inulinase production at an initial pH 5.0 and incubation temperature 30 °C for 120 h. Under these conditions, the maximum inulinase concentration of 131.6 U/ml was achieved. SDS PAGE revealed that the molecular weight of the enzyme was around 90 kDa. Further study revealed that *Fusarium* sp. could produce inulinase as well as invertase. Thin layer chromatography was carried out to analyse the FOS production and their oligomeric properties. Inulin as well FOS can be used as prebiotics as they are selectively fermented by *Bifidobacteria* and lactic acid bacteria and thus imparting health benefits.

Keywords: Inulin; Fructooligosaccharide; Fermentation; Prebiotics

1. INTRODUCTION

Inulin is a polymer of fructose produced by plants which are used in the food and pharmaceutical industry. Inulin is reserve carbohydrate found in Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Chicories intibuca*), dahlia (*Dahlia pinata*), dandelion (*Taraxacum officinale*), garlic (*Allium sativum*) and onion (*Allium cepa*) in valued quantities[1]. The chain length of inulin is usually 25 to 35 residues and the enzymes active on inulin and on sucrose are fructofuranosidases (inulinases). Two types of inulinases, i.e., exo- and endo- are produced by microorganisms. The action of exo-inulinase splits the terminal fructose units from inulin whereas, endo-inulinases (belong to the group of fructanohydrolase) hydrolyse internal β- 2, 1 fructofuranosidic linkages to yield inulo-oligosaccharides like inulotriose, inulotetrose, inulopentose as the main products[2].

The chemical approach for fructose production is associated with some drawbacks[3,4] as the chemical hydrolysis of inulin is found to be non- specific and to produce more undesirable by-products (such as difructose anhydride) than enzymatic hydrolysis[5,6]. Total or partial hydrolysis of inulin leads to the production of fructose or fructooligosaccharides; these fructose syrups produced through enzymatic processes can compete as sweeteners with the available high-fructose corn syrups[7] and ultra-high-fructose glucose syrups, prepared from starch by enzymatic hydrolysis and separation of fructose with ion exchange resin. Most of the inulinases are exo-

enzymes that split fructose units from the fructose end of the inulin molecule. A few endo-enzymes have been reported to liberate oligofructosides as primary products of hydrolysis[8,9].

Inulin and oligofructose are classified as food or food ingredients in several countries where, they are in use. Inulin and oligofructoses are amongst the most studied and well-established prebiotics. They are indigestible in the upper gastrointestinal tract and reach the large intestine intact. This property makes them as ideal sugars for fermentation in the colon by the saccharolytic resident microbiota. Among all the hydrolytic enzymes, inulinase is of interest as it can be used in the production of high fructose syrup, fuel ethanol and inulooligosaccharides production from inulin.

In the study, authors isolated inulin hydrolysing fungi which could be used for the production of fructooligosaccharides or fructose based on the hydrolysis conditions. Since fructose is a GRAS sweetener and sweeter up to 1.5 times than sucrose and also cost effective. In addition, fructose has functional properties known to enhance flavour and colour of food. The process parameters were also optimised for enhanced production of the enzyme.

2. MATERIALS AND METHODS
2.1 Medium

The culture and isolation media had the following composition (per litre): $NaNO_3$, 3.0 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 1.0 g, KCl, 0.5 g; and inulin, 10.0 g. The pH was adjusted to 5.0. The inulin was sterilised at 110 °C (30 min),

and the salts solution was sterilised at 121 °C (15 min). The medium for the culture broth and slant experiments was similar, except that it contained an additional 15 g of agar per litre of medium.

2.2 Isolation of the Microorganism

Inulinase producing fungi were isolated from Dahlia rhizosphere soil collected from Defence Food Research Laboratory, Mysuru garden. Serial dilutions of the soil were carried out in 0.9 % NaCl and plated on inulin containing plates as sole carbon source. The plates were incubated at 30 °C for 72 h and then flooded with Lugol's iodine solution (1.5 % KI and 1 % I$_2$) for 2 min to check for the inulinase production. Strain forming a clear halo around the colony was recorded as inulinolytic, whereas strain that did not form halo was recorded as noninulinolytic. The colonies showing zone of clearance were selected for further studies. The isolated colonies were maintained on agar slants at 4 °C.

2.3 Strain Identification

The potential isolate was selected and systematically identified by sequencing the ITS region of the fungi using primers ITS1 TCCGTAGGTGAACCTGCGG and ITS4 TCCTCCGCTTATTGATATGC in a thermocycler, Eppendorf. The PCR amplifications were carried out in a reaction mixture for 50 μl containing 5 μl 10 × PCR buffer, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.4 μM each primer, and 1 U Taq DNA polymerase. The amplified PCR products were purified using PCR purification kit, Quigen, cloned in p-drive vector, and sequenced at Ultra Lab Products, Bangalore. Further, the sequences were analysed using NCBI-BLAST homology search (National Centre for Biotechnology Information) program and identified the fungi as *Fusarium* sp.

2.4 Effect of Carbon and Nitrogen Source on Inulinase Production

The effect of different carbon sources such as glucose, galactose, fructose, sucrose and maltose was studied at 1 % (w/v) instead of 1 % (w/v) inulin in the media for production of inulinase. The effect of nitrogen sources on inulin production was also studied using peptone, yeast extract, casein peptone and soya peptone at 0.2 %. The pH of the medium was adjusted to 5.0 with 1.0 N HCl before autoclaving. Carbon sources were sterilised separately and added in the medium. All the experiments were carried out under sterile conditions. The flasks were kept at 30 °C for incubation at 100 rpm for 5 days.

2.5 Effect of Temperature and pH on Inulinase Production

To study the optimum incubation temperature for growth of inulinase-producing fungi was incubated at different temperatures such as 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C at pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0.

2.6 Inulinase Production and Extraction

The isolated culture was grown in the medium containing gL^{-1}: NaNO$_3$, 3.0 g; MgSO$_4$.7H$_2$O, 0.5 g; KH$_2$PO$_4$, 1.0 g,

KCl, 0.5 g; and inulin, 10.0 g for 5 days. The mycelium was separated from the culture broth by centrifugation at 10000 rpm for 15 min. The enzyme was precipitated from the supernatant using an equal volume of cold acetone and centrifuged at 10000 rpm for 15 min. The pellet was dissolved in 0.1 M sodium phosphate buffer, pH 6.0. The enzyme solution was applied to a Sephadex G-100 column (2.0 × 85 cm) previously equilibrated with 50 mM phosphate buffer pH 6.0 for further purification. Bound proteins were eluted from the column at a flow rate of 24 ml/h in the same buffer, and fractions (2 ml each) were collected.

2.7 Inulinase Activity

Enzyme activity was performed by adding 0.5 ml of the crude enzyme to 0.5 ml of 30 % (w/v) inulin dissolved in phosphate buffer (50 mM, pH 6.0) followed by incubation at 45 °C for 30 min. Each reaction was inactivated immediately by putting the reaction mixtures on the ice, adding 5.0 ml of DNS reagent to each reaction mixture and keeping them in boiling water for five minutes. The inactivated enzyme crude extracts with the same mixtures were used as blank controls. A number of the reducing sugars released from inulin in each reaction mixture was assayed using DNS method[10] by measuring the absorbance at 540 nm. One unit of inulinase was defined as the amount of enzyme that produced 1.0 μmol of fructose per minute under standard assay conditions. The standard curve was prepared for DNS using fructose. All enzymatic reactions were performed in triplicates. The molecular mass of inulinase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by comparing the size of the major band with standard protein markers (Puregene PG-PMT0782) according to the method of Laemmli[11]. Proteins in the polyacrylamide gel were visualised by Coomassie brilliant blue R-250 staining. Native PAGE was carried out in a similar way without denaturing the enzyme. After completion of the electrophoresis, the gel was washed with de-ionised water and overlaid on a plate containing inulin. The plate was incubated at 30 °C for 2 h and flooded with Lugol's iodine solution as mentioned above.

2.8 Effect of Temperature and pH on Enzyme Activity

The optimum temperature for the inulinase activity was determined by incubating the enzyme and inulin (30% w/v) mixture at 35.0 °C – 85.0 °C in 50 mM phosphate buffer, pH 6.0. Temperature stability of the enzyme was tested by pre-incubating it for 30 min at temperatures ranging 35.0 °C – 95.0 °C at pH 6.0, and the relative activity of inulinase was measured. The pH stability of inulinase was assessed by pre-incubating the enzyme for 12 h with buffered inulin ranging from pH 3.0 to pH 10.0, pH 3.0-5.0 (sodium acetate buffer), pH 6.0-8.0 (phosphate buffer), and pH 9.0 and pH 10.0 (glycine–NaOH buffer) at 4 °C. The residual enzyme activity was determined under standard assay conditions. The inulinase activity of the pre-incubated sample at 4 °C was taken as 100 per cent. All the experiments were performed in triplicate, and the error was expressed as the standard deviation of the three measurements.

2.9 Thin Layer Chromatography for Catalytic Nature Determination of the Inulinase

The products obtained from inulin hydrolysis with purified inulinase from *Fusarium* sp. were determined by performing the thin layer chromatography (TLC). The enzyme reaction 30 % (w/v) inulin in 50 mM phosphate buffer (pH 6.0), purified inulinase was added and incubation carried out at 45 °C and pH = 6.0 for 24 h. Precoated TLC plates (Silica gel 60, Merck) spotted with samples were developed with the solvent system, butanol/propanol/water (2:6:2, by volume). Fructose (Sigma -Aldrich) was used as a standard. The TLC plates were developed using detection reagent containing 5 % (by volume) sulphuric acid in methanol and heating them at 100 °C for 5 min till brown spots develop to detect sugars.

2.10 Statistical Analysis

One- way analysis of variance (ANOVA) was used for statistical analysis. Data were expressed as mean $\pm$ standard deviation from three independent parallel experiments. Student's t-test was used to compare the mean values. Significance was set at $p < 0.05$ at 95% confidence level.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Inulinase Producing Fungi

A total of 15 inulinase producing fungi were isolated from Dahlia rhizosphere soil. Based on the enzyme activity (data not shown) *Fusarium* sp. was further selected for the inulinase production and its optimisation as shown in Fig. 1.
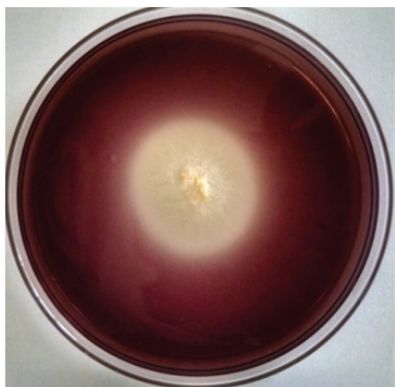

Figure 1. *Fusarium* sp. showing inulinolytic acitivity.

3.2 Effect of Carbon and Nitrogen Sources on Inulinase Production

Sugars such as glucose, galactose, fructose, sucrose and maltose were used as carbon source for inulinase production. Maximum inulinase production was noticed in the presence of sucrose 80.0 U/ml as the sole carbon source in comparison with other sugars (Fig. 2). Among the organic nitrogen sources tested at 0.2 % (w/v), yeast extract supported the highest inulinase production 90.8 U/ml when used as the sole nitrogen source in comparison with peptone and soya peptone (Fig. 3). These findings are in accordance with the findings reported by Fiedurek[12], *et al.* wherein *Aspergillus niger* strain 13/36 was studied using sucrose at 6.66 g/L along with yeast extract as sole nitrogen source at 3.94 g/l yielded inulinase at 79.8 U/ml.
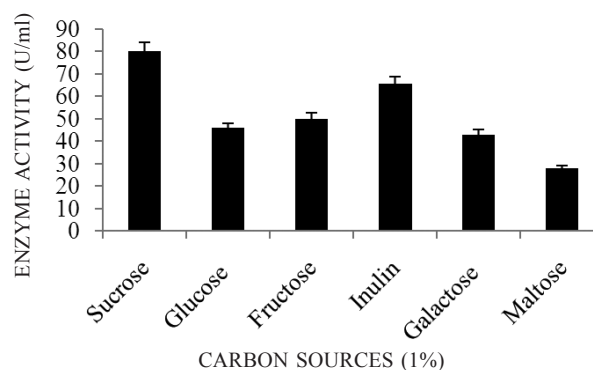

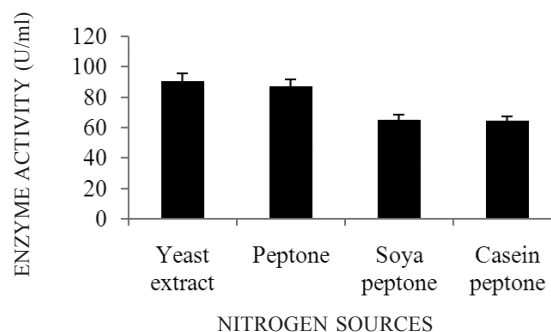Figure 2. Effect of various carbon sources on inulinase production.

Figure 3. Effect of various nitrogen sources on inulinase production.

Among the substrates, inulin and sucrose have been used as the preferred carbon source. In general, if the microbial strain showed only inulinase activity, inulin was the best substrate but if the microorganism exhibited inulinase activity along with invertase activity, sucrose served as a better source for enzyme production[13]. Kochhar[14], *et al.* reported that inulinases purified from various sources retain some invertase activity even after a number of chromatographic processes, suggesting that the active site of inulinase might have two parts, one implicated in sucrose hydrolysis and the other in inulin breakdown.

3.3 Effect of pH and Temperature on Inulinase Production

Effects of pH and temperature on inulinase production were assessed and found that the optimal pH and temperature were pH 5.0 and 30 °C, respectively (Figs. 4 and 5). Under these conditions, inulinase activity of about 131.6 U/ml was achieved which was about 2-fold higher than that of the screening conditions.

3.4 Characterisation of Inulinase

The purified inulinase used in the present study showed a single major band of approximately 90 kDa on 10 % SDS-PAGE gels (Fig. 6). The protein band was found to show a zone of clearance in the Native PAGE confirming active inulinase. Previous reports on inulinase indicated different molecular weight from different organisms *Penicillium janczewskii* (80 kDa), *Arthrobacter* sp. (75 kDa), *Bacillus stearothermophilus* KP1289 (54 kDa), *Kluyveromyces marxianus* var. *bulgaricus*
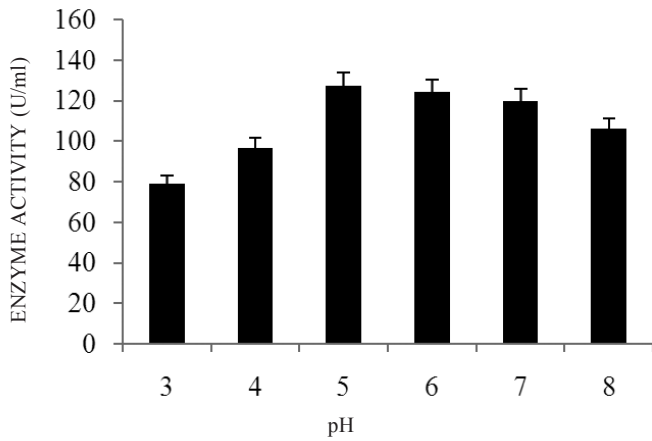
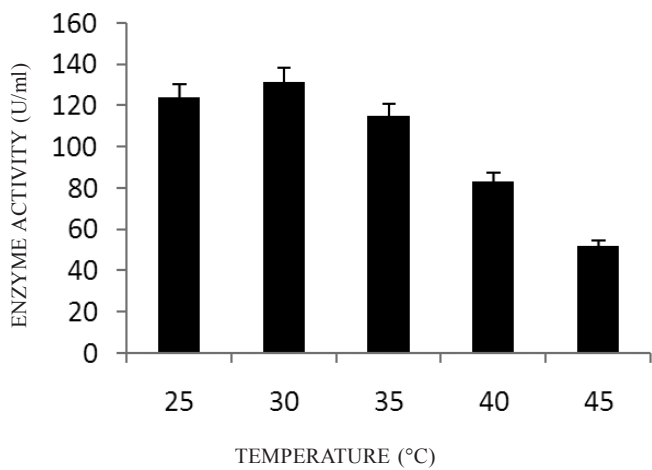**Figure 4. Effect of pH on inulinase production.**

Figure 5. Effect of temperature on inulinase production.
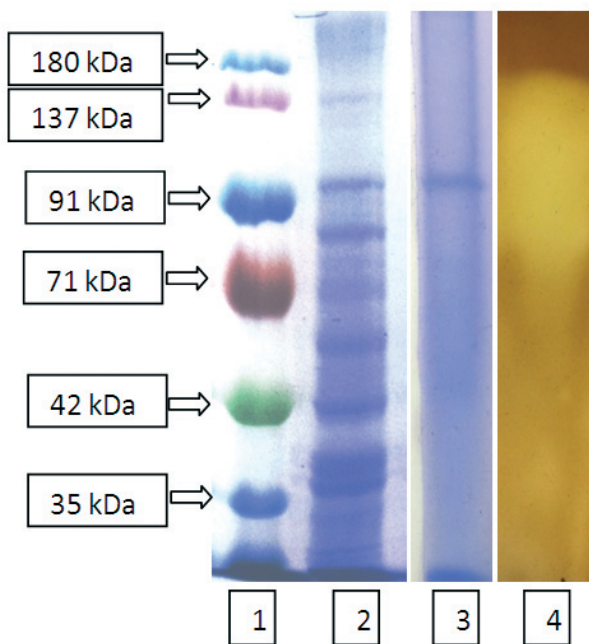

Figure 6. Lane 1: SDS – PAGE Marker, Lane 2: Crude enzyme, Lane 3: Purified enzyme, and Lane 4: Native PAGE showing the active inulinase.

(57 kDa), *Penicillium* sp. TN-88 (68 kDa), *Aspergillus candidus* (54 kDa), and *Streptomyces* sp. (45 kDa)[15,16]. The optimal conditions for enzyme activity in terms of temperature and pH for the enzyme were investigated based on the fructose released by the enzyme from inulin. The influence of temperature on inulinase activity was examined at pH 5.0, and the optimum temperature was found to be 55 °C. The enzyme activity at 45 °C was 60–70 % and less than 40 % above 65 °C. The temperature stability drastically reduced on incubation above 55 °C for 20 min (Figs. 7(a)-7(b)). It is considered to be active at moderately high temperature when compared to inulinases from *Aspergillus niger* Mutant 817 (40 °C)[12], *Bacillus polymyxa* (35°C)[17] and *Cryptococcus aureus* G7a (50 °C)[18]. The enzyme had optimal pH 6.0, however it was still quite active at broad pH 4.0 to pH 7.5 (Fig. 8) and stable at broad range of pH 5.0 to pH 9.0 for 30 min whereas inulinases from several microorganisms were reported to be optimally active at acid pH including *Aspergillus niger* Mutant 817 (pH 5.0)[13], *Bacillus smithii* T7 (pH 4.5)[19] *Kluyveromyces marxianus* var. *bulgaricus* (pH 4.75)[20] and *Fusarium oxysporum* (pH 4.0 - 5.5)[21].
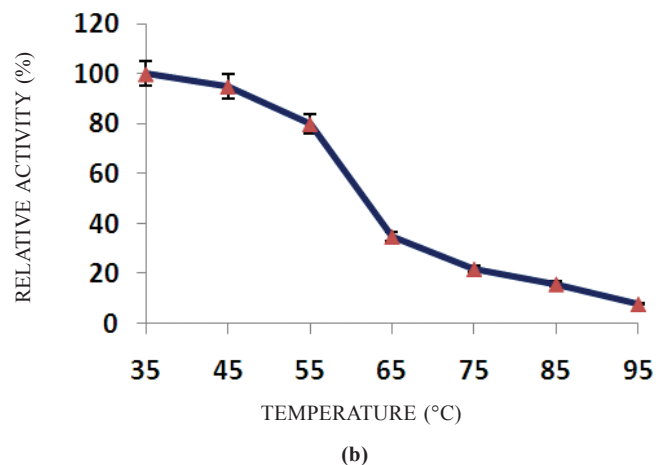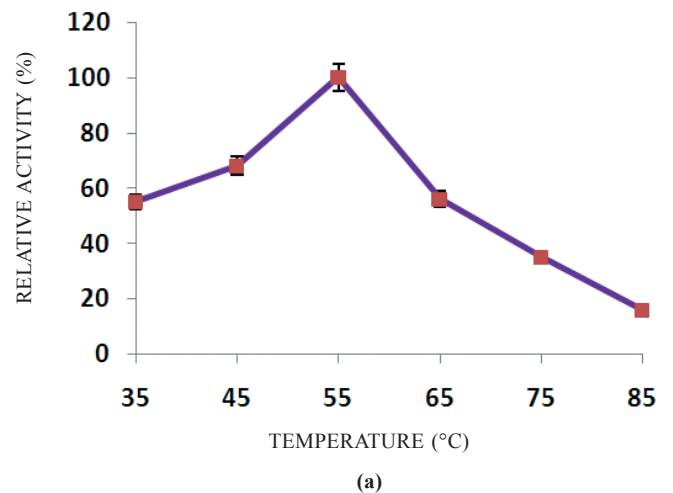

(a)

(b)

Figure 7. Effect of temperature (a) on relative activity of inulinase and (b) on relative stability of the inulinase.
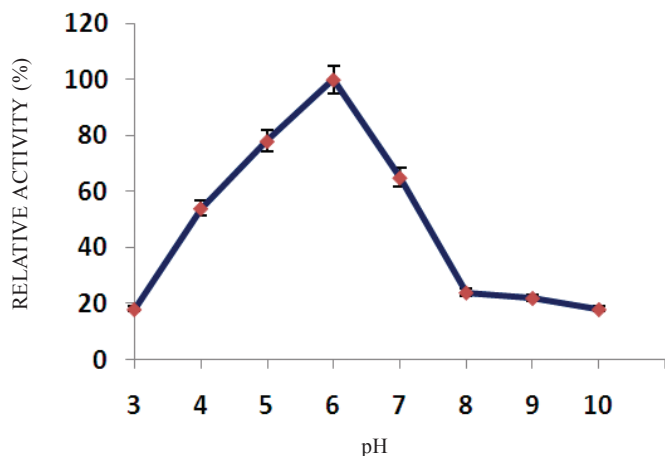
Figure 8. Effect of pH on relative activity of inulinase.

3.5 Product of Inulin Hydrolysis by Purified Inulinase

Thin layer chromatography analysis of products of inulin hydrolysis showed that fructose was the predominant end product during hydrolysis for 30 min (Fig. 9). Fructose was obtained as major hydrolysis product suggesting the presence of an active inulinase. Inulin hydrolysis in *Rhizopus* sp. by an extracellular inulinase resulted in the production of fructose and oligosaccharides after 24 h of incubation[22]. Fructose formation was completely absent when inulin was hydrolysed with crude endoinulinase of *X. oryzae* No. 5[23]. The native endoinulinase produced by *Arthrobacter* sp. S37 hydrolysed inulin at optimal pH 7.5 and 50 °C primarily into inulotriose, inulotetraose and inulopentaose[24]. Inulobiose was found to be the major product of the activity of immobilised endoinulinase by *Pseudomonas* sp. No. 65 or immobilised recombinant *E. Coli* which possessed endoinulinase gene[25].
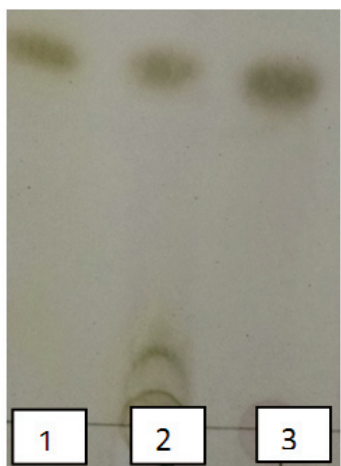

Figure 9. TLC of hydrolysis product of inulin Lane 1: Fructose, Lane 2: After 30 min, Lane 3: After 60 min.

4. CONCLUSIONS

Inulin hydrolysing *Fusarium* sp. from Dahlia rhizosphere was isolated and harvested for the production of fructo-oligosaccharide. The physical and chemical parameters were optimised for the enzyme production. The optimised medium with sucrose as a carbon source, yeast extract as a nitrogen

source at 30 °C and pH 6.0 showed an enzyme activity of 131 U/ml. The molecular weight as determined by SDS- PAGE was found to be around 90 kDa. The enzyme can be used for the production of fructose which is an alternative to sucrose and also high fructose syrup. The enzyme has a great potential in food and pharmaceutical industry.

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He conceptualised the work carried out and also proof reading of the manuscript.