International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 395-404
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Characterization of Inulinase from *Aspergillus niger* AM270052.1 and Scale Up of Conditions for Mass Production

V. Parivuguna[1], P. Saravana kumari[2] and S. Shanmugham[3]

[1]Research and Development Center, Bharathiar University, Coimbatore and Associate Professor, Dept. of Microbiology, Rathnavel Subramaniam College of Arts and Science, Coimbatore.

[2]Associate Professor, Dept. of Microbiology, Rathnavel Subramaniam College of Arts and Science, Coimbatore.

[3]Assistant professor, PG and Research Department of Microbiology, Presidency College, Chennai.

Received: 23 Mar 2019 / Accepted: 25 Apr 2019 / Published online: 1 Jul 2019
Corresponding Author Email: parivugunarvs@gmail.com

Abstract

Microbial inulinase are of considerable interest both in fundamental studies and in industrial applications. The increasing potential of inulinase application prompts screening for newer inulinase producing microorganisms using cheap substrates which can meet the conditions favourable to the industrial applications. A soil isolate, *Aspergillus niger* AM270052.1 was confirmed as a potential candidate for the large-scale production of inulinase using agave tuber as a cheaper substrate. Using the isolate, the optimum pH and temperature for enzyme production was determined as 4.5 and 55°C. Inulin content in easily available agave tuber was comparatively equal to the inulin content available in the economically costlier substrate such as chicory, bulbs of onion and garlic. So, agave tubers were suggested in the study for the mass production of inulinase. The maximum yield of pure inulinase enzyme was obtained by DEAE-Sephadex ion-exchange chromatography purification technique from the fermented substrate under optimized conditions was 23.2%. Zymogram analysis showed the presence of single band with approximate molecular weight of 93 KDa. Further confirmation on inulinase activity affirmed by presence of glucose, fructose and sucrose from agave medium treated with enzyme by TLC technique. So, the study concludes that agave tuber as the cheaper substrates for production of inulinase for their applications in industrial purposes.

Keywords

Inulinase, agave tuber, enzyme activity, optimization, characterization of inulinase.

*****

1. INTRODUCTION

Inulin occurs as a reserve of carbohydrate in the roots or tubers of plants such as *Helianthus tuberosus* L, *Cichorium intybus L., Jerusalem artichoke* and *Dahlia variabilis*. Inulins are a group of naturally occurring polysaccharides produced by many types of plants. Inulin is a widespread naturally occurring polyfructan in plants,[1] consisting of linear chains of β (2, 1)- linked fructose residues attached to a terminal sucrose molecule.[2] They belong to a class of fibres known as fructans. Inulin is used by some plants as a means of storing energy and is typically found in roots or rhizomes. Most plants that synthesize and store inulin do not store other materials such as starch. Complex form of inulin usually converted into simple sugars, such as glucose, fructose by the enzyme inulinase; thereby inulin can be used as the potential source of simple sugars in various industries. Day-by-day the demand for these sugars are increasing in pharmaceutical and food industries. It can be satisfied by use of inulinase to convert inulin into simple sugars by using cost-effective method.

Microbial inulinases are of considerable interest both in fundamental studies and in industrial applications. The production of ß-fructofuranosidases (Inulinases) is widely distributed in different groups of microorganisms and its synthesis is dependent on growth conditions, mainly the carbon source. These enzymes are classified according to their mode of action on inulin. Endo-inulinases (ß-D-fructan: fructan hydrolase, EC 3.2.1.7) produce inulo-oligosaccharides from inulin, and exo-inulinases (ß-D-fructopyranoside fructohydrolase, EC 3.2.1.8) release fructose from the fructosyl terminal of inulin. The use of an industrial medium represents a good alternative to produce inulinase at low cost, since the activity may improve or at least remain the same, as that obtained using a synthetic medium. Selection of suitable substrates for fermentative processes has mainly been centred on agro-industrial residues because of their potential advantages. In addition, the utilization of these agro-industrial wastes, provides an alternative substrate and also helps in solving pollution problems, which otherwise may cause their disposal.[3]

The increasing potential of inulinase application prompts screening for newer inulinase producing microorganisms using cheap substrates which can meet the conditions favourable to the industrial applications.[4] Keeping in mind the potential applications of inulinase, the present study was undertaken with the following objectives to explore the inulinase production ability of *Aspergilllus niger* using cheap substrates.

2. MATERIALS AND METHODS

2.1. Isolation and identification of inulinase producing fungi

Inulinase producing organisms are rich in agriculture soil. A soil plate was prepared by transferring a small amount of the surface soil sample of about 0.005–0.015 gm into a sterilized petri dish and then approximately 8–10 ml of cooled potato dextrose agar medium was added and the soil particles was adequately dispersed throughout the agar by shaking and rotating the plate before the agar solidified.[5] The plate was incubated at room temperature for 3 days. After incubation, morphologically unique fungal isolates were subcultured on Czapek dox agar, then the established growth of isolates were stored at 4°C. The isolate was subjected to 18s rRNA sequencing, amplified, compared and identified by BLASTN program.

2. 2. Screening for inulinase production

The isolates were sub-cultured on Czapek Dox agar plates and were incubated at 28°C for 72hrs. After incubation, the plates were flooded with 0.01% of Triphenyl tetrasolium chloride suspended in 0.5M NaOH. The plates were kept for incubation for 20 minutes in dark. The plates were washed with 0.1M acetate buffer (pH 5) for the observation of red zone of hydrolyses by the inulinase producing fungal isolates.[6]

2. 3. Quantitative assay on inulinase production

The isolates showing red colour zone formation were subcultured separately on Czapek Dox broth and cultured under submerged fermentation at 28°C for 4 days. About 50 ml of culture suspension was taken and was extracted in 50ml of 80% of absolute alcohol for 12 hours to remove free sugars. From the dried sample, 500mg was taken in a 100ml conical flask. It was made up to 20ml and heated in a water bath at 90°C for 10min.The extract was collected and made up to 70ml with distilled water. The flask was replaced for another 30min with occasional shaking to dissolve the fructosan and then it was removed and cooled at room temperature. The extract was filtered and made up to 100ml in a standard flask. The quantity of fructose present in the sample was estimated using standard graph developed using resorcinol method. Standard fructose solution was prepared. The extinction was read within 30min at 520nm in ELICO spectrophotometer. The amount of inulin is expressed in terms of fructose concentration. The quantity of enzymes

concentration was measured by Lowry *et al*., method.

2. 4. Optimization of conditions for inulinase production

The suitable media for mass production of inulinase was ascertaining by inulin content assay. The percentage of inulin present in 1g of dry powder of agave tuber, onion bulbs, garlic bulbs and chicory (tuber) were determined by resorcinol method.

The isolates showing maximum enzyme production was cultured in 250 ml of Czapek Dox broth for submerged fermentation at 28°C for 4 days. Production of inulinase by the isolate was determined from the day of inoculation to 8 days of incubation by resorcinol method.

The optimum pH on Inulinase production was determined at pH between 3.5 and 9.0, adjusted using 100 mM sodium acetate (pH 3.5-5.5), sodium phosphate (pH 6.0-8.0) and Tris - HCl (pH 8.5-9.0) buffers. The enzyme activity was measured by resorcinol method.

The effect of temperature on enzyme activity was measured at pH 4.5 in 100 mM sodium acetate buffer, over a temperature range of 35-80°C. After incubation reducing sugars were estimated by DNS method. Thermo stability in the absence of substrate was determined by measuring the residual activity of the enzyme at different intervals after incubation at various temperatures.

2. 5. Purification of enzyme

Under aseptic conditions, the isolate was cultured in 250 ml of Czapek dox broth under optimized condition. After incubation, the mycelium was separated by filtration. To the 5ml of filtrate, ammonium sulphate (20-80%) was added slowly with constant stirring and the mixture was kept aside at 4°C for 2h. The suspension was centrifuged at 10000 rpm for 10min and the supernatant was collected. The pellet was dissolved in 100µl of sodium acetate buffer (0.1M, pH 4.5). The fraction showing higher specific activity was taken to the next step of purification. The enzyme precipitate transferred to a dialysis bag and sealed. The bag was suspended for 3 hours in buffer. Dialysed sample was run on anion exchange chromatography column packed with DEAE-Cellulose column (10 cm height) which was then pre-equilibrated with 0.05M acetate buffer. The sample was loaded. The column was eluted with a step gradient of NaCl from 0 to 0.4 M of acetate buffer at a flow rate of 0.6 ml/min. Absorbance was monitored at 280 nm. The elution profile revealed three major peaks showing inulinase activity on inulin, which was designated as A, B, C by the order of elution. The three active fractions of A, B and C were pooled, dialysed against 0.025M acetate buffer, lyophilised, dissolved in a small volume of acetate buffer.[7]

2. 6. Determination of molecular weight of purified enzyme

To monitor the progress of purification of the enzyme, polyacrylamide gel electrophoresis was performed under native conditions. The concentration of the upper gel was 4.5% and the lower was 10%. The protein was stained with Coomassie Brilliant Blue G-250 and the decolorant used was methanol. The molecular weight of the purified protein was determined by SDS-PAGE. [8]

2. 7. Analysis of inulinase activity by TLC

In order to analyse the products of inulin hydrolysis, the crude enzyme was incubated at 55°C for 24h with 0.17% inulin in 0.1M sodium acetate buffer pH 4.5. The hydrolysis products were analysed by thin layer chromatography (TLC). Pre-coated TLC plates (Merck, Germany) spotted with samples were developed using n-butanol: acetic acid: water (2:1:1 v/v/v) as a solvent system and the sugars were visualized by heating the plates for 30 min at 80°C after spraying with orcinol sulphuric acid reagent.[3]

2. 8. Zymogram for pure inulinase

For detecting and localizing the inulinase activity on native- polyacrylamide gel, an agar replica was prepared with 1.0% agarose and 0.2% pure inulin. After Native-PAGE of inulinase, the gel was treated with acetate buffer. The gel was then placed on the agar replica and incubated at 55°C for 45 min. Then the gel was soaked in congo red solution for 30 min and washed with 1M NaCl solution for 2 h at room temperature. The clear zone of the inulinase activity was visualized in the coloured background.

3. RESULTS AND DISCUSSION

3. 1. Isolation of inulinase producing fungi

From the collected soil samples, fifteen different wild type strains of fungi were isolated and used for the study. Isolates were named serially as S1, S2, till S15 according to the order of isolation.

3. 2. Qualitative and quantitative assay of inulinase production by the isolate

Isolated fungi were evaluated for the production of inulinase by Triphenyl tetrasolium chloride method. Among the 15 different fungi, about 20% of organisms were unable to produce inulinase (-), whereas remaining 40% of organisms showed lower level of inulinase production (+), whereas another 40% of organisms showed large zone of colour formation (+++). Of the inulinase producing organisms labelled as S3 showed the maximum production. The qualitative assay results highly

correlated with the quantitative assay and the organism S3 showed maximum inulinase production by 0.876 U/ml (Table 2).

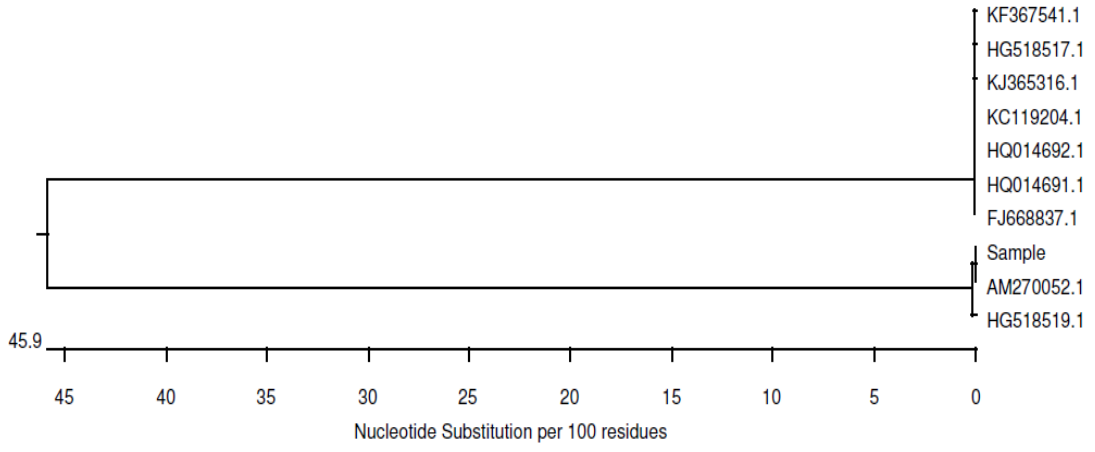
3. 3. Identification of isolate

The maximum producing organism was subjected to microscopic and biochemical tests for identification. On Czapek dox agar media, black colour powdery radial colonies with powdery spores were observed and recorded. By lactophenol cotton blue staining, radiating bi-seriate conidia with columnar in the conidiophore was confirmed. According to Raper and Fennall (1975)[9] scheme of identification, the isolate was revealed as *Aspergillus niger*. Inulinase was produced from *Aspergillus niger* using tap roots of dandelion (*Taraxacum officinale*) as substrate.[10] The maximum inulinase producing fungi was identified and confirmed by molecular studies as *Aspergillus niger* by 18S rRNA sequencing as the isolate was showing 95% of similarity with *Aspergillus niger* AM270052.1 (Fig. 1).

TABLE 1. INULINASE ACTIVITY ISOLATED FUNGI

Isolate No.	Activity (Qualitative)	Inulinase production U/ml
S 1	+	0.23
S 2	---	0.000
S 3	+ + +	0.876
S 4	+	0.045
S 5	+	0.064
S 6	+ + +	0.087
S 7	+	0.043
S 8	---	0.000
S 9	+ +	0.409
S 10	---	0.000
S 11	+	0.024
S 12	+ + +	0.567
S 13	+	0.060
S 14	+ +	0.319
S 15	---	0.000

FIG.1. PHYLOGENETIC TREE OF ISOLATED SPECIES OF *ASPERGILLUS NIGER*



3. 4. Assay on substrate for mass production of inulinase

Comparatively, higher inulin content was observed with agave next to garlic and chicory. These results confirmed that use of agave tuber powder as cheap carbon source for inulinase production next to other commercial sources such as garlic and chicory. Usually, chicory tubers are used for the commercial production of inulinase which showed maximum inulin content of 94%.[11] Percentage of inulin content in selected plant substrates were given in table 2.

TABLE 2. Analysis of Inulin content of different substrate

Substrates	Inulin content (%)
Agave tubers	6.19
Garlic bulbs	7.73
Chicory tubers	7.62
Onion bulbs	0.99

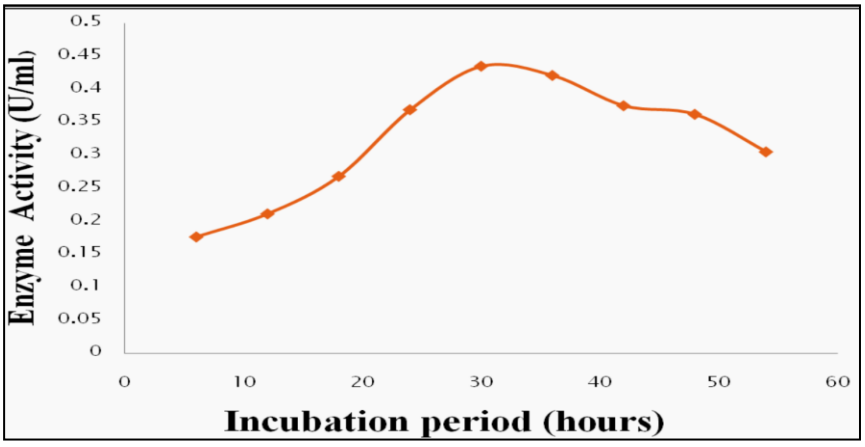
Compared to pure substrates, inulin-containing plant materials offer more advantages. Different natural substrates like rye, barley, banana, wheat, garlic, chicory and dahlia were used in inulinase production from *Streptomyces* sp.[3] Garlic and onion peels were used for the production of inulinase from *Xanthomonas campestris pv. phaseoli* in solid state fermentation.[12] Yacon (*Polymnia sanchifolia*) extract was also as a substrate to produce inulinase by *Kluyveromyces marxianus* var. *bulgaricus.*[13] Inulinase was produced from *Aspergillus niger* using Tap roots of dandelion (*Taraxacum officinale*) as substrate.[9]

In the present study, Inulinase production was attempted using agave tuber as substrate which was not reported earlier. Inulinase produced by *A. niger*, using agave as carbon source showed maximum activity of 435 IU/L which was comparatively better than inulinase produced by *Panaeolus papillonaceous* (230 IU/L),[9] by *Aspergillus fumigatus* (400 IU/L), *Penicillium* sp., TN-85 (560 IU/L),[14] *Kluyveromyces marxianus* (470 IU/L), *Staphylococcus* sp., (615 IU/L).[15] Compared to pure inulin (310 IU/L), the inulinase activity was 1.4 fold higher in agave tuber. The enzyme activity was relatively good compared to the activity obtained from earlier reports using different substrates including rye (313 IU/L); barley (244 IU/L); banana (283 IU/L); wheat (320 IU/L); chicory (328 IU/L); garlic (524 IU/L) and onion (300 IU/L).[16] So, the study recommends the use of agave tubers as a cheap alternative source for industrial production of inulinase.

3. 5. Optimization of incubation temperature on inulinase production

The maximum activity (435 IU/L) was obtained at the 30th hour of culturing, which was suitable property for industrial processes (Fig. 2).Growth time for the maximum inulinase activity reported in different microorganisms; *Fusarium oxysporum* (8 IU/L after 9 days);[17] *Aspergillus fumigatus* (5 days);[18] *Streptomuces* sp., (2 days);[16] *Actinomycetes* strain (24 h);[19] *Penicillium purpurogenum* and *Aspergillus niveus*;[20] *Pichia guilliermondii* (15 days);[21] *Aspergillus niger* and *Candida guilliermondii*(48 h);[22] *Pichia pastoris* (10 days);[23] *Kluyveromyces bulgaricus*(48 h);[24] *Aspergillus ficuum* (5 days); *Penicillium janczewskii* (8 days);[25] *Rhizopus* sp., strain TN -96 (5 days).[26]

FIG.2. EFFECT OF INCUBATION TIME ON INULINASE ACTIVITY

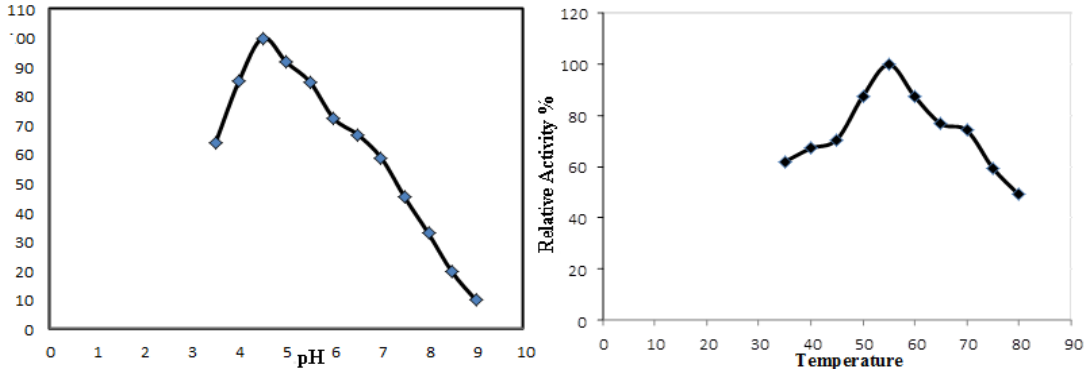


3. 6. Effect of pH and temperature on enzyme activity

Inulinase from *A. niger AM2700* 52.1 was found to be active at a pH range between 3.5 to 9.0 (Fig.3), the optimum being pH 4.5 with inulin as substrate in acetate buffer. Sheng *et al.,* in 2008, [27] proposed that the optimal pH of the purified inulinases from fungi and yeasts ranges 4.5–6.0. Similar conclusion was made by studies on inulinase production by *Kluyveromyces* sp., Y-85 and indeed most yeasts like

Pichia guilliermondii have optimum pH in the range of 5.0 to 6.0. [28] Inulinase from *Staphylococcus* sp., has an optimum pH of 6.5 and temperature at 37°C.[15] Inulinase from *A. niger* AM270052.1 was found to be maximum at 55°C temperature (Fig.3). Similar observation was obtained from *K. fragilis* and *K. marxianus.*[29] It was also observed that the inulinase activity increased as the temperature increased, reaching the optimum at 60°C. The maximum inulinase activities for *K. marxianus* var. *bulgaricus* were observed at 50°C and 60°C.[13] High temperatures were also observed by Wenling *et al.,* 1998[28] being 50°C for free inulinases and 55°C for immobilized inulinase.So the maximum inulinase production achieved by *A. niger* AM270052.1 using agave as substrate at 55°C, and was stable over wide pH range (3.5-6.0) (Fig. 4). The enzyme was thermostable and exhibited stability over the range 40-65 °C (Fig. 5). The results were in accordance with the study on *Aspergillus ficuum*, inulinase showed stability over wide pH range (4.0-8.0) at 60 °C and there was decrease in activity beyond that range.[30]

Fig. 3. EFFECT OF pH AND TEMPERATURE ON INULINASE ACTIVITY



3. 7. Purification of inulinase

The purified inulinase from *A. niger* AM270052.1 was purified on the DEAE-Sephadex column. Inulinase eluted from the column with 0.5 M NaCl was purified over 3.01-fold a yield of 23.2 %. The elution profile showed two peaks. The higher inulinase activity was obtained with peak area and the samples were lyophilised. By purification of extracellular inulinase from *Rhizopus* sp., TN -96 afforded 12 fold purified enzyme with yield of 0.57 % by ion exchange chromatography. The purification of inulinase obtained with *Streptomyces* sp., using ion exchange and affinity chromatographies showed 18-fold purification with the yield of 4.8%.[3] Figure 6 shows SDS–PAGE of the purified inulinase from *A. niger*. The purified inulinase fractions were homogenous, since only a single band was observed on native- and SDS-PAGE. On native-PAGE the purified inulinase protein showed a mobility corresponding to about 93.0 KDa.

FIG. 4. pH STABILITY ON INULINASE FROM *ASPERGILLUS NIGER*

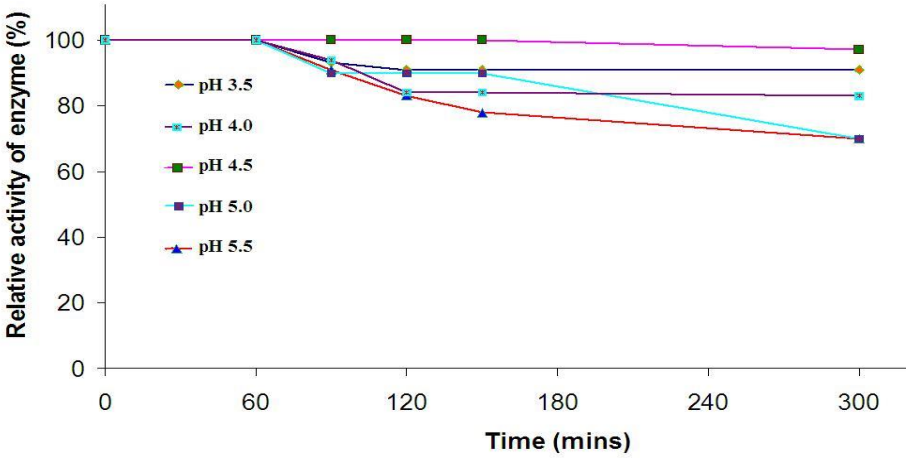
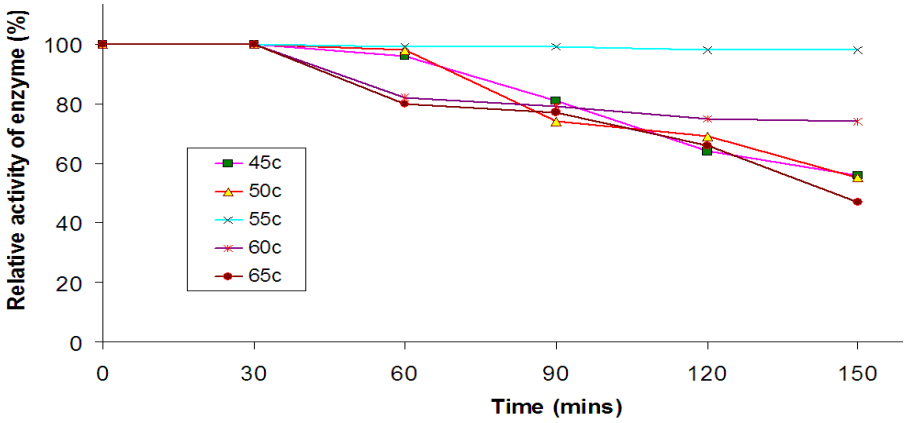


FIG.5. TEMPERATURE STABILITY ON INULINASE FROM *ASPERGILLUS NIGER*

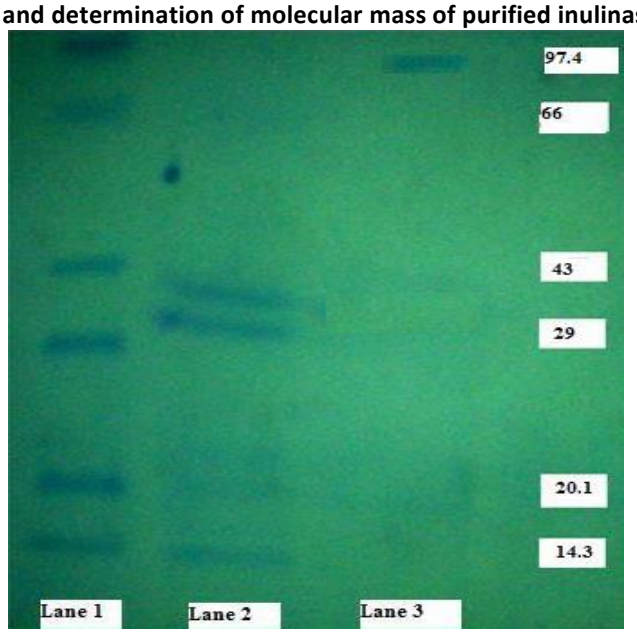


3. 8. Confirmation of Inulinase enzyme activity by TLC and Zymogram

To determine the exo- or endo- nature of the crude inulinase, TLC analysis was done with the inulin treated with purified inulinase (Fig. 7). Fructose was the major sugar produced during hydrolysis, supported the view that inulinase was an end group cleaving enzyme. Therefore, it was concluded that crude enzyme preparation contained exo-inulinases.[16] From the TLC results, concluded that fructose, sucrose and glucose like sugars were absent in agave tuber, therefore the enzyme treatment was responsible for conversion of complex inulin into simple sugars. Similar results reported by Ohta *et al.,* 2002[33] that fructose was the primary product results from the hydrolysis of inulin by active inulinase. Hydrolysis of inulin by extracellular inulinase produced by *Rhizopus* sp., resulted in fructose and other oligosaccharides after 24 h of incubation.

SDS – PAGE analysis showed the presence of single band with approximate molecular weight of 93kDa. Inulinase, from various sources were purified and previous studies on inulinase from a similar filametous fungus, *Penicillium janczewskii* proven to have 96 KDa,[34] and from *Fusarium* sp., determined as 90 KDa.[35] Zymogram analysis result also confirmed the homogeneity and of clearance caused by the purified inulinase obtained from SDS-PAGE (Fig. 8).

Fig. 6. Separation and determination of molecular mass of purified inulinase on SDS – PAGE



Lane 1: LMW – SDS marker
Lane 2: Partially purified inulinase by $(NH_4)_2SO_4$ precipitation
Lane 3: Purified inulinase by Column chromatography

Fig. 7. Zymogram for detecting the inulinase activity

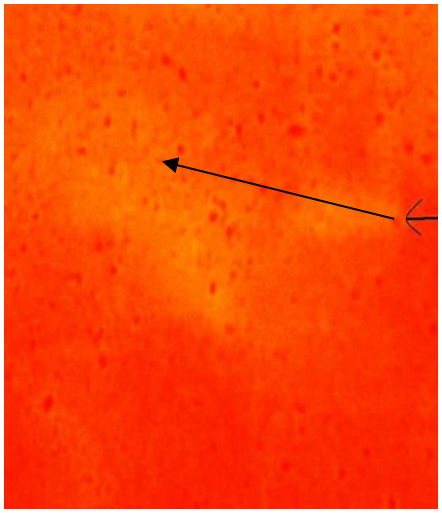
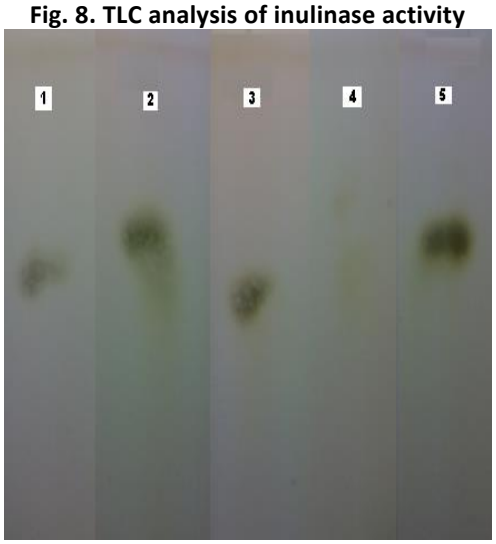


Fig. 8. TLC analysis of inulinase activity



Key note:
Lane 1: Separated band formed from standard glucose
Lane 2: Separated band formed from standard fructose
Lane 3: Separated band formed from standard sucrose
Lane 4: Separated band formed from agave extract without enzyme treatment
Lane 5: Separated band formed from agave after enzyme treatment showing fructose

4. CONCLUSION

From this study, it was found that *Aspergillus niger* AM270052.1 was confirmed as a potential candidate for the large-scale production of inulinase using agave tuber powder than chicory as substrates which was cheaper and easily available. Of the 15 isolates screened for inulinase production, 3 isolates showed higher inulinase activity. Comparatively, *Aspergillus niger* AM270052.1 resulted in highest inulinase production. In order to optimize the conditions for mass production, various parameters were analysed. It was found that the optimum pH and temperature was 4.5 and 55°C. Even though the optimum range of enzyme production was extreme for the survival of vegetative cells, the enzyme was highly stable and active there by satisfies the industrial requirement for enzyme activity. Addition of agave tuber to culture media can enhance the production of inulinase as it consists of comparatively same quantity of inulin. The maximum yield of pure inulinase 23.2% of enzyme was obtained by DEAE-Sephadex ion-exchange chromatography. Zymogram analysis showed the presence of single band with approximate molecular weight of 93kDa. Further confirmation on inulinase activity was affirmed by the presence of glucose, fructose and sucrose from agave medium treated with enzyme by TLC. So, the study concludes that agave tuber as the cheaper

substrates for production of inulinase for their applications in industries.

5. REFERENCES

1. Marchetti, G. Inulin and Falcons, Industry Alimentary, 32:945–949. (1993).
2. Vandamme EJ, Derycke DG. Microbial inulinases; fermentation process, properties and applications', Advances in Applied Microbiology, 29: 139–176. (1983).
3. Arun DS, Prabhjot KG. Purification and characterization of heat-stable exo-inulinase from *Streptomyces* sp. Journal of Food Engineering, 79: 1172–1178. (2007).
4. LuminitaGeorgescu, LA, Irina Stoica. Studies concerning the dynamic hydrolyse on the *Jerusalem artichoke* (*Helianthus tuberosus*) inulin. Journal of food technology, 6:77-80. (2005).
5. Warcup JH. The Soil-Plate Method for Isolation of Fungi from Soil. Nature, 166:117–118. (1950).
6. Hyun-Ju Kwon et al. Cloning and Characterisation of an exoinulinase from *Bacillus polymyxa*, Biotechnology letter, 25:155-159. (2003).
7. Han QC, Xiao MC, Yin L, Jing W, Zheng J, Xue-Ming X, Jian-Wei Z, Tian-Xiang C. Purification and characterisation of exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06. Food Chemistry, 115:1206–1212. (2009).
8. Laemmli, UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 227(5259): 680–685. (1970).
9. Naveen Kango, Production of inulinase using tap roots of dandelion (*Taraxacumofficinale*) by *Aspergillus niger.* Journal of Food Engineering, 85: 473-478. (2007).
10. Raper KB, Fennell DI. The genus Aspergillus. Baltimore: Williams and Wilkins, 238 -245. (1965).
11. Glyn MF, Kil JP, Fernando PB, SylvioLH. Evaluation of desorption isotherms, drying rates and inulin concentration of chicory roots (*Cichoriumintybus L.*) with and without enzymatic inactivation, Journal of Food Engineering, 63:273-380. (2004).
12. Ayyachamy M, Khelawan K, Pillay D, Permaul K, Singh S. Production of inulinase by *Xanthomonas campestris* pv. phaseoli using onion (*Allium cepa*) and garlic (*Allium sativum*) peels in solid state cultivation. Lett Appl Microbiol 45:439–444. (2007).
13. Cazetta, ML, Martins PM, MontiR, and Contiero J. Yacon (*Polymniasanchifolia*) extract as a substrate to produce inulinase by *Kluyveromyces marxianus* var. *bulgaricus.*, Journal of Food Engineering,66:301-305. (2008).
14. NakamuraT, Shitara A, Matsuda S, Matsuo T, Suiko M, and Ohta K. Production, purification and properties of an endoinulinase of *Penicillium* sp. TN-88 that liberates inulotriose. Journal of Fermentation and Bioengineering, 84:.313–318. (1997).
15. Selvakumar and Ashok pandey. Comparative studies on Inulinase synthesis by S*taphylococcus sp.* and *Kluyveromyces marxianus* in submerged culture', Journal of Bioresource technology, 69:123-127. (1999).
16. Arun DS, Sandeep K., Prabhjo TKG. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. Journal of Food Engineering.**,** 77: 486–491. (2006).
17. Kaur N, Kaur M, Gupta A, Singh R. Properties of β-fructosidases (invertases and inulinases) of *Fusarium oxysporum* grown on aqueous extract of *Cichoriumintybus* roots, Journal of Chemical Technology and Biotechnology, 53: 279–284. (1992).
18. Sharma AD, Kainth S, Gill PK. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. Journal of Food Engineering**,** 77:486-491. (2006).
19. PrabhjitKaur Gill, Arun Dev Sharma and Rajesh Kumari Harchand. Effect of media supplements and culture conditions on inulinase production by an *Actinomycete* strain. Journal of Bioresource technology, 87:.359-362. (2003).
20. Nandagopal S, and Ranjitha KBD. Enhancement of inulinase production from chicory and Fenugreek Rhizosphere soil. American-Eurasian, Journal of Agricultural and Environmental Science, 1(3):225-228. (2006).
21. Ning GFG, Zhenming CJS, Jing L. Enhanced inulinase production in solid state fermentation by a mutant of the marine yeast *Pichia guilliermondii* using surface response methodology and inulin hydrolysis. Journal of Industrial Microbiology and Biotechnology, 36:499–507. (2009).
22. SaretaSirisansaneeyakul, Luangpipat T, Vanichsriratana W, Srinophakun, T, Chen HHH, Chisti Y. Optimization of lactic acid fermentation by immobilized*Lactococcuslactis*IO-1 using Taguchi method. J. Ind. Microbiol. Biotechnol. 34: 381 – 391. (2007).
23. Tong ZFG, Ying PZC. Optimization for high-level expression of the *Pichia guilliermondii* recombinant inulinase in *Pichia pastoris* and characterization of the recombinant inulinase', Process Biochemistry, 44:1335–1339. (2009).
24. DarijaVrane, Bender, and Zagreb. Inulinase Production by *Kluyveromyces bulgaricus*, Food Technol. Biotechnol, 40 (1): 67–73. (2002).
25. Pessoni RAB, Braga, MR, and Cassia RL, (2007). Purification and properties of exo-inulinases from *Penicillium janczewskii* growing on distinct carbon sources. *Mycologia* 99: 493–503. doi:10.1080/15572536.2007.11832543.
26. Kazuyoshi, O, Norio S, ToyohikoN. Purification and properties of an extracellular inulinase from *Rhizopus* sp. strain TN-96. Journal of Bioscience and bioengineering, 94(1):78-80. (2002).
27. Sheng J, Chi ZM, Gong F, Li J. Purification and characterization of extracellular inulinase from a marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the purified inulinase. Applied Biochemand Biotechnology, 144:111–121. (2008).

28. WenlingW, ZhonghuiZ, Yueying I,Xinsheng Z. Optimizing the Culture Conditions for Higher Inulinase Production by *Kluyveromyces sp*. Y-85 and Scaling-Up Fermentation. 86: 395-399. (1998).
29. Kushi R., Mont R, Contiero J. Production, purification and characterization of an extracellular inulinase came from *Kluyveromyces marxianus* var. *bulgaricus*. Indian journal of Microbiology Biotechnology, 25: 63–69. (2000).
30. Kochhar A, Kaur N, Gupta AK. Inulinase from *Aspergillus versicolor* a potent enzyme for producing fructose from inulin, Journal of scientific and industrial research. 56:721–726. (1997).
31. Gong F, Chi ZM, Sheng J, Li J, Wang XH. Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and Inulin hydrolysis by the purified inulinase. Biotech and Bioprocess Engineering; 13: 533–539. (2008).
32. Chen HQ, Chen XM, Li Y, Wang J, Jin ZY, Xu, XM, Zhao JW, Chen TX, Xie ZJ. Purification and characterisation of exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06. Food Chem; 115, 1206 – 1212. (2009).
33. Ohta K, Norio N, Nakamura T. Purification and properties of an extracellular inulinases from *Rhizopus* sp. strain TN−96. J Biosci Bioeng; 94:78–80. (2002).
34. Rosemeire AB, Marcia R Braga, Rita de Cassia L Figueiredo Ribeiro. Purification and properties of exo-inulinase from *Penicillium janczewskii* growing on distinct carbon sources, Mycologia, 99(4): 493 – 503. (2007).
35. Neera KV Ramana, Gopalan N, Sharma RK. Production of inulinase by *Fusarium* sp., and its application for fructo oligosaccharide production for use as prebiotics. Defence life science Journal, 3(1): 45 – 50. (2018).