Progress Report

Optimization and Scale-up of Process Parameters for High Fructose Syrup Production Using *A. niger* OP-3 and *Penicillium* sp. NFCCI 2768 Inulinase

(PA-23/656 dt. 22.11.2012)

Sponsored by

MADHYA PRADESH BIOTECHNOLOGY COUNCIL
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Submitted By

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Section A: Project Details

A1.	**Project Title:**	Optimization and scale up of process parameters for high fructose syrup production using *A. niger* OP-3 and *Penicillium* sp. NFCCI 2768 inulinase
A2.	**Name of Principal Investigator**	Prof. Naveen Kango
A3.	**University**	Dr. Harisingh Gour Vishwavidyalaya (*A Central University*)
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A5.	**MPBTC Sanction letter no. and date:**	PA-23/656 Dated 22.11.2012
A6.	**Total cost (Non recurring + Recurring)**	Rs. 6, 58, 000/-
A7.	**Duration of the project**	3 Years

A8. Approved objectives of the project

Year	Objectives
I Year	Optimization of inulinase production by select fungal strains using response surface methodology (RSM). Scale-up of inulinase production under optimized conditions in 5-litre laboratory fermenter.
II Year	Partial purification of inulinase using precipitation and ion exchange chromatography. Extraction of inulin from locally available plants. Application of crude and partially purified inulinase for hydrolysis of commonly available inulin (Asparagus, Chicory, Dandelion and Dahlia) and crude inulin extracts.
III Year	Immobilization of inulinase using alginate beads, glutaraldehyde and chitosan etc. Optimization of process parameters (pH, temperature, dosage, substrate concentration) for inulin hydrolysis using free and immobilized inulinase. Evaluation of efficacy of inulinase preparation in terms of product yield.

A9. Specific recommendations made by the PAC (if any) NA

Section- B: Scientific and Technical progress

B1. Brief introduction

Inulin is a linear β-(2→1)-linked fructose polymer that occurs as a reserve carbohydrate in many members of liliaceae and compositae including garlic, asparagus root, dandelion, Jerusalem artichoke, dahlia tubers and chicory root. Inulinases are fructofuranosyl hydrolases produced by a wide array of organisms including plants, bacteria, molds and yeasts. The general reaction mainly involves action of two enzymes: (i) Exoinulinase (EC 3.8.1.80) which splits of the terminal fructose units from inulin and (ii) Endoinulinase (EC 3.2.1.7) that breaks down inulin into inulooligosaccharides (IOS). The yield in such process can be up to 75–85% fructose solution. The high-fructose syrup obtained from enzymatic hydrolysis of inulin can be used for production of ethanol. Inulinase obtained from microbial sources have a promising application in obtaining high-fructose syrup from plant-inulin. Dandelion (*Taraxacum officinale*) is a perennial herb of the family Asteraceae and native to temperate areas with large amount of inulin (12-15%) and oligofructans in its tap roots and leaves that have various ethno-pharmacological relevancies and regarded as anti-tumor, anti-diabetic, anti-rheumatic, anti-inflammatory and anti-cardiogenic disorder. Dandelion, a common flowering weed and a rich source of fructan can be exploited for generation of fructose using microbial inulinase (Kango, 2008; Chi *et al.*, 2011; Qian *et al.*, 2014; Weber and Wigg, 2014; Liu *et al.*,2014; Rawat *et al.*,2015a; Martinez *et al.*, 2015).

Nowadays, fructose is becoming an important food ingredient in pharmaceutical, soft drink and energy industries for the production of fruit beverages, ice creams, dairy products, baby food, carbonated soft drinks and bio-ethanol.The use of fructose in place of sucrose is helpful

in avoiding problems associated with corpulence, cariogenicity, atherosclerosis and diabetes (Chi *et al.*, 2011; Kango and Jain, 2011).

Enzyme immobilization has received greater attention because it renders the enzyme reusable, provides greater stability and catalytic control, prevents product contamination, allows continuous product formation and thus has great potential for industrial applications. Reusability of the immobilized enzymes also increases cost-effectiveness and helps in actual realization of enzyme application at industrial level. Enzyme entrapment using calcium alginate is one such technique that is widely used for generation of industrial important products (Gupta *et al.*, 1992; Catana *et al.*, 2005; Richetti *et al.*, 2011; Silva *et al.*, 2013; Ganaie *et al.*, 2014a).

Response surface methodology (RSM) provides information about optimum levels of each variables, interaction between them and their effects to generate product with higher-rate (Dilipkumar *et al.*, 2013; Mutanda *et al.*, 2008; Rao and Satyanarayana, 2003). A packed-bed reactor (PBR) can continuously generate products of enzyme action under controlled environment (Nakamura *et al.*, 1995; Jung *et al.*, 2011; Ganaie *et al.*, 2014a; Detofol *et al.*, 2015). The present study describes production and characterization of exoinulinase from *A. niger* NFCCI 3879 and use of inulin rich extract obtained from dandelion root as an alternative material for continuous generation of fructose syrup using PBR.

B2. Review of literature

Microbial inulinases are an important class of industrial enzymes that hydrolyze β-fructosyl linkages. Inulinases are fructofuranosyl hydrolases produced by a wide array of organisms including plants, bacteria, molds, and yeasts. exoinulinase splits the terminal fructose units from inulin and endoinulinase that breaks down inulin into inulooligosaccharides (IOS). The former can be used for production of high fructose syrup from natural inulins

(saccharification), and the latter can be used for producing inulooligosaccharides of varying lengths. A number of yeast, filamentous fungi and bacteria are reported to produce inulinases (Kango and Jain 2011). Inulinases are receiving attention as they can be used for the production of high-fructose syrup from abundant and renewable inulin. Conventional fructose production from starch needs at least three enzymatic steps involving α-amylase, glucoamylase and glucose isomerase and yields only 45% fructose. In contrast, enzymatic hydrolysis of inulin is a single step process yielding 90-95% (w/w) fructose (Vandamme and Derycke 1983). Apart from being a low calorie sweetener, fructose has other important applications such as in the production of ethanol, acetone and butanol, gluconic acid, sorbitol and fructo-oligosaccharides (Singh and Gill 2006). Inulooligosaccharides (IOS) produced by action of endoinulinase are reported to have similar physiological functions to fructooligosaccharides (FOS). These are regarded as prebiotics which positively influence the composition of the gut microflora (Roberfroid 1993; Kaur and Gupta 2002).

Production of inulinase is affected by medium components and type of the organism used for fermentation. To compensate the high cost of inulin, various inulin rich plant materials like Jerusalem artichoke, dahlia, chicory; kuth roots, dandelion extract etc. have also been used for the production of inulinases (Vandamme and Derycke 1983; Kango and Jain 2011; Jain 2011). Dahlia tubers, asparagus roots, garlic bulbs and onion bulbs are rich source of inulin containing 15-20%, 10-15%, 9-16% and 2-6% of inulin (% fresh weight) (Singh and Singh 2010; van Loo *et al.* 1995). *Kluyveromyces marxianus* and *Aspergillus niger* are the most commonly used microorganisms for inulinase production (Kango 2008). Inulinase production by *Penicillium* has been a subject of extensive survey and several such strains have been reported. These include *Penicillium* sp. strain1 (Nakamura and Nakatsu 1977), *Penicillium purpurogenum* (Onodera and Shiomi 1988), *Penicillium trzebinskii* (Onodera and Shiomi 1992), *Penicillium*

palitans, *Penicillium cyclopium* (Balayan *et al.* 1996) and *Penicillium* strain TN-88 (Nakamura *et al.* 1997). Other strains of this genus reported for inulinase production include *Penicillium janczewskii* (Pessoni *et al.* 2007) and *Penicillium subrubescens* (Mansouri *et al.* 2013). In the present study production and properties of inulinase by newly isolated *Penicillium* sp. NFCC 2768 on media containing inulin rich plant extracts is studied.

Statistical experimental design techniques are very useful tools for the selection of nutrient, as they can provide statistical models which help in understanding the interactions among the process parameters at varying levels and in calculating the optimal level of each parameter for a given target maximal enzyme production (Reddy *et al.,* 2003). The application of statistical experimental design techniques in fermentation process development can result in improved product yields, reduced process cost, confirmation of the output response and reduced development time (Elibol 2004). RSM is used to enhance enzyme production by optimizing the culture media has been reported. On the other hand, studies regarding to optimization of culture medium in SSF for the production of inulinase are still required scientific consideration. Optimization of bioprocess by statistical experimental designs is very useful as it helps in understanding the interactions among the process parameters at varying levels and in calculating an optimal level of each parameter for the maximal product yield (Reddy *et al.*, 2003; Trivedi *et al.*, 2012).

The optimization of a process or formulation of a medium by classical methods involving the change of one-variable-at-a-time is extremely time consuming and expensive, when a large number of variables are considered. This method does not. Statistical experimental design techniques are very useful tools for the selection of nutrients. They can provide statistical models which help in understanding the interactions among the nutrients at various levels and in calculating the optimal level of each nutrient for a given target (Selvakurmar and Pandey,

1999; Reddy *et al.*, 2003). The application of statistical experimental design techniques in a fermentation process development can improve product yield, reduce process variability and process time, and can make the process more cost effective, etc. (Elibol, 2004). Response surface methodology (RSM) is a model consisting of mathematical and statistical techniques, mostly used to study the effect of several parameters and to know the optimum conditions for a multivariable system. Furthermore, central composite design (CCD) is widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments.

Optimization of fermentation conditions for inulinase production has significantly affected on the productivity of the enzyme and thus production cost (Hounsa *et al.*, 1996; Francis *et al.* 2003; Xiong, Jinhua, and Dongsheng, 2007; Mazutti *et al.*, 2007). The present study was focused on optimization of process parameters for inulinase production on low cost substrate (asparagus, dahlia, dandelion etc.) under submerged fermentation (SmF) and solid state fermentation (SSF) using a newly isolated fungi *Aspergillus niger* OP-3 and *Penicillium sp.* NFCCI 2768. Properties of crude inulinase were also studied to predict the end products using TLC and HPLC analysis. In the present work optimization of medium composition for exoinulinase production by *Aspergillus niger* OP-3 *and Penicillium sp.* NFCCI 2768 at shake-flask level using a Plackett-Burman design was carried out.

B3. Progress made against the approved objectives, targets and timeline during the reporting period

Objectives of I Year

Optimization of inulinase production by select fungal strains using response surface methodology (RSM). Scale-up of inulinase production under optimized conditions in 5-litre laboratory fermenter.

(1) Optimization of inulinase production by selected fungal strains using Plackett-Burman

Test organisms: *Penicillium* sp. NFCCI 2768 and *Aspergillus niger* OP-3

(i) Cultivation and maintenance of fungi:

Fungal culture ware grown on Czapek's Dox Agar plates ($NaNO_3$: 2.0g; K_2HPO_4: 1g; KCl: 0.5g; $MgSO_4.7H_2O$: 0.5g; $FeSO_4$: 0.1g; Sucrose: 30g; Agar: 20g; Distilled water: 1000 ml) and incubated at 28°C. Potato dextose agar slant was used for maintaing fungal culture.

(ii) Preparation of inulin rich plant extracts

200g of the tubercles/ root/ leaf were washed in running water and crushed in a blender with 1000 ml of distilled water. The slurry obtained was allowed to stand for sedimentation of particulate matter. Afterwards, it was filtered through muslin cloth and the filtrate was used in media formulation.

(ii) Extracellular inulinase production at shake-flask level using various C-sources

Media containing different inducers (2% w/v asparagus/ dandelion/ dahlia/ chicory inulin/ sucrose/ onion extract/ polysaccharides and monosaccharides etc.) were prepared in 50 ml of sterile distilled water in 250 ml Erlenmeyer flasks and yeast extract (2% w/v) was used as nitrogen source. These were inoculated with two mycelial discs of seven days old fungal culture. Shake-flask cultivations were carried out at 28°C under shaking (150 rpm) for 72 hours. The contents were used then flittered by using Whatman filter paper No 1.

(iv) Enzyme Assay

Inulinase/ Invertase activity:

For evaluation of inulinase activity, 0.2 ml of appropriately diluted enzyme (culture filtrate) was added to 1.8 ml of inulin (1% w/v dissolved in 200 mM sodium acetate buffer, pH 5.0) and incubated at 50°C for 15 min. After incubation, total reducing sugars liberated from inulin were measured by adding 3 ml DNS reagent and boiling for 5 min (Kango, 2008). Samples were allowed to cool and their absorbance was read at 540 nm. Invertase activity was measured using sucrose solution (1% w/v) in place of inulin. One nanokatals (nkat) of inulinase/invertase activity was defined as the amount of enzyme which produced 1nano mole of fructose/glucose per second under the assay conditions as described above.

(V) Optimization of culture conditions for Inulinase production

Best carbon source supporting maximum titres of inulinase by *Penicillium* sp. 2768 and *A. niger* OP-3 were then selected for optimization studies. Accordingly effect of varying best carbon source and yeast extract, temperature, rpm, pH (total five parameters) were studied using Placket-Burman design (**Table 1**).

(VI) Chromatographic analysis of inulin hydrolysate

End products of inulinase action were visualized using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). 200 µl of undiluted enzyme (culture filtrate) was added to 200 µl of inulin (5% w/v in 200 mM sodium acetate buffer, pH 5.0) and incubated at 50°C in a water bath. Aliquots of 3 µl were withdrawn at different time intervals and spotted on TLC plate (Merck, UV_{254}). Plates were developed with the solvent system containing isopropyl alcohol: ethyl acetate: water (2:2:1 by volume). Sugar spots were developed with reagent containing 0.5% α-naphthol and 5% sulfuric acid in absolute

ethanol and by heating the plate at 100°C for 10 min (Kango, 2008). Fructose (F), kestose (GF_2) and nystose (GF_3) were used as sugar standards. Inulin hydrolysis was also studied by high performance liquid chromatography (HPLC), (Waters) using the Sugar Pak I column and the Waters 410 RI detector. Distilled water was used as the mobile phase.

Results

Table 1: Effect of mono, di, polysaccharide and crude inulin rich carbon sources on inulinase and invertase production

S. No.	C- Source Used	*Penicillium* sp. 2768		*A. niger* (OP-3)	
		Inulinase	Invertase	Inulinase	Invertase
1	Fructose	5.89	16.8	**100.0**	80.3
2	Dextrose	48.3	25.8	40.1	55.9
3	Mannitol	3.80	8.66	17.9	59.2
4	Sucrose	5.19	43.8	28.9	84.1
5	Maltose	25.9	Nil	94.9	94.3
6	Raffinose	7.98	32.7	1.14	61.0
7	Starch	9.96	11.4	58.12	250.3
8	C M Cellulose (CMC)	1.98	6.35	25.08	65.34
9	Pure Chicory Inulin	11.4	56.8	**243.2**	530.3
10	Solka flock	6.59	5.18	29.37	174.3
11	Dandelion Leaf Extract	19.6	5.82	19.19	158.3
12	Dahlia Tuber Extract	52.9	35.5	105.5	463.3
13	DR Extract	9.70	50.5	30.56	297.7
14	Asparagus root (AR) powder	13.4	31.2	**162.8**	878.1
15	Onion Extract	6.53	22.5	37.95	284.3
16	Sugarcane Bagasse	1.83	34.8	18.06	328.2
17	Wheat Bran (WB)	9.38	8.16	22.89	359.5

Table 2: Optimization using a Plackett–Burman design* (Variable Levels)

Nutrient and physical factor	Variables	
	Low (−1)	High (+1)
Dahlia tuber powder (DTP) or Asparagus root powder	2.0 g/50ml	5.0 g/ 50 ml
Yeast extract	1.0 g/50ml	5.0 g/50ml
Temperature	28°C	30°C
RPM	120	160
pH	5.0	6.0

Table 3: Plackett-Burman (PB) design for optimization of various nutrients and physical factors for inulinase production by *Penicillium* sp. NFCCI 2768

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Inulinase activity (nkat/ml)
	DTP (g/50ml)	Yeast extract (g/50ml)	Temp (°C)	pH	RPM	dummy	
1	5	3	25	6.0	130	-1	27.69
2	2	1.5	25	6.0	160	1	31.47
3	2	3	25	5.0	130	1	38.33
4	5	1.5	28	6.0	130	1	54.89
5	2	3	28	5.0	160	-1	39.7
6	2	1.5	25	5.0	130	-1	20.19
12	**5**	**3**	**28**	**5.0**	**160**	**1**	**64.3**
8	5	3	25	6.0	160	-1	51.28
9	2	1.5	28	6.0	160	-1	29.78
10	5	1.5	25	5.0	160	1	33.4
11	2	3	28	6.0	130	1	13.77
7	5	1.5	28	5.0	130	-1	41.25

DTP-Dahlia tuber powder (Values are mean of three experiments) *Design Expert 6.0, State-ease Inc., USA

Table 4: Plackett-Burman (PB) design for optimization of various nutrients and physical factors for inulinase production by *Aspergillus niger* (OP-3)

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Inulinase activity (nkat/ml)
	ARP (g/50ml)	Yeast extract (g/50ml)	Temp (°C)	pH	RPM	dummy	
1	**5.00**	**1.00**	**30.00**	**6.00**	**120.0**	**1**	**180**
2	2.00	1.00	27.00	5.00	120.0	-1	127
3	5.00	1.00	30.00	5.00	120.0	-1	98
4	2.00	1.00	30.00	6.00	150.0	-1	147
5	5.00	3.00	27.00	6.00	120.0	-1	91
6	5.00	3.00	27.00	6.00	150.0	-1	59
7	2.00	3.00	30.00	5.00	150.0	-1	47
8	2.00	3.00	27.00	5.00	120.0	1	98
9	5.00	1.00	27.00	5.00	150.0	1	104
10	2.00	1.00	27.00	6.00	150.0	1	111
11	2.00	3.00	30.00	6.00	120.0	1	84
12	5.00	3.00	30.00	5.00	150.0	1	116

ARP-Asparagus root powder (Values are mean of three experiments)

Table 5: Inulinase activity in the selected isolates after optimization

S. N.	Fungus	Unoptimized	After Optimization
1.	***Penicillium sp.*** **NFCCI 2768**	**52.9**	**64.3**
2.	***Aspergillus niger*** **OP-3**	**162.8**	**180**

Inulinase activity (nkat/ml)

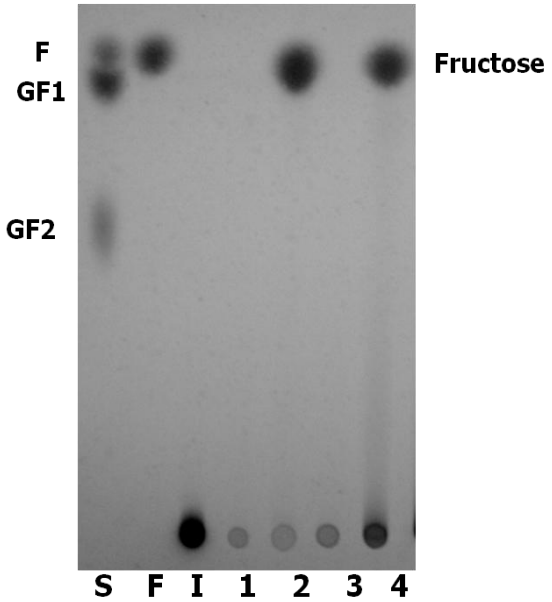
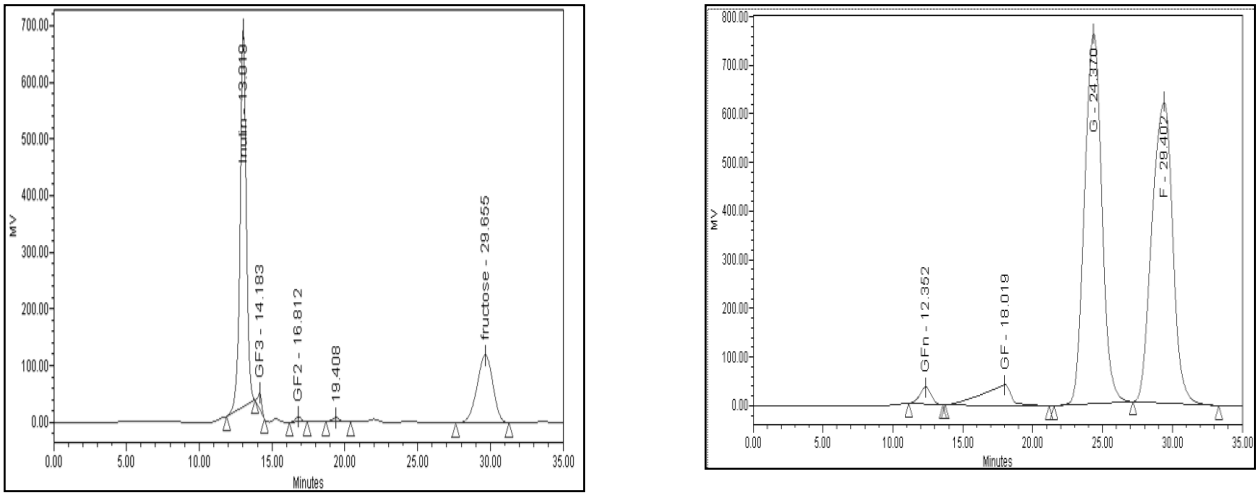


Figure 1: S-Standards (F-fructose, GF1-sucrose and GF2-raffinose); F-fructose; I- chicory inulin L1, L2 *Penicillium* sp. NFCCI 2768 and L3, L4 *A. niger* OP-3
Culture filtrate was incubated with pure chicory inulin (2% w/v, pH 5.0) at 50°C for 1 hr.



A. Inulin hydrolysis ***B. Sucrose hydrolysis***

Figure 2: Liberation of fructose from inulin and sucrose by *Penicillium sp.* NFCCI 2768 inulinase A. Inulin Chicory 10 % (24 h) B. Sucrose 10 % (24 h)

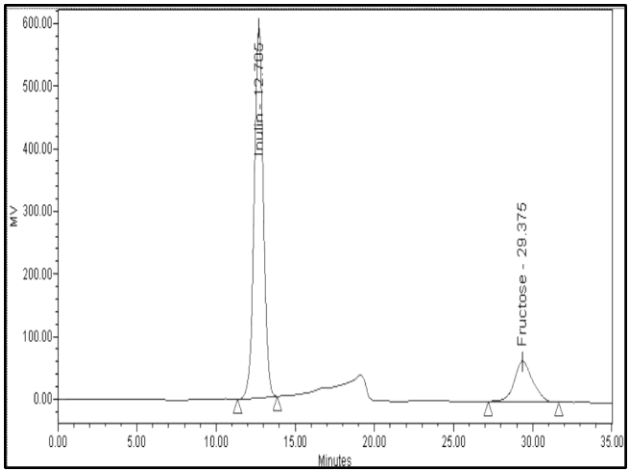


Figure 3: Liberation of fructose from *Penicillium* sp. 2768 inulinase using dahlia tuber extract as substrate after 24 h

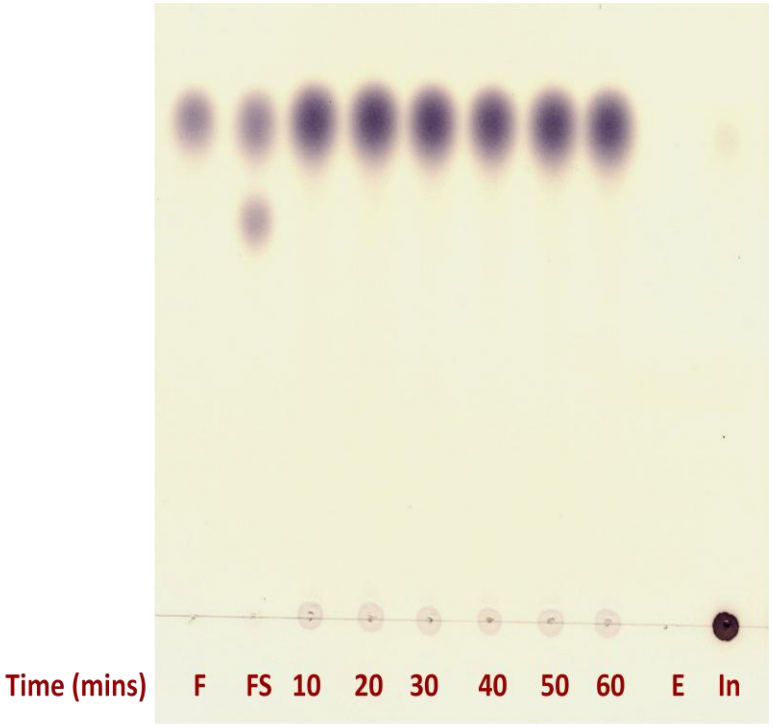
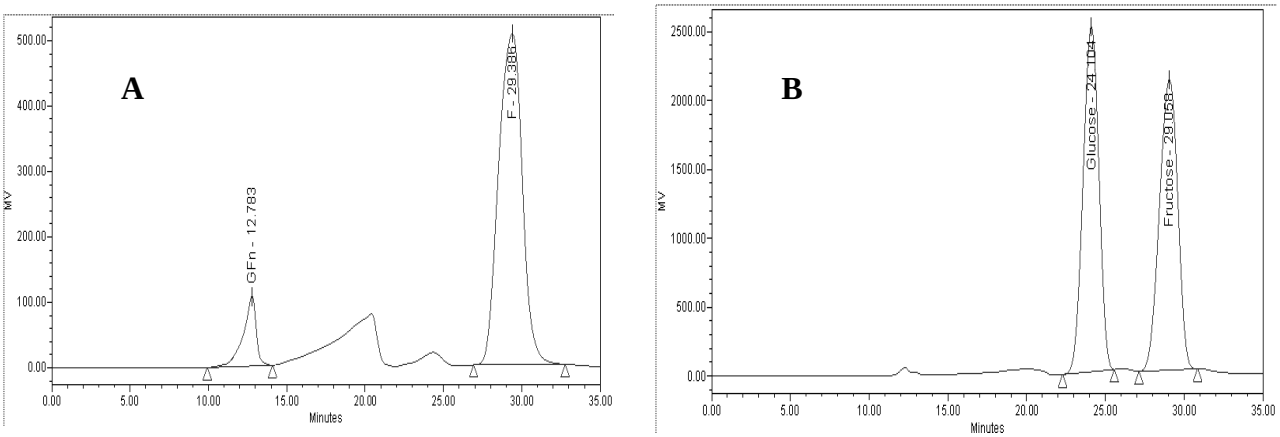


Figure 4: Time dependent hydrolysis of chicory inulin (2% w/v, pH 5.0) by *A. niger* OP 3 inulinase at 50°C up to 60 minutes



A. Inulin hydrolysis ***B. Sucrose hydrolysis***

Figure 5: Liberation of fructose and other sugars from inulin by *A. niger* OP-3 A. Sucrose 25 % (24 h) B. Inulin Chicory 10 % (24 hours)

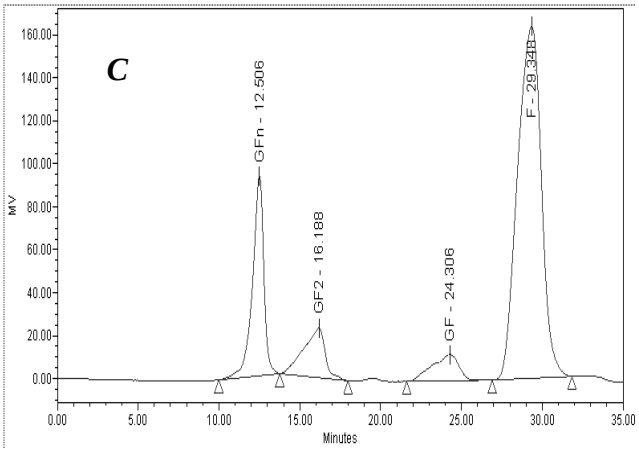


Figure 6: Liberation of fructose and other sugars by *A. niger* OP-3 inulinase from dandelion root extract (24 hours)

Scale-up of inulinase production under optimized conditions in 5-litre laboratory fermentor

Enzyme production was carried out in 5-L laboratory scale fermentor (Bio-Spin, Bioage). Fixed volume batch experiment was performed by adding asparagus root powder yeast extract solution as substrate as described below.

Table 6: Medium used for fermentor study

S. No.	Fermentation condition	per litre
1	**Asparagus root Powder (10% w/v)**	**100g**
2	**Yeast extract**	**20g**
3	**Temperature**	**30°C**
4	**Time**	**96h**
5	**RPM**	**120**

Organism used: *Aspergillus niger* (OP 3)

Submerged Fermentation **5 L-Fermenter**

Figure 7: Showing the scale-up from shake flask level to fermenter level

Table 7: Inulinase yield obtained in 5 L fermenter study

S. N.	Fungus	Yield (Units / litre)
1.	*Aspergillus niger* OP-3	10797

Solid State Fermentation (SSF)

Inulinase production by the two select strains was studied in SSF. Four different inulin rich plant materials alongwith wheat bran were examined for this purpose. Czapek's Dox Mineral Salt Solution without sucrose was used as moistening agent.

Table 8: Production of inulinase in SSF by *Penicillium* sp. NFCCI 2768

S No	Substrate used	Inulinase Activity (nkat/gds)	Invertase Activity (nkat/gds)
1	Wheat Bran	34	147
2	**Dandelion Root powder**	**205**	**367**
3	Asparagus root powder	50	300
4	Dandelion leaf powder	35	665
5	Dandelion Root powder	38	140

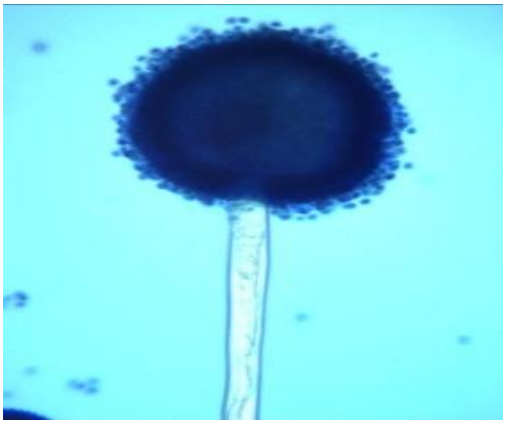
Czapek mineral salt solution without sucrose used as moistening agent

Table 9: Production of inulinase in SSF by *Aspergillus niger* OP- 3

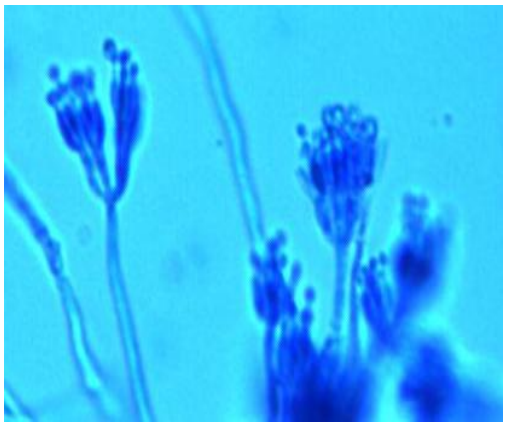
S N	Substrate used	Inulinase Activity (nkat/gds)	Invertase Activity (nkat/gds)
1	Wheat Bran	48	511
2	Dandelion Root Powder	245	2067
3	**Asparagus Root Powder**	**620**	**3150**
4	Dandelion Root Powder	193	853
5	Dandelion Leaf Powder	319	1053

Czapek's mineral salt solution without sucrose used as moistening agent

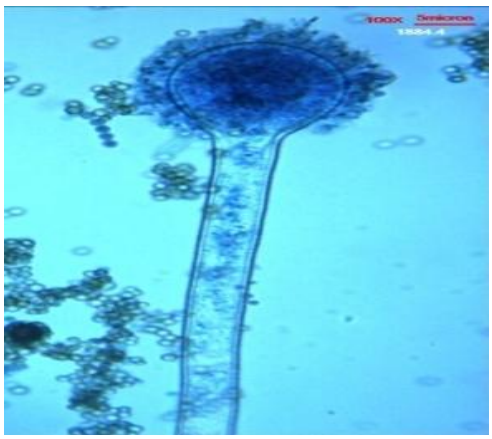
Figure 8: Photographs of Microscopic Image Projection System (MIPS)



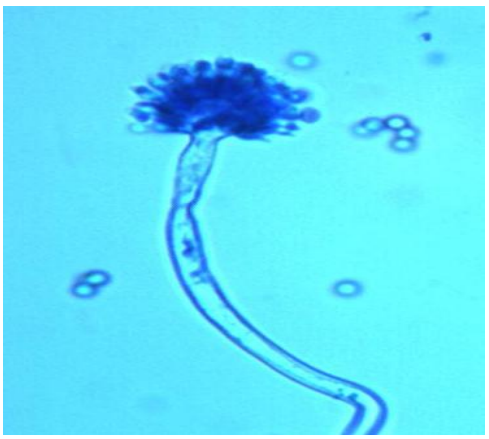
***Aspergillus niger* OP-3**



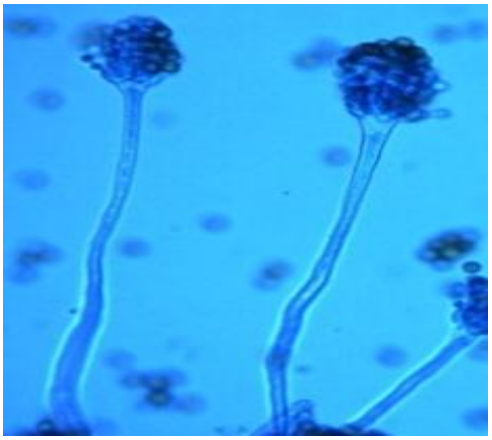
Penicillium sp. 2768



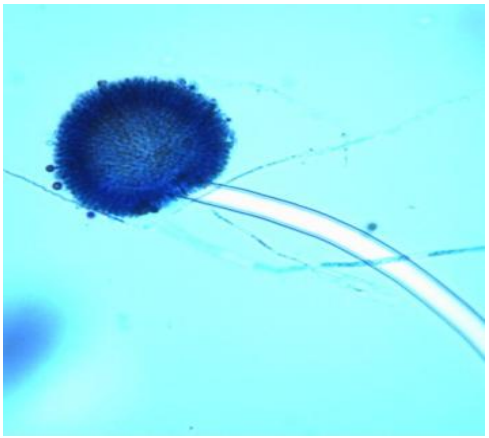
***Aspergillus flavus* 2364**



Aspergillus versicolor



***Aspergillus parasiticus* MTCC 2796**



***Aspergillus ficuum* MTCC 7591**

Figure 9: Photographs of different fungal species by Microscopic Image Projection System

Conclusions

- The inulinulolytic systems studied in *Penicillium* sp. NFCCI 2768 and *Aspergillus niger* OP-3 were found to produce varied enzyme titres on different carbon sources (**Table 1**).
- Plackett-Burman design experiments showed wide variations in inulinase activity with changes in variables. These variations reflected the importance of optimization for obtaining higer production yield (**Table 2,3,4**).
- Optimization studies resulted in 11% and 21% increase in inulinase yield in case of *Penicillium* sp. NFCCI 2768 and *Aspergillus niger* (OP-3), respectively. Further studies on optimization using Central composite design are underway.
- Analysis of end products by TLC and HPLC confirmed that fructose is generated exclusively using *Aspergillus niger* OP-3 and *Penicillium* sp. NFCCI 2768 inulinase. The inulinase can be used for generating fructose from crude inulin extracts (**Figure 1-6**).
- The optimum values for different variables were used inulinase production in 5 L-laboratory fermenter by *A. niger* OP-3. The fungus produced 10797 nkat/l of inulinase (**Table 6,7 and Figure 7**).
- These strains also produced inulinase in solid state cultivation on various crude vegetal substrates (**Table 8,9**).

Objectives of II Year

- Partial purification of inulinase using precipitation and ion exchange chromatography.
- Extraction of inulin from locally available plants.
- Application of crude and partially purified inulinase for hydrolysis of commonly available inulin (Asparagus, Chicory, Dandelion and Dahlia) and crude inulin extracts.

Methods

Materials

Inulin rich substrates used in this study were collected from local sources. Dahlia tubers, garlic bulbs, onion bulbs and asparagus root powder (locally known as *safed musli* or *shatavari*) were obtained from local market. Inulin (Chicory; MW-(162.14)n, hygroscopic in nature, white powder, Cat. No. I2255-25G Sigma), fructose, 3,5-dinitrosalicycilc acid (DNS), kestose, nystose and corn steep liquor (CSL) were obtained from Sigma Chemical Co., U.S.A., Silica gel plates UV_{254} were obtained from Merck, Germany.

Taxonomic studies

The fungal strain was isolated from dahlia rhizosphere and was grown on Czapek's agar at 28°C. It was identified on the basis of its morphological features using taxonomic description (Pitt 1973; Raper & Thom 1949). The identity of the culture was confirmed at National fungal culture collection (NFCC), Agharkar research institute, Pune, India as *Penicillium* sp. NFCC 2768.

Enzyme production

Penicillium sp. NFCC 2768 was cultivated on media containing inulin rich extracts in shake cultures and the culture filtrates were assayed for inulinase activity. Asparagus root medium contained 2% (w/v) dried root powder while production medium with pure inulin contained 2% (w/v) chicory inulin. Media containing dahlia, garlic and onion were prepared by crushing 200g of

tubers or bulbs in 1000 ml of distilled water (20% fresh w/v). The slurry was filtered through a muslin cloth and used in media preparation. Yeast extract 2% (w/v) was used as nitrogen source in all the media formulations. Erlenmeyer flasks (150 ml) containing 50 ml aliquots of medium were autoclaved (20 min, 121°C) and inoculated with two mycelial discs (7mm) cut from 7 days old culture of *Penicillium* sp. NFCC 2768. Flasks were incubated at 30°C on a rotary shaker (150 rpm) and were withdrawn at regular intervals of 24 h. The content of the flasks was filtered through Whatman filter paper No.1 and clear filtrate was used for enzyme assays. The biomass (dry weight in grams/50 ml) and final pH were also recorded. All the experiments were carried out in triplicate and mean values ± standard deviation (SD) are reported.

Enzyme assays

For evaluation of inulinase activity, 0.2 ml of appropriately diluted enzyme (culture filtrate) was added to 1.8 ml of inulin (1% w/v dissolved in 200 mM sodium acetate buffer, pH 5.0) and incubated at 50°C for 15 min. After incubation, total reducing sugars liberated from inulin were measured by adding 3 ml DNS reagent and boiling for 5 min (Kango 2008). Samples were allowed to cool and their absorbance was read at 540 nm. Invertase activity was measured using sucrose solution (1% w/v) in place of inulin. One nanokatals (nkat) of inulinase/invertase activity was defined as the amount of enzyme which produced 1nano mole of fructose/glucose per second under the assay conditions as described above.

Enzyme purification

Precipitation of total proteins was done by adding two volumes (2:1 v/v) of chilled ethanol to the culture filtrate obtained from garlic bulb medium under constant stirring for 30 min at 4°C. The precipitate was obtained by centrifuging the mix at 8000 g for 10 minutes at 4°C. Protein

precipitate thus obtained was suspended in sodium acetate buffer (200 mM, pH 5.0) and was used finding pH optima, temperature optima and other characteristics of the inulinase. Inulinase was apparently purified by total precipitation using ethanol followed by fractionation using DEAE-Cellulose-52 (Hi-media, Mumbai) ion exchange chromatographic column of size 2.5×50 cm (internal diameter×height). Five gram DEAE-cellulose-52 with the charge density of 0.80-1.10 meq/g dry weight was added to 300 mL of 0.2M NaOH with slow stirred for overnight. Discard the NaOH solution and washed the slurry with double distilled water until pH achieved 5.5 to 6.5. Then the 0.2M HCL was used to reach the pH 2.0 and mild stirred for 12 hour. After that slurry was washed with double distilled water and poured the slurry in column. Purification of inulinase from 100 ml crude enzyme was carried out at 4°C. The culture filtrate was precipitated using ethanol precipitation. 1 mL of concentrated enzyme sample was loaded on to pre-equilibrated column with the sodium acetate buffer (pH 5.0; 0.2 M). The proteins were eluted with a linear gradient of 1 M NaCl at a flow rate of 0.2 mL/min. Optical densities of eluted column fractions were measured at 280 nm and these were examined for protein concentration (Lowry 1951) and inulinase activity (Kango 2008). Fractions showing activity were pooled and excess amount of water was removed by lyophilization.

Gel electrophoresis:

SDS-PAGE was performed at a concentration of 10 % (w/v) polyacrylamide according to the method of Laemmli (1970). Bands were visualized using Coomassie brilliant blue R-250 (0.25% w/v). Standard protein markers used were phosphorylase b (98 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), glutathione S-trasferase (29 kDa) and lysozyme (16 kDa).

Effect of pH and temperature on inulinase activity

The effect of pH on inulinase activity was determined by incubating 0.2 ml of suitably diluted enzyme and 1.8 ml of inulin (1% w/v in different buffers; 200 mM sodium acetate buffer: pH 4.0 and 5.0; 100 mM phosphate buffer: pH 6.0, 7.0 and 8.0; 100 mM Tris-HCl buffer: pH 9.0; 200 mM Glycine-NaOH buffer: pH 10.0) for 15 min at 50°C. The effect of temperature was determined by incubating 0.2 ml of suitably diluted enzyme and 1.8 ml of inulin (1% w/v in 200 mM sodium acetate buffer: pH 5.0) for 15 min at different temperatures.

Effect of metal ions and other reagents on inulinase activity

Effect of metal ions and enzyme inhibitors such as phenylmethanesulfonyl fluoride (PMSF), p-chloromercuribenzoic acid (pCMB), ethylene diamine tetra acetic acid (EDTA) on inulinase activity was examined by treating the enzyme sample (protein precipitate) with their solutions for 1 hour at 30°C. 1 mM solutions of $MgSO_4$, $ZnSO_4$, $HgCl_2$, $BaCl_2$, $MnSO_4$, $CaCl_2$, $CuSO_4$, $FeCl_3$ and aforesaid enzyme inhibitors in 50 mM sodium acetate buffer (pH 5.0) were used. The residual activity of inulinase was then determined and compared to the control i.e. untreated enzyme sample.

Chromatographic analysis of inulin hydrolysate

End products of inulinase action were visualized using thin layer chromatography (TLC) as described earlier (Kango 2008). 200 µl of undiluted enzyme (culture filtrate) was added to 200 µl of inulin (5% w/v in 200 mM sodium acetate buffer, pH 5.0) and incubated at 50°C in a water bath. Aliquots of 3 µl were withdrawn at different time intervals and spotted on TLC plate (Merck, UV_{254}). Plates were eluted with the solvent system containing isopropyl alcohol: ethyl acetate: water (2:2:1 by volume). Sugar spots were developed with reagent containing 0.5% α-naphthol and

5% sulfuric acid in absolute ethanol and by heating the plate at 100°C for 10 min. Fructose (F), kestose (GF_2) and nystose (GF_3) were used as sugar standards. End products formed after inulin hydrolysis were also analyzed by HPLC (Waters) using the Sugar Pak I column (6.5 × 300 mm) and the Waters refractive index (RI) differential detector (RI 2414). Distilled water was used as the mobile phase. The samples were filtered by using 0.45µm Millipore syringe filters before injecting in 20µl valve. Column temperature was maintained 70°C by oven column (Dyna, Mumbai). Calculation and analysis were performed using Empower-2 software Build 2154, Waters (Ganaie *et al.* 2014).

Results and discussion

Effect of carbon-sources on inulinase production

Use of low-cost complex substrates as carbon source has been reported to ameliorate enzyme production (Kango *et al.* 2003). In the present study *Penicillium* sp. NFCC 2768 was found to utilize different carbon sources for its growth. Growth on dahlia tuber extract was profuse and a decrease in the turbidity of the medium was also noticed. Inulinase and invertase activities noticed on different carbon sources were noticed. Maximum inulinase activity was seen in dahlia extract medium (64.54 nkat/ml) followed by 45.2 nkat/ml and 41.3 nkat/ml in asparagus root powder and garlic extract medium, respectively (**Table 1**). Dahlia tuber extract can be an interesting alternative, since it is a relatively cheap and easily available substrate that can serve as a feedstock for large-scale fermentation. Recently, Jain *et al.* (2012) have used tubers of dahlia for production of inulinase by *Kluyveromyces marxianus* and have observed higher inulinase activity (25.3nkat/ml) as compared to pure inulin (17.8nkat/ml). Nakamura *et al.* (1997) reported 9.9 U/ml of inulinase production by *Penicillium* sp. TN-88 using inulin as carbon source. Trivedi *et al.* (2012) have indicated use of low-cost substrates such as wheat bran and corn steep

Table 1 Inulinase and invertase production by *Penicillium* sp. NFCC 2768 on different medium containing plant extracts. Results represent mean ± S.D. of three experiments

C-source	Inulinase (nkat/ml)	Invertase (nkat/ml)	I/S ratio
Inulin[§]	17.76 ± 1.98	33.79 ± 2.38	0.53
Asparagus	45.23 ± 3.31	75.80 ± 5.51	0.60
Dahlia	64.54 ± 6.92	110.09 ± 5.98	0.59
Garlic	41.32 ± 4.72	40.67 ± 3.12	1.02
Onion	13.65 ± 1.75	20.62 ± 2.47	0.66

[§] Pure inulin from chicory

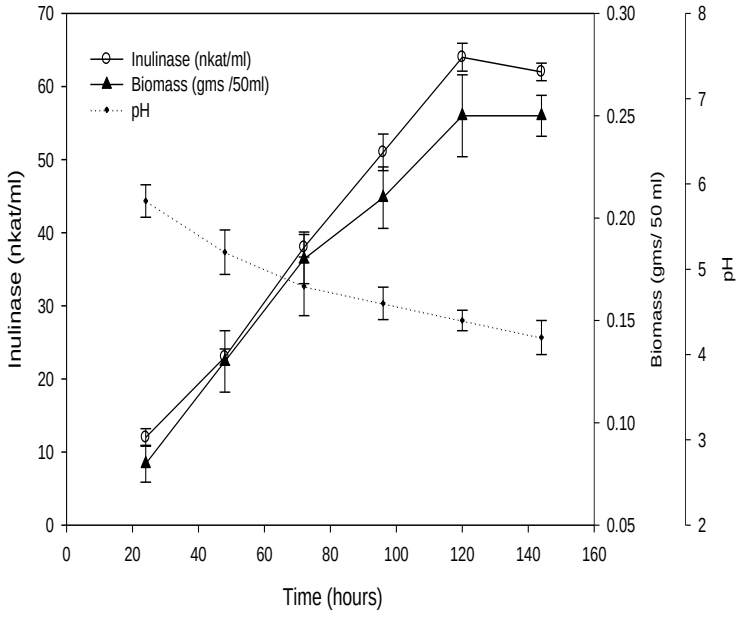


Fig. 1 Time course of inulinase production by *Penicillium* sp. NFCC 2768 grown in flasks containing 50 ml dahlia extract with yeast extract (2% w/v) at 30°C and 150 rpm. Results represent mean of three experiments.

liquor in the production of inulinase by newly isolated *Aspergillus tubingensis* CR16. Catalytic activity of inulinase is described in terms of I/S ratio which represents ratio of the activity of enzyme preparation on inulin and sucrose (Vandamme & Derycke 1983). Invertase activity was also found to vary in fashion similar to inulinase (**Table 1**). I/S ratio were found in the range of 0.53-0.66 except in case of garlic extract medium where it was 1.02. A range of I/S ratios between 0.02 and 7.9 for various microbial inulinases has been reported by workers previously (Moriyama *et al.* 2002). Contrastingly, much more higher *I/S* ratios, ranging from 4.7 to 9.5, with respect to nitrogen source, have been observed with *Penicillium* sp. TN-88 (Nakamura *et al.* 1997).

Effect of incubation period

Time course of inulinase production by *Penicillium* sp. NFCC 2768 on dahlia tuber extract medium is shown in **Table 1**. Inulinase activity was found to increase with the incubation period reaching 64.54 nkat/ml on fifth day with a pH shift from 6.2 to 5.4. A corresponding increase was noticed in the biomass of the fungus. Kango (2008) have found inulinase production by *A. niger* to reach maximum on the fourth day of growth on dandelion tap root extract medium. Nakamura *et al.* (1997) have found inulinase production by *Penicillium* sp. TN-88 to reach maximum (9.9 U/ml) after 4 days. Inulinase activity in *Penicillium janczewskii* is reported to reach maximum (3.2 U/ml) in relatively longer period of 12 days on medium containing inulin or sucrose (Pessoni *et al.* 2007). Initial pH (6.0) of the medium decreased to 4.5 after six days of incubation (**Fig. 1**). Similar decrease in pH has also been noticed in case of *A. niger* grown on crude dandelion extract (Kango 2008).

Effect of nitrogen source

Yeast extract was found to be the best nitrogen source for inulinase production (64.54 nkat/ml) followed by peptone (57.11 nkat/ml). Kango (2008) and Nakamura *et al.* (1997) have also found

Table 2 Partial purification of extracellular inulinase from *Penicillium* sp. NFCC 2768

Steps	Total Activity (nkat)[a]	Total protein (mg)	Specific activity (nkat/mg)[b]	Yield of activity (%)	*I/S* ratio[c]	Purification factor
Culture filtrate[d]	4132	238	17.3	100	1.02	1
Ethanol precipitate[e]	1199	28.1	42.6	29	1.37	2.4
DEAE cellulose-52[f]	219	2.68	81.7	5.3	1.84	4.7

[a] One nanokatals (nkat) of inulinase activity was defined as the amount of enzyme which produced 1nano mole of fructose/second at 50°C, pH 5.0.
[b] The specific activity is expressed in nkat of enzyme activity per milligram of protein in an assay condition.
[c] *I/S*, inulinase/sucrase activity.
[d] Culture filtrate was 100 ml.
[e] Protein precipitate was resuspended in 10 ml of 200 mM sodium acetate buffer.
[f] Fractions, excess amount of water was removed by lyophilization.

yeast extract to be the best nitrogen source for inulinase production. Among inorganic nitrogen sources, $NaNO_3$ was found to support 14.8 nkat/ml inulinase activities. Nakamura *et al.* (1997) have observed NH_4Cl as the best inorganic nitrogen source (5.91 IU/ml) for *Penicillium* sp. TN-88. The fungus produced higher levels of inulinase on organic nitrogen sources while much lower activity was noticed with inorganic nitrogen sources (**Fig. 2**).

Inulinase Purification

Inulinase was purified from cell free culture filtrate of *Penicillium* sp. grown on garlic bulb extract medium. The culture filtrate had 41.32 nkat/ml inulinase with I/S ratio 1.02. 29% of inulinase activity was recovered in ethanol precipitate showing 2.4 fold purification and an enhanced I/S ratio of 1.37. Finally, inulinase was purified to apparent homogeneity with 4.7 fold purification and I/S ratio increased 1.84. Enhancement in I/S ratio indicated reduction in contaminating invertase activity with increase in degree of purification (**Table 2**). Nakamura *et al.* (1997) have reported I/S ratio in the range of 11.2-2210 using purified *Penicillium* sp. TN-88 inulinase. Following results summarize two step purification of inulinase. Total protein precipitated from the culture filtrate was dissolved it in 0.2 M sodium acetate buffer (pH 5.0) and was fractionated using DEAE-cellulose-52 column (El-souod *et al.* 2014). The elution profile indicated binding and separation of inulinase. SDS-PAGE analysis of the pooled active fractions showed a single band with apparent molecular weight of 68 kDa (**Fig 3**). Similar to our findings, Fawzi (2011) reported the comparative account of two purified inulinase from *Thielavia terrestris* and *Aspergillus foetidus* using DEAE-cellulose-52 chromatography and obtained MW of protein was 72 & 78 kDa respectively. DEAE-cellulose-52 and Sepharose CL-6b columns were used for purification and characterization of *Aspergillus ficuum* JNSP5-06 exo and endo-inulinases. The obtained MW of purified inulinase was range between 31kDa to 70 kDa with optimum activity at 45°C and pH optima between 4 to 8 (Chen et al, 2009). Chen *et al.* (2012) have reported molecular

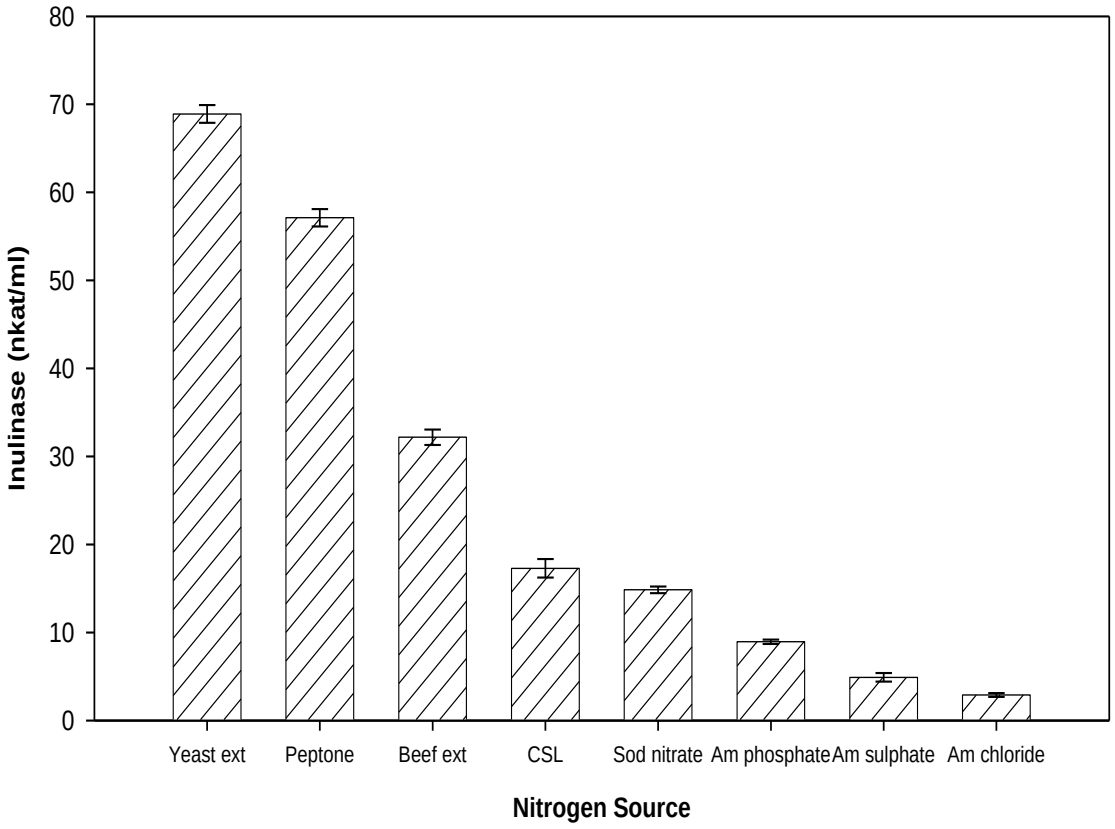


Fig. 2 Effect of nitrogen source on inulinase production by *Penicillium* sp. NFCCI 2768. Medium contained dahlia extract and nitrogen source (2% w/v). Results represent mean of three experiments ± SD.

Table 3 Effect of metal ions and other enzyme inhibitors on activity of inulinase of *Penicillium* sp. NFCC 2768.

Compound	**Relative activity (%)**
Control (Untreated)	100.00 ± 3.78
Mg^{2+}	79.29 ± 2.89
Zn^{2+}	91.25 ± 3.19
Ba^{2+}	74.58 ± 2.48
Mn^{2+}	106.58 ± 3.58
Ca^{2+}	102.25 ± 3.39
Cu^{2+}	94.25 ± 2.91
Fe^{3+}	71.58 ± 2.61
Hg^{2+}	21.59 ± 1.03
PMSF	21.25 ± 1.34
pCMB	37.15 ± 1.78
EDTA	61.25 ± 1.81

Fig. 3a

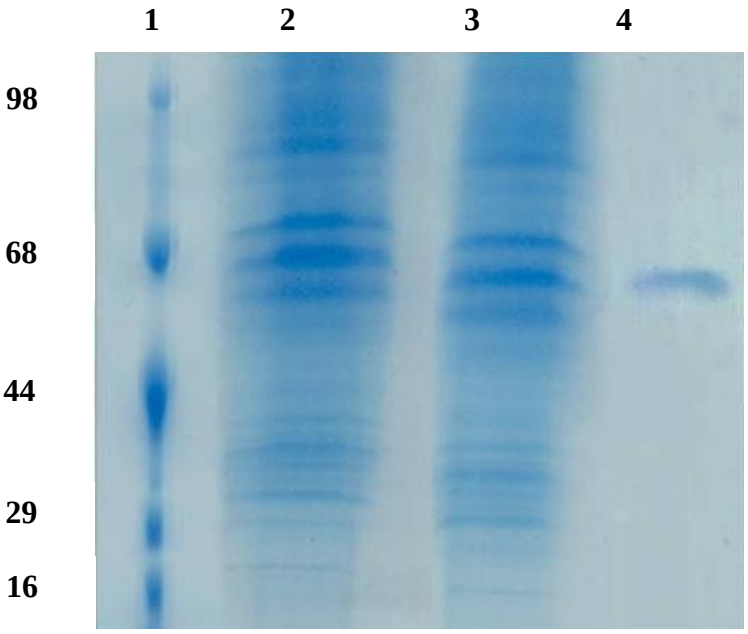


Fig. 3b

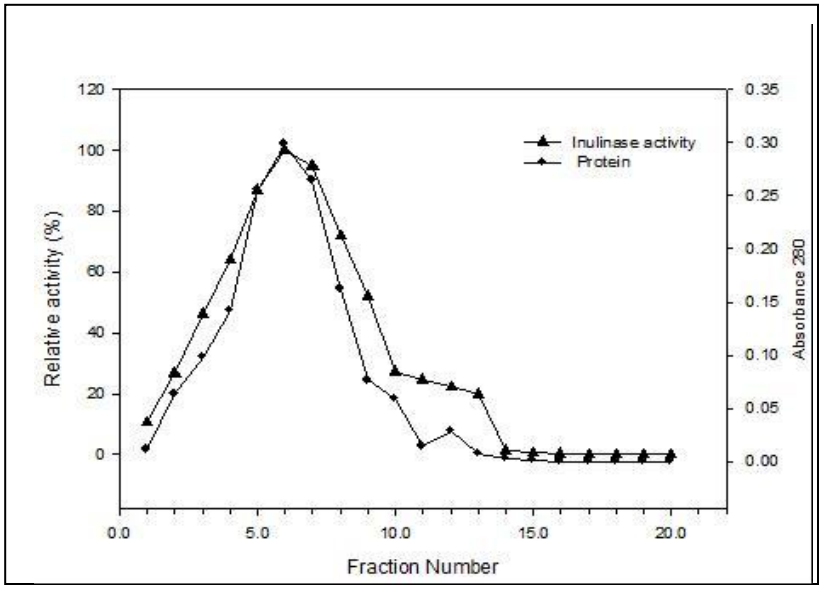


Fig. 3 3a. SDS-PAGE analysis of purified inulinase of *Penicillium* sp NFCC 2768. Lane 1: Standard marker; lane 2: crude enzyme; lane 3: ethanol precipitate; lane 4: purified inulinase.
3b. Elution profile of the protein in DEAE-Cellulose using binding buffer of pH 5.0.

mass of endoinulinase obtained from *Aspergillus ficuum* JNSP5-06 as 60.0 kDa and optimum pH and temperature of this enzyme were 5.0 and 60°C, respectively. Exoinulinase obtained from *Penicillium janthinellum* strain B01 purified and the molecular weight of purified inulinase was 100 kDa. The optimal pH and temperature of the purified exoinulinase were 4.5 and 50°C, respectively (Wang *et al.*, 2011). Goosen et al, (2008) purified exoinulinase sourced from *Aspergillus niger* N402 having molecular weight 57 kDa and the enzyme hydrolyzed inulin into fructose, sucrose and oligosaccharides.

Effect of metal ions and other reagents

Effect of various metal ions and reagents on inulinase obtained from *Penicillium* sp. NFCC 2768 is presented in **Table 2**. Activity of inulinase was increased slightly in presence of Mn^{2+} and Ca^{2+} ions, whereas Zn^{2+} and Cu^{2+} ions were marginally inhibitory. Mg^{2+}, Ba^{2+} and Fe^{3+} showed significant reduction in the activity. Hg^{2+}, which is known to affect -SH- groups, strongly inhibited inulinase activity. Singh *et al.* (2007) have also reported Mn^{2+} and Ca^{2+} to increase the enzyme activity by 2.4 and 1.2 folds, respectively, while Hg^{2+} and Ag^{+} completely inhibited the activity of *Kluyveromyces marxianus* YS-1 inulinase. In the present study EDTA partially inhibited the inulinase activity while PMSF and pCMB strongly inhibited the enzyme (**Table 3**). These results are in accordance with those noticed for inulinases of *Penicillium* sp. TN-88, *Chaetomium* sp., *A. fumigatus*, *Cryptococcus aureus* G7a, *Bifidobacterium infantis* and *Kluyveromyces* sp. Y-85 (Chi *et al.* 2009).

Effect of pH and temperature on enzyme activity

The activity of enzyme was measured at different pH ranging from 4.0-10.0. The optimum pH for inulinase activity was found to be 5.0 (**Fig. 4**). *Penicillium janczewskii* inulinase had optimum pH between 5.0-5.5 (Pessoni *et al.* 2007). The optimum pH for inulinase of *Penicillium* sp. strain TN-

88 was noticed as 5.2 (Nakamura *et al.* 1997). *Penicillium* sp. NFCC 2768 inulinase showed optimal activity at 50°C (**Fig. 5**). Temperature optimum at 50°C has also been reported for *Penicillium* sp. TN-88 (Nakamura *et al.* 1997) and many other fungal inulinase (Kango & Jain 2011).

Chromatographic analysis of the hydrolysate end products

Nature of inulinase from *Penicillium* sp. NFCC 2768 was ascertained by analyzing the end products of enzyme action using TLC. Liberation of fructose and inulo-fructosaccharides from chicory inulin was noticed. Amount of these sugars enhanced with incubation time as witnessed by the increasing intensity of the spots. Formation of 4 to 5 oligosaccharides with apparent degree of polymerization (DP) between 2 to 5 along with fructose indicated presence of inulinase activity.

Endoinulinase from *Penicillium purpurogenum* produced F_3, F_4 and F_5 oligosaccharides (Onodera & Shiomi 1988) while endoinulinase of *Penicillium* sp. TN-88 liberated only F_3 (Nakamura *et al.* 1997). In the present study *Penicillium* sp. NFCC 2768 produced a mixture of exo- and endoinulinase leading to production of both fructose and oligosaccharides from chicory inulin. The enzyme preparation obtained from *Penicillium* sp. NFCC 2768 had resemblance with that of *A. niger* NK-126 (Kango 2008) in being a mixture of exo- and endo-inulinase, however, number of oligosaccharides was more in case of former. End product profile as visualized by TLC (**Fig. 6**) was also confirmed by HPLC analysis. Result showed glucose, fructose, sucrose, kystose (GF_2), nystose (GF_3) as the prominent end products of inulinase action on chicory inulin (**Fig. 7**).

Conclusions

In the present study production of inulin hydrolyzing enzyme using low value inulin rich vegetal infusions has been demonstrated by a newly isolated *Penicillium* sp. NFCC 2768. The inulinase

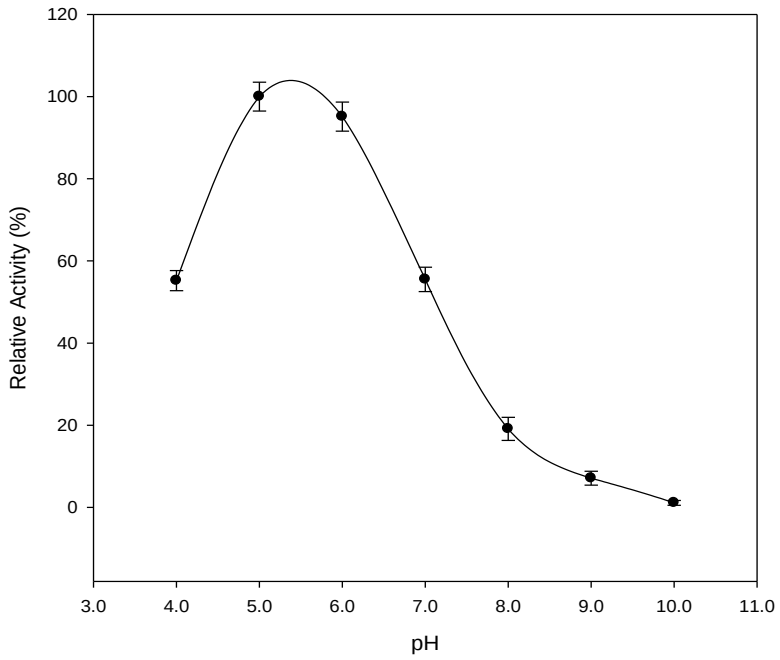


Fig. 4 Effect of pH on activity of inulinase of *Penicillium* sp. NFCC 2768.

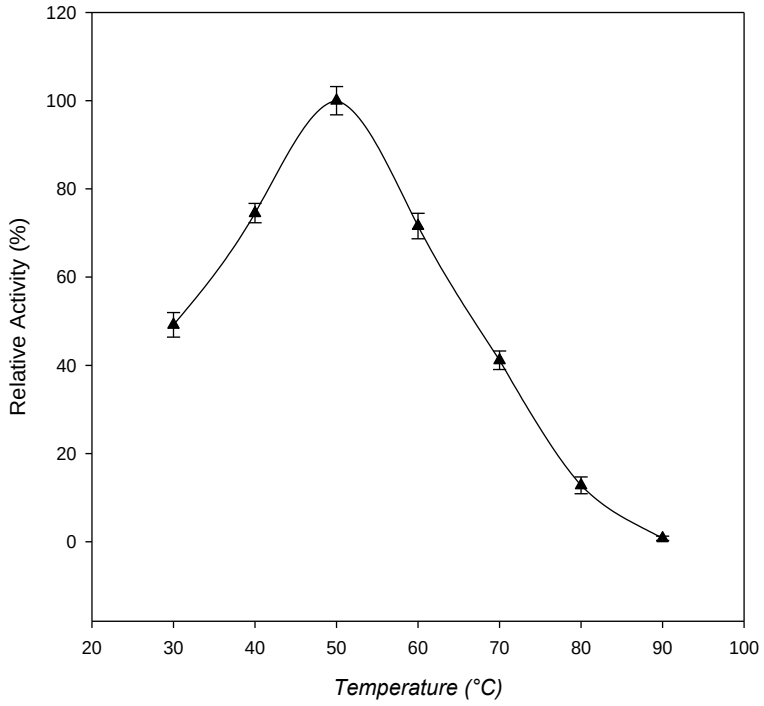


Fig. 5 Effect of temperature on activity of inulinase of *Penicillium* sp. NFCC 2768.

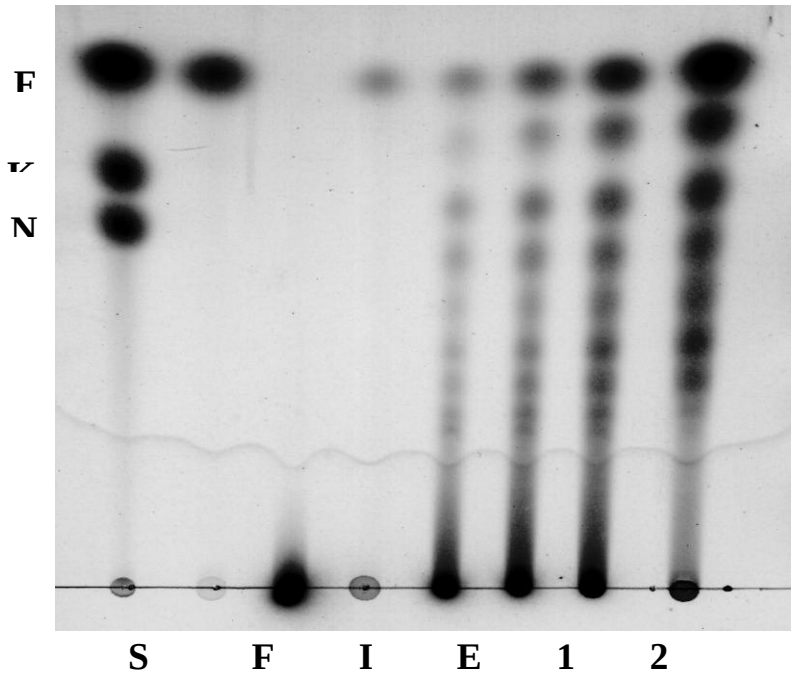


Fig. 6 End product analysis of inulinase preparation of *Penicillium* sp. NFCC 2768 by TLC. S- Standards (F-Fructose, K-Kestose and N-Nystose); F-Fructose; I- Chicory inulin (5% w/v); E-Enzyme sample of *Penicillium* sp. NFCCI 2768, Lanes 1 to 4 end products of inulin hydrolysis after 5, 15, 30 and 60 minutes, respectively.

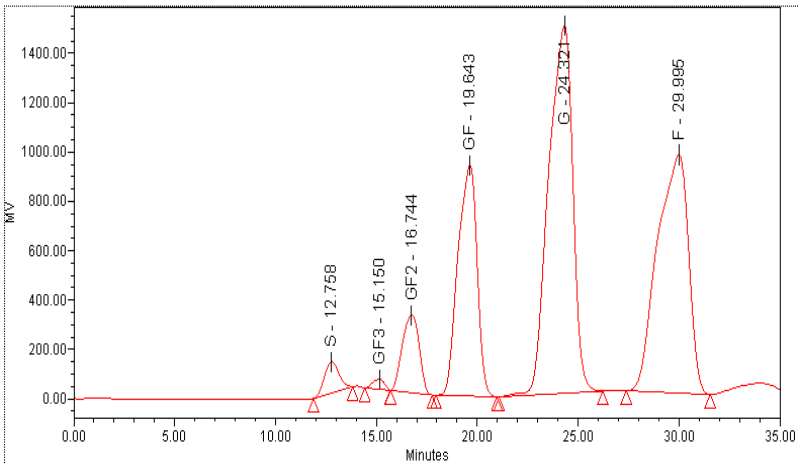


Fig. 7 HPLC analysis of end products of *Penicillium* sp. NFCC 2768 inulinase action on Chicory inulin (S- Standard inulin, GF_3-Nystose, GF_2-Kestose, GF- Sucrose, G-Glucose, F- Fructose)

preparation liberated fructose and inulooligosaccharides from inulin. The maximum yield of inulinase achieved was 64.54 nkat/ml. The apparent molecular weight of purified inulinase as determined by SDS-PAGE was 68 kDa. The optimum pH and temperature of this enzyme were 5.0 and 50°C, respectively. The report signifies use of low value inulin rich infusions in production of inulinase and describes its properties.

Progress of III Year

- **Immobilization of inulinase using alginate beads, glutaraldehyde and chitosan etc.**
- **Optimization of process parameters (pH, temperature, dosage, substrate concentration) for inulin hydrolysis using free and immobilized inulinase.**
- **Evaluation of efficacy of inulinase preparation in terms of product yield.**

Materials and methods

Materials

Inulin rich substrates dandelion tap root, dahlia tubers and asparagus root powder used in this study were collected from local sources. Pure inulin (chicory), fructose, 3, 5-dinitrosalicylic acid (DNS), glutaraldehyde and sugar standards were obtained from Sigma Chemical Co., U.S.A. Sodium alginate (molecular mass 10,000-600,000) and calcium chloride (anhydrous) were obtained from Finar, India. Glass column reactor (60cm × 10cm) was used in the present study (ASGI, India). Other chemicals were purchased from Hi-Media and Merck, India.

Microorganism

The fungal strain was isolated from dandelion rhizospheric soil of local garden and was grown on Czapek's dox agar at 28°C. It was identified on the basis of its morphological features using taxonomic description (Onions *et al.*, 1981). The identity of the culture was confirmed as *Aspergillus niger* and it was deposited in National Fungal Culture Collection of India (NFCCI) at Agarkar Research Institute (ARI), Pune, India with an accession number NFCCI 3879 and Gour Nodal Culture Collection (GNCC), departmental culture collection centre, Sagar, (M.P.).

Substrates and carbohydrate analysis

Fresh dandelions (complete plant with tap roots) were collected from local fields of Sagar (MP, India) district. Roots and leaves were separated and tap root was used for the extraction of inulin.200 gram of fresh solids (20% w/v) root was mixed in 1000 ml of distilled water using grinder. Inulin was extracted from this slurry by hydrothermal treatment (10-20 psi) of the formed slurry. The slurry was filtered through a muslin cloth to obtain clear solution (containing soluble inulin) and used in media preparation (Kango, 2008). Total carbohydrate and inulin content were estimated by HPLC analysis.

Enzyme production

Aspergillus niger NFCCI 3879 was cultivated on media containing inulin rich extracts in shake cultures and the culture filtrates were assayed for inulinase and invertase activity. Media

containing extracts of dandelion root, dahlia tubers, asparagus roots, onion and garlic bulb were used as crude substrate and pure chicory inulin (2% w/v each) (Sigma) for inulinase production (**Table 6.1**). Yeast extract 2% (w/v) was used as nitrogen source in all the media formulations. Fungus was also cultivated on solid substrates for enzyme production. Solid state fermentation (SSF) was performed using various crude substrate (5g each; moisture ratio 2:1) employed in SSF study. Erlenmeyer flasks (250 ml) containing 50 ml/ 5g aliquots of medium were autoclaved (20 min, 121°C) and inoculated with two mycelial discs (7 mm) cut from 7 days old culture of *Aspergillus niger*. Flasks were then incubated at 28°C and samples were withdrawn at regular intervals of 24 h upto 5 days. The content of the flask was filtered through Whatman filter paper No.1 and clear filtrate was used for enzyme assays. All the experiments were carried out in triplicate and mean values ± standard deviation (SD) are reported.

Experimental design for the determination of optimum values of yeast extract and dandelion extract

Both yeast extract (A) and dandelion tap root extract (B) were selected to find their optimum values for inulinase production by *A. niger* NFCCI 3879 using RCCD. The ranges and levels of the variables taken for RSM are listed in **Table 2**. According to RCCD, the total number of experimental combinations is $2^k + 2k + no$, where k is the number of independent variables and no is the number of repetitions of the experiments at the centre point. A total of 13 set of experiments including five center points were conducted along with different combination of physical parameters. Each numeric factor was varied over 5 levels, that is, plus and minus alpha (axial point), plus and minus one (factorial points), and zero (center point).

Statistical analysis and validation of experimental modeling

The data obtained from RSM was subjected to analysis of variance (ANOVA) for analysis of regression coefficient, prediction equations, and case statistics. Analysis of data was performed using Design-Expert software (Version 7.0). The experimental results of RSM were fitted using the second order polynomial equation:

$$Y = \beta 0 + \sum_{i} \beta iXi + \sum_{ii} \beta iiXi2 + \sum_{ij} \beta ijXiXj \qquad \text{(Eq. 1)}$$

In this polynomial equation, Y is the predicted response, Xi Xj are independent variables, β0 is the intercept term, βi is the linear coefficient, βii is the quadratic coefficient, and βij is the interaction coefficient. The statistical model was validated with respect to all variables within the design

space. A random set of 2 experimental optimized combinations were used to study the inulinase production under submerged fermentation.

Enzyme assays

For evaluation of inulinase activity, 0.2 ml of appropriately diluted enzyme sample was added to 1.8 ml of chicory inulin (0.5% w/v dissolved in 200 mM sodium acetate buffer, pH 5.0) and incubated at 50°C for 15 min. For estimation of immobilized inulinase activity, 1 g of Ca-alginate beads was added to 3 ml of inulin/ sucrose (0.5 and 1% w/v, respectively) solution. After incubation, reducing sugar liberated from inulin/ sucrose was measured by adding 3 ml DNS reagent and boiling for 5 min. Samples were allowed to cool and their absorbance was read at 540 nm. One unit of inulinase/invertase activity was defined as the amount of enzyme which produced 1 micromole of fructose/glucose per minutes under the assay conditions as described above (Rawat *et al.*, 2015a).

Partial purification of enzyme

Precipitation of total proteins was done by adding two volumes (2:1 v/v) of chilled ethanol to the culture filtrate (100 ml) obtained after growth on dandelion root extract medium under constant stirring for 30 min at 4°C. The precipitate was obtained by centrifuging the mix at 9000*g* for 10 minutes at 4°C. Protein precipitate thus obtained was suspended in appropriate volume of sodium acetate buffer (200 mM, pH 5.0).Inulinase was partially purified by precipitating total protein using ethanol followed by fractionation using DEAE-Cellulose-52 (Hi-media, Mumbai) ion exchange chromatographic column of size 2.5×50 cm (internal diameter × height) (Rawat *et al.*, 2015b). Optical densities of eluted column fractions were measured at 280 nm and these were examined for protein concentration (Lowry, 1951) and inulinase activity. Fractions showing activity were pooled and excess amount of water was removed by lyophilization. Partially purified enzyme was used in finding pH and temperature optimum, thermo-stability, metal ions, inhibitors and other characteristics of the inulinase.

Enzyme immobilization

Sodium alginate slurry was prepared by mixing 3 g (w/v) of sodium alginate in 99 ml of warm distilled water (50°C) to which 1 ml of glutaraldehyde (25%) was added. Approximately 2337nkat/ml of inulinase obtained in free form was added to the appropriate volume of alginate slurry. For entrapment of exoinulinase, enzyme was mixed in the slurry and extruded drop-wise through needle (1D, 1.0 mm) into 0.2 M chilled $CaCl_2$solution by peristaltic pump. To maintain the spherical

conformation Ca-alginate beads were cured for 2h in $CaCl_2$ and stored in sodium acetate buffer (pH 5.0) for further study (Ganaie *et al.*, 2014a).

Effect of pH, temperature and thermostability profile

The effect of pH on free and immobilized inulinase was determined by incubating suitable enzyme with 1.8 ml of inulin (0.5% w/v) in different buffers (200 mM sodium phosphate citrate: pH 2.0 and 3.0, 200 mM sodium acetate buffer: pH 4.0, 5.0 and pH 6.0; 200 mM phosphate buffer: 7.0 and 8.0; 200 mM Tris-HCl buffer: pH 9.0; 200 mM, Glycine-NaOH buffer: pH 10.0) for 15 min at 50°C. The effect of temperature was determined by incubating suitably diluted enzyme with 1.8 ml of inulin (0.5% w/v) (200 mM sodium acetate buffer: pH 5.0) for 15 min at different temperatures (30 - 80°C). Thermo stability profile of enzyme sample was estimated by measuring the relative activity of free and Ca-alginate entrapped inulinase after different incubation period at different temperatures (15 - 80°C).

Effect of metal ions and inhibitors on enzyme activity

Effect of various metal ions on enzyme activity was assessed by pre-incubating the inulinase (free and immobilized) with2 mM solutions of $MgSO_4$, $ZnSO_4$, $BaCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, $FeCl_3$, and $HgCl_2$. The effect of inhibitors (2 mM) such as phenylmethanesulfonyl fluoride (PMSF), ethylene diaminetetraaceticacid (EDTA), and pCMB on inulinase activity was examined by treating the enzyme sample with their solutions for 1 hour at 30°C. The effect of metal ion was compared to untreated control and is presented as relative activity of inulinase.

Kinetic parameters

Kinetic characteristics (K_m, V_{max} and K_{cat}) of free and immobilized inulinase were studied and compared. For confirming the rate of reaction, different substrate concentrations ranging from 0.1 to 2.0% mg/ml was used for inulinase activity. The rate of reaction versus substrate concentration was plotted to understand whether the enzyme followed Michalis-Menten kinetics and K_m and V_{max} parameters were analyzed by Lineweaver-Burk plot.

Packed-bed reactor (PBR) studies

Ca-alginate immobilized beads were used in packed-bed reactor (PBR) for continuous hydrolysis studies of dandelion root extract and pure inulin (chicory and dahlia). 50 ml of dandelion root extract (20% fresh w/v), chicory and dahlia inulin (Sigma, 2% w/v) prepared in 0.2 M sodium acetate buffer (pH 5.0) were poured in designed reactor contained packed-bed of immobilized beads under constant temperature 50°C maintained using thermostat. In order to test

the practicability of this PBR system samples flowing through it were collected after regular incubation intervals i.e. beginning from 30 minutes up to 30 days.

Chromatographic analysis of inulin hydrolysate

End products formed after action of immobilized inulinase on dandelion root, pure chicory and dahlia inulin was analyzed by TLC and HPLC. Samples were withdrawn at different time intervals and 3 µl was spotted on pre-coated TLC glass plate (Merck). These were developed with the solvent system containing isopropyl alcohol: ethyl acetate: water (2:2:1 by volume). Sugar spots were developed with reagent containing 0.5% alpha-naphthol and 5% sulfuric acid in absolute ethanol and by heating the plates at 100°C for 10 min. Fructose (F), Sucrose (GF), Kestose (GF2) and nystose (GF3) were used as sugars standards. HPLC (Waters) was performed by using the Sugar Pak I column (6.5 × 300 mm) and the refractive index differential detector (RI 2414). Distilled water was used as the mobile phase. Samples were filtered using 0.45µm Millipore syringe filters before injecting in 20µl valve. Column temperature was maintained at 70°C by oven column (Dyna, Mumbai). Calculations and analysis were performed using Empower-2 software Build 2154, Waters (Rawat *et al.*, 2015a).

Results and discussion

Enzyme production

Aspergillus niger NFCCI 3879 grew well on various inulin-rich plant extracts and elaborated varied levels inulinase. Dandelion tap root extract supported maximum inulinase production (984 nkat/ml) followed by asparagus, garlic and dahlia extracts (**Table 1**). Complex inulin rich plant extracts supported high inulinase activity while much lower activity was seen on pure chicory inulin. Use of inulin rich infusions in media formulation makes the process economical thus making enzyme application cost-effective. Jing *et al.* (2003) have found maximum inulinase (25 U/ml) activity of *A. ficuum* after 5 days of incubation. Previous study reported the use of various crude inulin rich plant materials *viz.* dandelion, dahlia, asparagus, sunflower, lettuce root and Jerusalem artichoke (Mansouri *et al.*, 2013; Housseiny, 2014; Singh and Singh, 2014; Rawat *et al.*, 2015b). Kango (2008) reported *A. niger* NK-126, an isolate from onion peels, to produce 55 U/ml of inulinase in four days using dandelion tap root extract. Many microbial preparations of inulinase possess remarkable invertase activity accompanying the inulinase activity. The *I/S* ratio was differed considerably ranging from 0.12 to 0.83.In the present study dandelion tap root extract supported higher inulinase activity in SmF (984 nkat/ml) while dahlia tuber powder supported maximum yield (972 nkat/gds) in SSF. Inulin containing plant

materials are being used to induce inulinase production (Cazetta *et al.*, 2005) and optimized production on such media ingredients can pave the way of formulation of a low-cost industrial medium.

Optimization of physical factors by response surface methodology (RSM)

In this investigation RSM was applied for the optimization of two crucial factors *viz.* dandelion root extract and yeast extract for inulinase production in order to study the importance of these factors at different levels. RSM involving a rotatable central composite design (RCCD) was adopted to optimize the physical parameters for inulinase production by *A. niger*. A set of 13 experiments including five center points was carried out. Each numeric factor was varied over five levels (-α, -1, 0, +1, +α). The full experimental plan with respect to their actual and coded forms is listed in **Table 2**. The response values (Y= inulinase activity) in each trial were the average of the triplicates. The experimental results of RSM were fitted using the following second order polynomial equation (1). In this study, the independent variables were coded as A (dandelion extract), and B (yeast extract). Thus, the second order polynomial equation can be represented as follows:

$$\text{Inulinase Activity} = +2330.20+33.79*A-6.10*B+42.50*A*B-512.04*A^2-523.29*B^2 \quad \text{(Eq. 2)}$$

For inulinase production, the correlation coefficient (R^2) of polynomial equation was found to be 0.99. The R^2 value indicates a measure of variability in the observed response values which can be described by the independent factors and their interactions over the range of the corresponding factor. So, quadratic model has been suggested for this analytical work. The "Predicted *R*-Squared" value of 0.99 is in reasonable agreement with the "Adjusted *R*-Squared" value of 0.99 because the predicted *R*-squared and the adjusted *R*-squared should be within 0.10 of each other (Soni *et al.*, 2015). This indicated a good agreement between the observed and predicted values. *F*-value is used for comparing lack of fit (LOF) variance with pure error variance and higher *F*-value indicated model is significant.

In this case A, B, AB, A^2 and B^2 were significant model terms. Moreover, "LOF *F*-value" of 1.12 implies that it was not significant relative to the pure error. Furthermore, value of prob>*F*, higher than 0.05 indicated their non significance. Non-significant LOF indicated a good fitness of model. The 3D response surface curve and their respective 2D contour plot determine the interaction of the physical factors and optimum value of each factor for maximum response. This plot was obtained from the pair-wise combination of independent factors. **Figure 1a** showed the

effect of yeast extract and dandelion extract on inulinase production. Increasing the value of both from significantly increased the inulinase

Table 1 Inulinase and invertase production by *Aspergillus niger* NFCCI 3879 on different substrates in submerged and solid state fermentation study

	Submerged fermentation (SmF)[a]			Solid state fermentation (SSF)[b]		
Substrate*	**Inulinase**	**Invertase**	***I/S* ratio[c]**	**Inulinase**	**Invertase**	***I/S* ratio**
Dandelion tap root	984.1 ± 14.3	1258.1 ± 25.4	0.78	867.6 ± 18.6	1038 ± 19.3	0.83
Asparagus root powder	570.0 ± 17.2	1956.2 ± 39.9	0.83	888.7 ± 19.7	1647 ± 29.4	0.53
Dahlia extract	182.4 ± 11.9	217.4 ± 19.7	0.22	972.9 ± 24.2	1467 ± 17.2	0.66
Garlic bulbs extract	220.3 ± 17.8	990.1 ± 31.8	0.16	892.4 ± 13.9	1179 ± 11.3	0.75
Onion bulb extract	108.2 ± 12.7	670.2 ± 25.8	0.12	347.2 ± 9.02	421 ± 8.61	0.82
Inulin[§]	31.7 ± 5.92	245.8 ± 13.6	0.30	-	-	-

Cultures were grown in 250 ml Erlenmeyer flasks containing 2% w/v (SmF) vegetal extract or 3-5g dry substrate (SSF) with yeast extract (2% w/v) as N - source. (SmF - 150 rpm; Temp - 28°C; SSF - Temp. 28°C; moisture - 1:2). [a]Enzyme activity in nkat/ml of culture filtrate; [b]Enzyme activity in nkat/gds; [c]*I/S* (inulinase/invertase) ratio; *fresh weight of extract (% w/v); [§]Chicory inulin (Sigma, USA); values represents mean values of three replicates ± SD.

Table 2 Experimental levels of the independent variables used for inulinase production using *Aspergillus niger* NFCCI 3879

Variables	**Experimental range and levels**				
	-α	**-1**	**0**	**1**	**+α**
Dandelion root extract (% fresh w/v)	34.14	10	20	30	5.8
Yeast extract (% w/v)	3.4	1	2	3	0.59

activity up to 2337 nkat/ml, but thereafter no significant increase in inulinase activity was observed. It was also observed that when the values were increased beyond level "0", the inulinase activity decreased (Std Run. 3, 4 and 8 in **Table 3**). Predicted *vs* actual plot (**Figure 1b**) represents a high degree of similarity that was observed between the predicted and experimental values. Analysis of variance (ANOVA) was used for analysis of regression coefficient, prediction equations and case statistics (**Table 4**).There are some previous studies in the literature in which inulinase were produced by different carbon sources using different microorganism to support present study (Treichel *et al.*, 2009; Trivedi *et al.*, 2012; Abd El Aty *et al.*, 2014; Dilipkumar *et al.*, 2014b).

Validation of the optimum condition defined by the model

The quadratic model was validated by conducting experiments under the optimum conditions predicted by the model. The optimum value for both factors was found to be dandelion extract (20%) and yeast extract (2%) for maximum inulinase production. The maximum predictable response was calculated using regression equation employing substituted level of factors and was experimentally verified. The average activities obtained from 3 replicates was 2337 ± 13.9 nkat/ml, which is in agreement with the predicted value of 2330 nkat/ml.

Partial purification of inulinase and immobilization studies

Inulinase was partially purified to homogeneity by ethanol precipitation followed by anion exchange chromatography. A summary of the purification steps for exoinulinase is presented in **Table 5**. Inulinase specific activity increased in comparison to crude enzyme leading to purification. The precipitated enzyme was partially purified by DEAE cellulose ion exchange chromatography. In this purification step, exoinulinase was partially purified 8.26 total fold with the yield of 34.9%. The purity of inulinase was realized as a single band on SDS-PAGE with molecular weight of 80 kDa approximately (**Figure 2**).

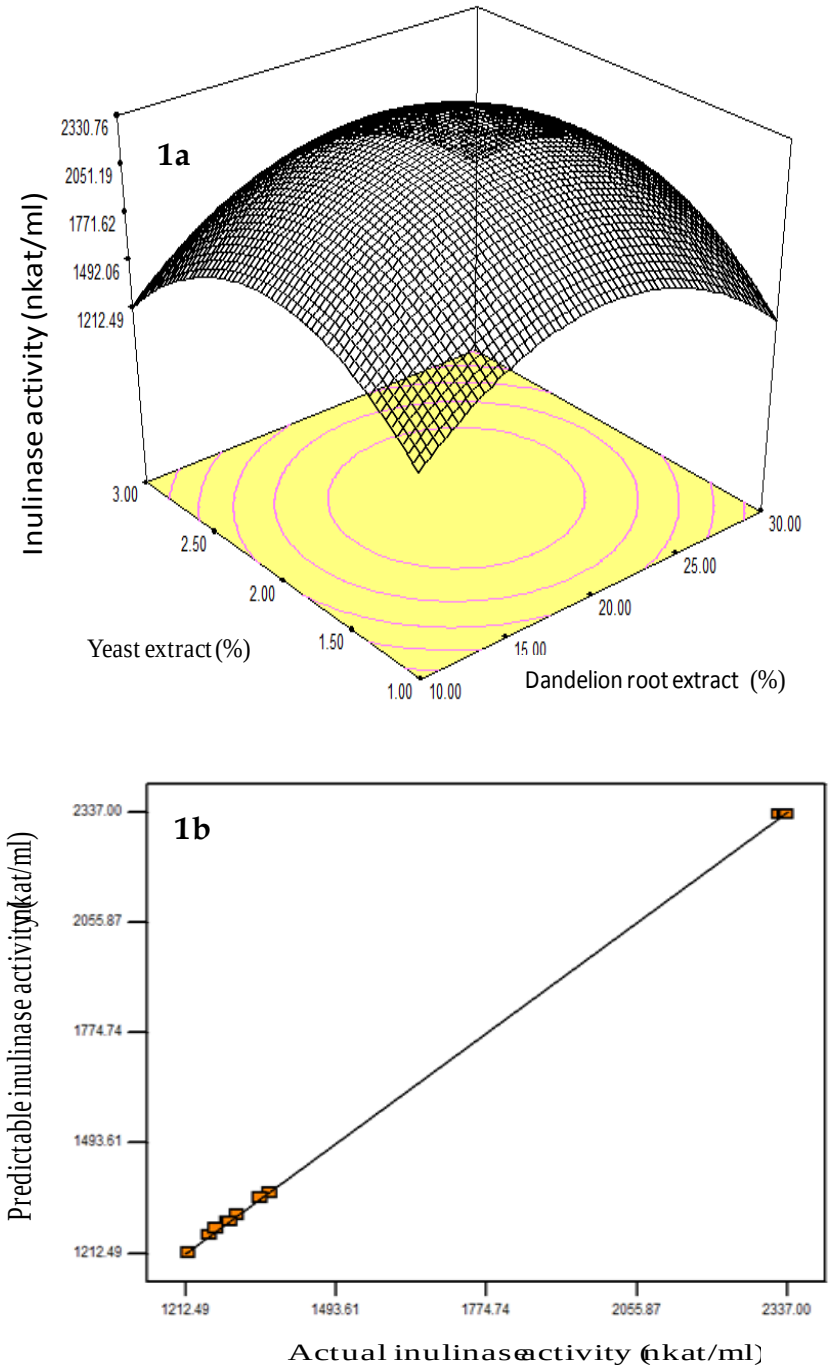


Fig 1 a) 3D-Response surface plot showing effect of concentration of dandelion tap root extract (% fresh w/v) and yeast extract (% w/v) on inulinase production (nkat/ml) in submerged fermentation by *Aspergillus niger* NFCCI 3879. b) Predicted *vs.* actual response plot of the quadratic model used for inulinase production yield (nkat/ml).

Table 3 Process variables used in the RCCD approach of RSM, showing the levels of variables and prediction and actual of inulinase production. Data points indicate the mean of triplicate values ± SD

Std. Run	Level	A: Dandelion root extract (% fresh w/v)	B: Yeast extract (%)	Inulinase Activity (nkat/ml)	
	-α	34.14	3.4		
	-1	10	1		
	0	20	2	Predicted	Actual
	+1	30	3		
	+α	5.8	0.59		
1		10	1	1309	1308 ± 8.2
2		30	1	1292	1291 ± 12.1
3		10	3	1212	1217 ± 9.3
4		30	3	1365	1370 ± 13.2
5		5.8	2	1258	1257 ± 11.2
6		34	2	1353	1352 ± 7.3
7		20	0.58	1292	1295 ± 5.2
8		20	3.4	1275	1269 ± 11.3
9		20	2	2330	2331 ± 22.6
10		20	2	2330	2327 ± 27.4
11		20	2	2330	2323 ± 19.4
12		20	2	2330	2333 ± 15.4
13		20	2	2330	2337 ± 10.6

Table 4 Analysis of variance (ANOVA) for the experimental results of the CCD used for inulinase production

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	3315312	5	663062.4	21592.44	< 0.0001	significant
A-pH	9136.16	1	9136.16	297.5165	< 0.0001	
B-Moisture	297.3087	1	297.3087	9.681774	0.017	
A^2	1823878	1	1823878	59394.07	< 0.0001	
B^2	1904903	1	1904903	62032.64	< 0.0001	
AB	7225	1	7225	235.2801	< 0.0001	
Residual	214.9565	7	30.70808			
Lack of Fit	98.15654	3	32.71885	1.120508	0.4399	not significant
Pure Error	116.8	4	29.2			
Cor Total	3315527	12				

Earlier reports suggest considerable variation in molecular weight from various Aspergilli and their exoinulinase in the range of 70-85 kDa *viz*. *Aspergillus niger* AF10 (83 kDa), *A. niger* (68.1 kDa), *A. ficuum* JNSP5-06 (70 kDa), *A. niger* AUMC 9375 (75 kDa) (Zhang *et al.*, 2004; Mutanda *et al.*, 2008; Chen *et al.*, 2009; Housseiny, 2014). An 83 kDa exoinulinase was purified from preparation of *Aspergillus niger* AF 10 using anion exchange (Hi-Trap-SP, Pharmacia) chromatography (Zhang *et al.*, 2004). Previously we have described purification of a 68 kDa inulinase from *Penicillium* sp. NFCCI 2768 using similar method (Rawat *et al.*, 2015b).

Inulinase preparation obtained after DEAE Cellulose-52 fraction containing 1166 nkat/mg was immobilized in Ca-alginate support by gel entrapment and cross-linking with glutaraldehyde. The immobilization yield was more than 80% and the amount of entrapped protein was higher as compared to other matrices such as chitosan and casein test for immobilization. Gill *et al.* (2006a) immobilized the purified inulinase from *A. fumigatus* on casein, alginate, chitin, DEAE-sephacel, QAE-sephadex, dowex and amberlite; however, no significant entrapment was achieved with calcium alginate. Inulinase sourced from *A. niger* AUMC 9375was immobilized in calcium alginate and was used upto 10 cycles for generation of high fructose syrup (Housseiny, 2014). In the present study, efficient entrapment of inulinase in Ca-alginate was achieved. Liquid containing fructose was separated from alginate beads and the end products were confirmed by TLC and HPLC.

Characterization of free and immobilized inulinase

Optimum pH, temperature and thermo-stability profile

Free and immobilized exoinulinase of *Aspergillus niger* NFCCI 3879 was selected to at different pH and temperatures revealed that the free and immobilized inulinase were optimally active at pH 5.0 and 50°C temperature (**Figure 3**).

Table 5 Partial purification of exoinulinase from *Aspergillus niger* NFCCI 3879

Fraction	Protein (mg/ml)	Total protein (mg)	Inulinaseactivity[a] (nkat/ml)	Total inulinase activity	Specific activity[b] (nkat/mg)	Yield % per step
Culture filtrate [c]	4.10	410	984.1	98410.0	240.0	100
Protein Precipitate[d]	1.44	14.4	1235.2	12352.3	857.7	21.3
DEAE-Cellulose-52[e]	0.74	3.70	862.7	4313.5	1166	34.9

[a]One nkat of inulinase activity was defined as the amount of enzyme which produced 1micro mole of fructose per minute at 50°C and pH 5.0.
[b]The specific activity is expressed in nkat/mg of enzyme activity per milligram of protein in an assay condition.
[c]Culture filtrate of dandelion tap root medium (100 ml).
[d]Protein precipitate was resuspended in 10 ml of 200 mM sodium acetate buffer pH 5.0.
[e] Fractions, excess amount of water was removed by lyophilization.

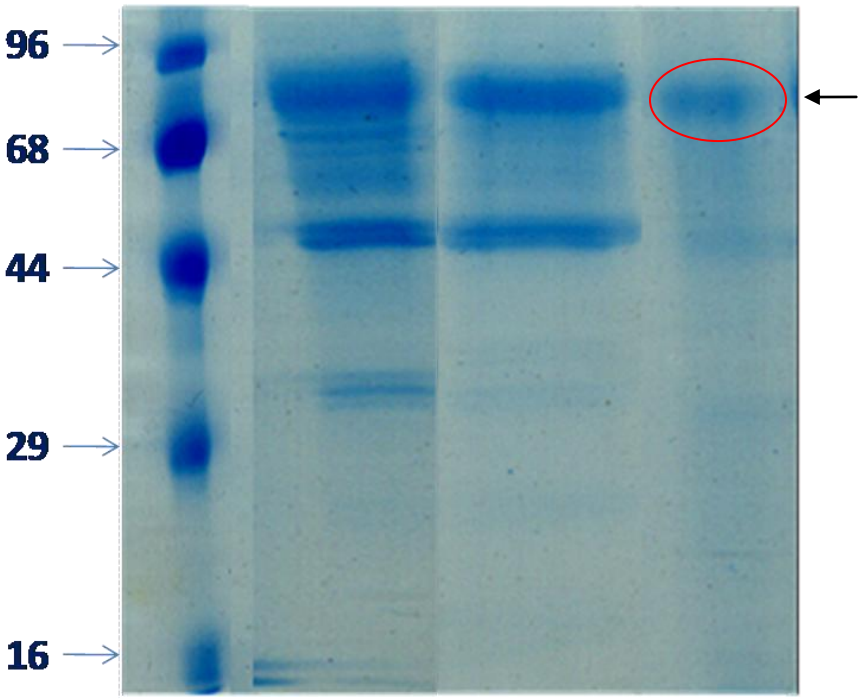


Fig. 2 SDS-PAGE analysis of purified inulinase of *Aspergillus niger* NFCCI 3879. Lane 1: Standard marker; Lane 2: precipitate enzyme; Lane 3: DEAE cellulose-52 column fraction of purified inulinase.

The purified exoinulinase was found to be suitable for long term stability purpose and had half life of 15 days at 50°C. Exoinulinase from *A. niger* AF10 (Zhang *et al.*, 2004) is reported to be optimally active at 55°C, which is higher to our findings. Again, the immobilized enzyme was relatively more reusable than free enzyme. Thermo-stability profile of temperature **(Figure 4a)** and pH confirmed that enzyme is highly stable at optimum temperature for long time (**Figure 4b**). The pH optimum of free inulinase was 5.0 which remained unchanged in case of immobilized inulinase also.

Similar results have been reported by Paula *et al.* (2008) and the effect of pH on free and immobilized inulinase was the same. These results are also comparable with other exoinulinases reported so far: *A. ficuum* JNSP5-06 (pH 4.5) (Chen *et al.*, 2009); *A. fumigatus* (pH 6.0) (Gill *et al.*, 2006b), and *A. niger* NK-126 (pH 5.0, 50°C) (Kango 2008). Present study described higher operational time and stability of immobilized biocatalyst makes this preparation more suitable for industrial application.

Effect of metal ions and inhibitors

The effect of various metal ions and inhibitors on *Aspergillus niger* inulinase is presented in **Table 6**. Activity of free and immobilized enzyme was strongly stimulated in presence of Mn^{2+},$Mg^{2+}$and Cu^{2+} while Hg^{2+} which is known to affect -SH- groups, strongly inhibited inulinase activity. Chen *et al.* (2013) and Singh *et al.* (2007) have reported Mn^{2+} to enhance the enzyme activity, while $Hg^{2+}$and Al^{3+} completely inhibited the activity of inulinase. In the present study, EDTA, PMSF and pCMB strongly inhibited the inulinase activity of free and immobilized inulinase. These results are in accordance with those noticed for exoinulinases of *Aspergillus ficuum, Aspergillus fumigatus* and *Sphingomonas* sp. JB13 (Chen *et al.*, 2009; Gill *et al.*, 2006a; Zhou *et al.*, 2015).

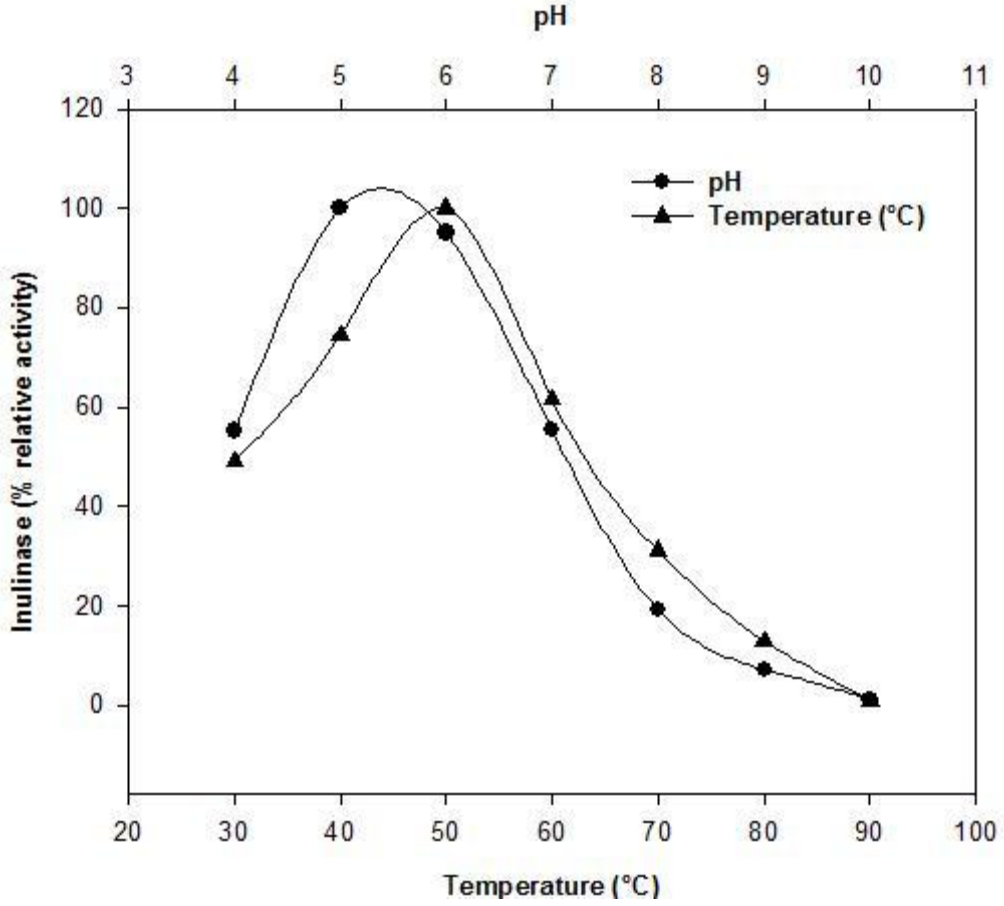


Fig. 3 Effect of pH and temperature on immobilized inulinase activity.

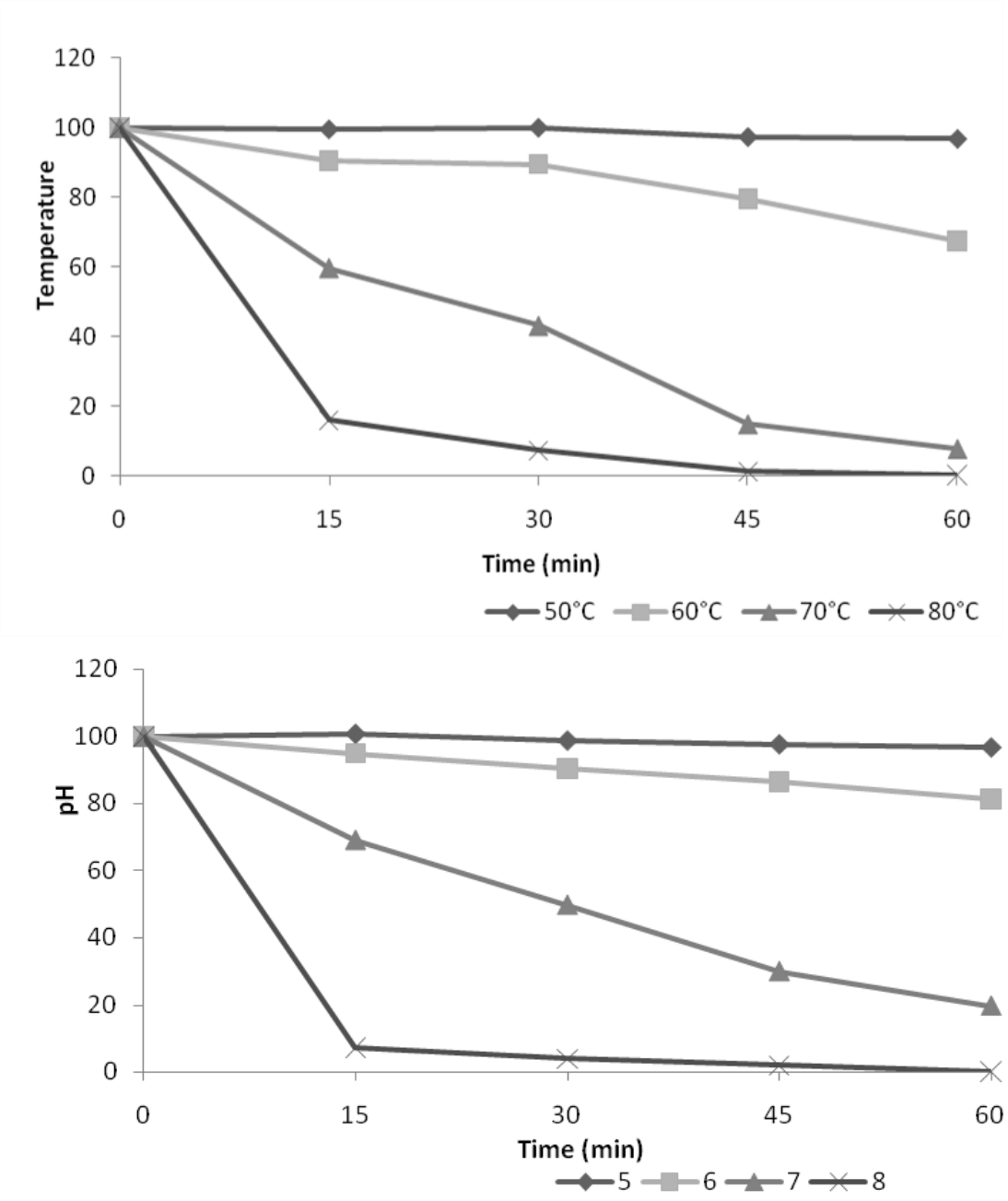


Fig. 4 Thermal stability of *Aspergillus niger* NFCCI 3879 immobilized inulinase at different (**a**) temperatures and (**b**) pH.

Continuous fructose production using packed-bed reactor (PBR)

Hydrothermally extracted inulin-rich extract of dandelion tap roots (20% fresh w/v), pure chicory and dahlia inulin (2% w/v) was continuously poured into the PBR (**Figure 5**) and the initial inulinase activity was maintained for over 15 days without changing the immobilized biocatalysts. Total fructose syrup was about <90% (w/w) and 85% (w/w) in case of dandelion root extract and chicory inulin hydrolysis, respectively. PBR employing alginate immobilized inulinase showed long term stability and efficient fructose generation. Inulinase preparation obtained after DEAE-cellulose fraction containing 0.74 mg/ml protein was immobilized in Ca-alginate support by covalent gel entrapment.

The immobilization yield was more than 85% and the amount of entrapped protein was also higher as compared to other matrices such as chitosan and casein tested for immobilization. Paula *et al.* (2008) immobilized cell free inulinase in gelatin for continuous hydrolysis of sucrose using fixed-bed column reactor. Immobilization yield of their study was 82% coming of glucose and fructose. Singh *et al.* (2007) have immobilized exoinulinae on duolite A568 after partial purification to generate high fructose syrup from crude asparagus inulin and yielded 39.2 g/L fructose in four hour. Inulinase sourced from *A. niger* AUMC 9375 was immobilized in calcium alginate and was used upto 10 cycles for generation of high fructose syrup (Housseiny, 2014). Trytek *et al.* (2015) immobilized inulinase and also reported efficient operational stability of inulinase in packed-bed reactor for continuous hydrolysis of inulin (0.5% w/v) to generate fructose (98%) period of 28 days.

In the present study the PBR resulted in 90% conversion producing fructose exclusively. Fructose containing fractions were collected at regular intervals from PBR and the hydrolysates were analyzed by TLC (**Figure 6a and 6 b)** and HPLC **(Figure 7**). Inulin rich extract obtained from dandelion tap roots was successfully hydrolyzed to fructose by the PBR.

Table 6 Effect of different metal ions and inhibitors on free and immobilized inulinase activity of *Aspergillus niger* NFCCI 3879

SN	Metal ion*	Concentration	Relative Activity	
			Free inulinase	Immobilized inulinase
1.	Control	-	100.0 ± 2.9	100.0 ± 1.8
2.	Mg^{2+}	2mM	102.8 ± 3.4	115.0 ± 2.6
3.	Zn^{2+}	2mM	94.3 ± 3.7	98.6 ± 3.9
4.	Ba^{2+}	2mM	81.1 ± 2.7	93.5 ± 2.1
5.	Mn^{2+}	2mM	112.1 ± 2.9	127.2 ± 3.2
6.	Ca^{2+}	2mM	91.9 ± 2.5	98.2 ± 2.5
7.	Cu^{2+}	2mM	96.5 ± 1.9	106.3 ± 2.1
8.	Fe^{3+}	2mM	69.2 ± 2.0	74.8 ± 3.4
9.	Hg^{2+}	2mM	14.1 ± 1.6	9.3 ± 0.8
10.	PMSF	2mM	12.2 ± 1.0	8.3 ± 1.2
11.	pCMB	2mM	7.94 ± 0. 9	9.67 ± 0.4
12.	EDTA	2mM	3.91 ± 0.6	3.47 ± 0.4

*Salts used for metal ions: Mg^{2+}: $MgSO_4.7H_2O$, Zn^{2+}: $ZnSO_4.7H_2O$, Hg^{2+}: $HgCl_2$, Ba^{2+}: $BaCl_2$, Mn^{2+}: $MnSO_4$, Ca^{2+}: $CaCl_2.2H_2O$, Cu^{2+}: $CuSO_4$, Fe^{3+}: $FeCl_3$

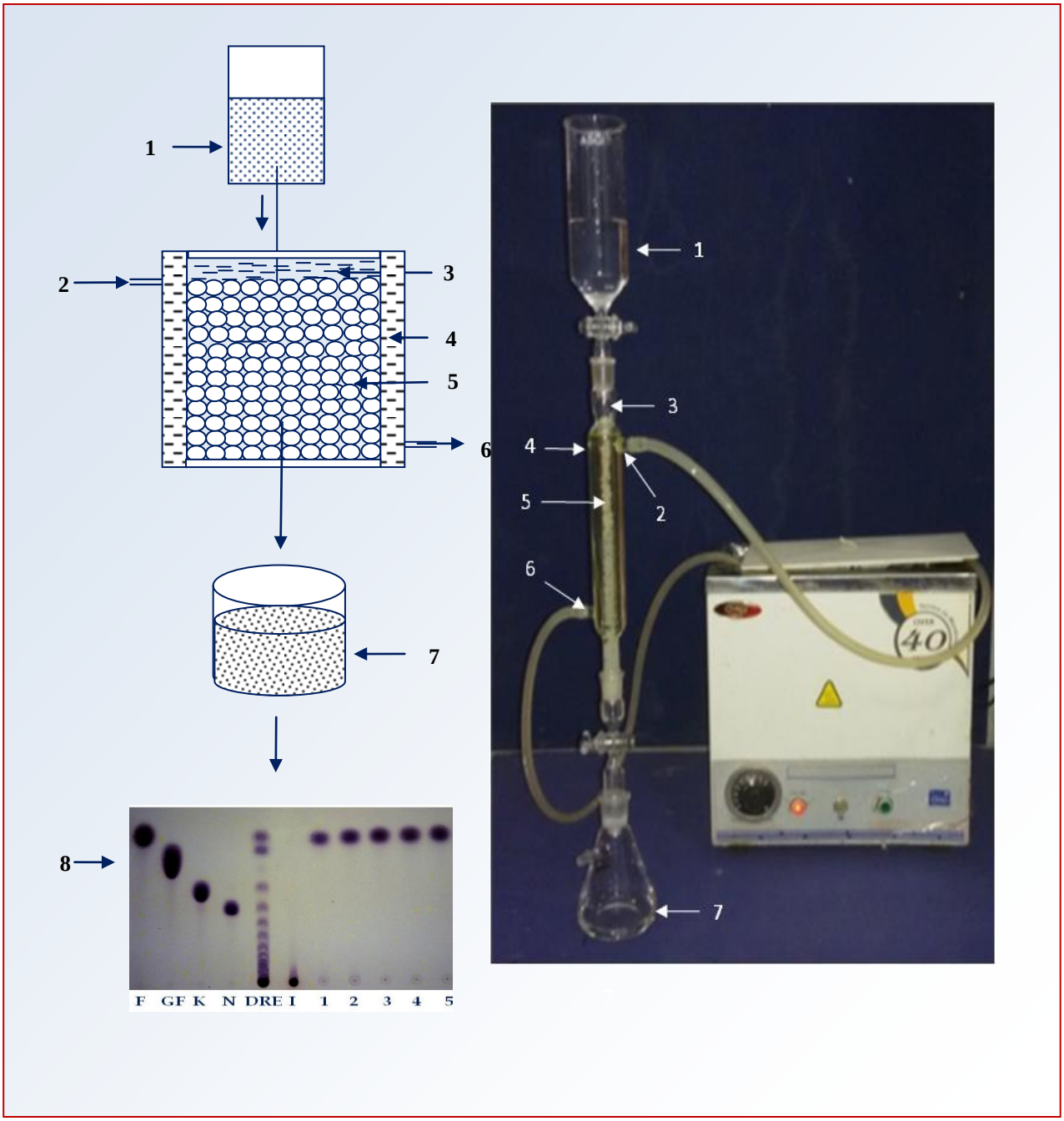


Fig. 5 Schematic diagram of packed bed bioreactor (PBR) used for continuous generation of fructose syrup from dandelion tap root extract (% fresh w/v) and pure chicory inulin using Ca-alginate immobilized *Aspergillus niger* NFCCI 3879 exoinulinase. Labels : 1.Dandelion tap root extract or pure chicory inulin; 2. Water inlet; 3. Substrate percolating on immobilized inulinase; 4. Glass vessel with water jacket; 5. Ca-alginate immobilized inulinase (biocatalyst) of *Aspergillus niger*; 6. Water outlet; 7. Generated fructose syrup; 8. TLC chromatogram showing fructose syrup generation (Conditions for enzyme catalysis: pH- 5.0, Tem- 50°C).

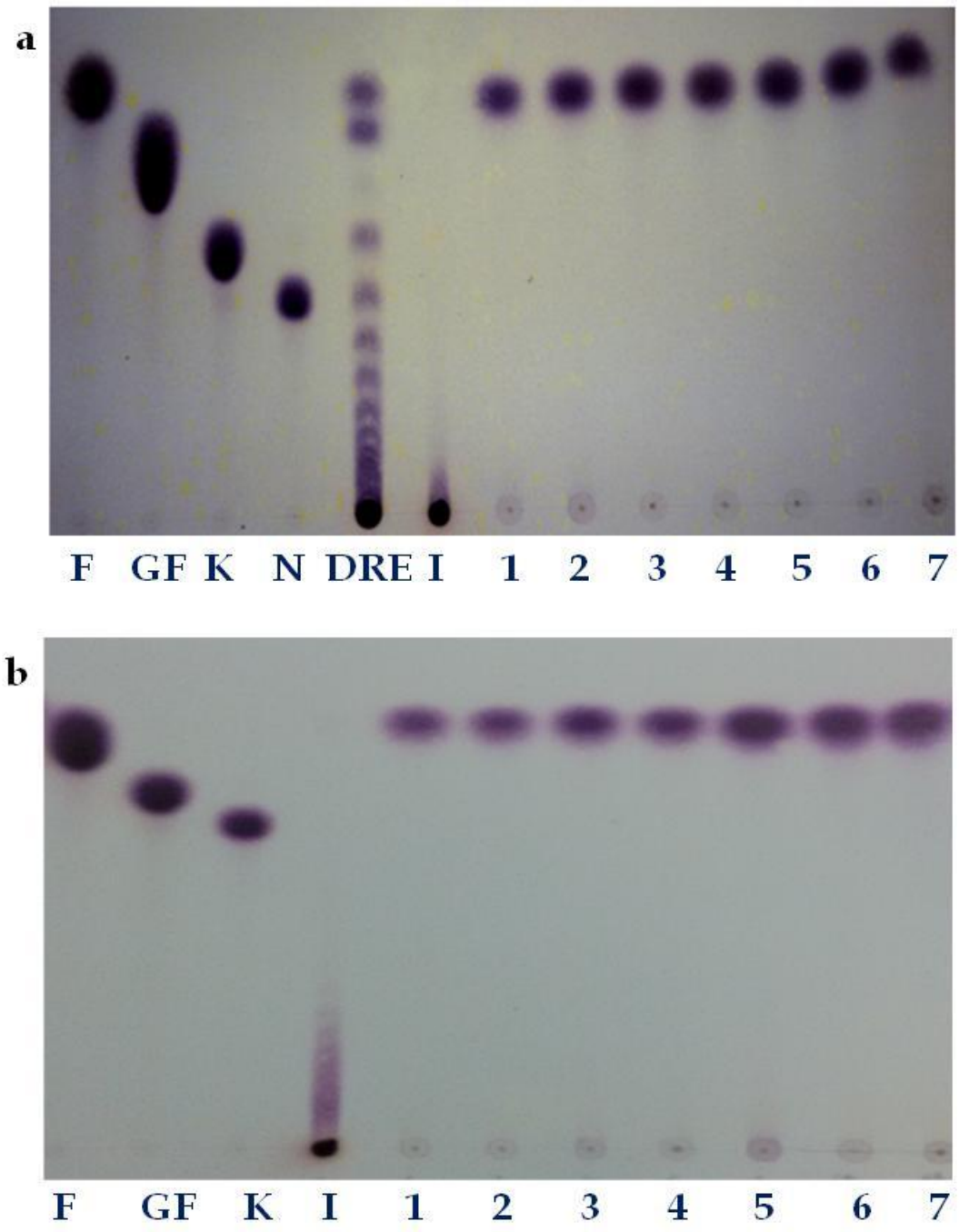


Fig. 6 TLC showing end product analysis of immobilized inulinase (*Aspergillus niger* NFCCI 3879) preparations employed in PBR for continuous hydrolysis (Standards: F- Fructose, GF- Sucrose, K- Kestose, N- Nystose, I- Pure chicory inulin and DRE- Dandelion root extract showing dandelion oligosaccharides, fructose and fructan). A) Lane 1-7, end product of dandelion tap root hydrolysis using PBR after 30 min to 120 h. B) Lane 1-7, end product of chicory inulin hydrolysis using PBR after 1h to 48 h.

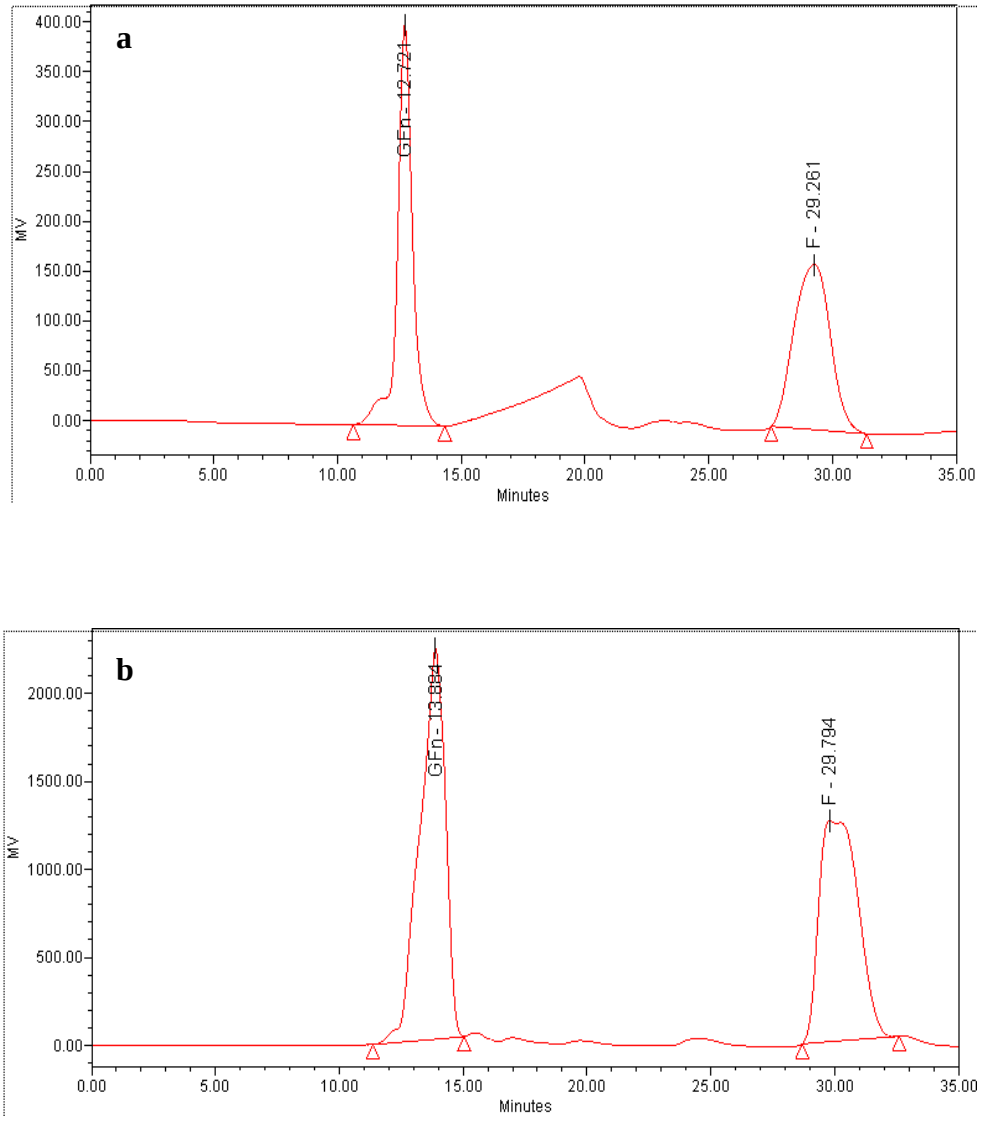


Fig. 7 End product analysis of PBR products immobilized inulinase of *Aspergillus niger* NFCCI 3879 a) Hydrolysis of dandelion tap root extract after 6h incubation; b) Hydrolysis of pure chicory inulin after 6 h incubation.

Continuous fructose production was obtained with crude dandelion root, pure chicory inulin and pure dahlia inulin using the PBR up-to 30 days (720 hrs). Maximum 90% of that the entrapped enzyme worked well on inulin available in dandelion tap root and other forms and such a system can be exploited in nutraceutical and pharmaceutical industries for continuous generation of fructose syrup. Mode of action of exoinulinase from Aspergillus niger NFCCI 3879 was ascertained by analyzing the end products of enzyme action using HPLC (Rawat *et al.*, 2015a). Major product of inulin hydrolysis was fructose in case of all kinds of inulins.

Conclusion

Production of exoinulinase from newly isolated *Aspergillus niger* NFCCI 3879 was statistically optimized using dandelion tap root extract (% fresh w/v) resulting in a ~2.5 fold (2337 nkat/ml) enhancement. Exoinulinase (1166 nkat/mg) immobilized in Ca-alginate beads was used for continuous generation of fructose from crude inulin extracted from *Taraxacum officinale* (dandelion) and chicory juice. Immobilized enzyme was optimally active at 50°C and pH 5.0 and its kinetic parameters were 3.74 mM (K_m) and 1.62 µm/min (V_{max}). Continuous generation of fructose syrup from low-cost dandelion root extract and chicory inulin using a packed bed bioreactor (PBR) prepared by exoinulinase immobilized in alginate beads was demonstrated. Successive degradation of dandelion oligosaccharides and fructans to pure fructose was observed over 30 days period. The bioreactor had a half-life of 15 days and more than 90% (w/w) yield of fructose was observed.

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B4. Summary and conclusions of the progress made so far (minimum 100 words)

Inulinase production of *Penicillium* sp. NFCC 2768 was studied using on media containing inulin-rich plant extracts. Maximum inulinase activity (64.54 nkat/ml) was observed with the tuber extract of dahlia (*Dahlia pinnata*). The molecular weight of the purified inulinase was 68 kDa. Inulinase liberated fructose, glucose, sucrose, kestose (GF2), nystose (GF3), and inulooligosaccharides (IOS). This study suggested the use of dahlia tuber extract and asparagus root powder as suitable substrates for inulinase production and its application in the generation of fructose and IOS.

Production of inulinase from newly isolated *Aspergillus niger* was statistically optimized using dandelion root extract (% fresh w/v) resulting in a ~2.5 fold (2337 nkat/ml) enhancement. The enzyme was an 80 kDa protein exo-acting in nature. It was successfully entrapped in ca-alginate and the preparation was stable to be employed in PBR. About 90% (w/w) of conversion of crude extract indicated high efficiency of the system.

Exoinulinase from *Aspergillus niger* immobilized in Ca-alginate beads was used for continuous generation of fructose from crude inulin extracted from dahlia, asparagus and *Taraxacum officinale* (dandelion) and chicory inulin.

B5. Details of new leads obtained, if any

Penicillium sp. NFCCI 2768 inulinase was purified and molecular weight of enzyme was 68k Da (Rawat *et al.*, 2015)

Production of exo-inulinase from newly isolated *Aspergillus niger* was statistically optimized using dandelion tap root extract resulting in a 2337 nkat/ml.

B6. Details of publications, patents and technology developed (*Reprints enclosed*)

1. Rawat H. K., Soni H., Treichel H. and **Kango N.** (2016) Biotechnological potential of microbial inulinases: Recent perspective **Critical Reviews in Food Science and Nutrition**. **Accepted** (Online issue) DOI: 10.1080/10408398.2016.1147419 (**IF 5.42**).
2. Rawat H. K., Ganaie M. A. and **Kango N.** (2015) Production of inulinase, fructosyltransferase and sucrase from fungi on low-value inulin-rich substrates and their use in generation of fructose and fructooligosaccharides. **Antonie van Leeuwenhoek**, 107: 799-811. Citation 11 (**IF 1.9**).
3. Rawat H. K., Jain S. C. and **Kango N.** (2015) Production and properties of inulinase from *Penicillium* sp. NFCC 2768 grown on inulin containing vegetal infusions. **Biocatalysis and Biotransformation**, 33: 61-68. (**IF 0.9)**
4. Rawat H. K., Soni H. and **Kango N.** (2015) Screening of inulinase producing fungi isolated from inulin containing sources and use of exoinulinase in generation of fructose. **Madhya Bharti Journal of Science**, 59: 10-14.

B7. Deliverable of the project

Inulinase was characterized from the native strains will be utilized by food and nutraceutical industries of M.P. Present study will be useful in case of industrial scale production of fructose syrup. An enzyme based process for fructose generation using microbial inulinase of selected fungal strains.

B8. A brief para about ‘‘application and use of research outcome - the technology aspect”

Immobilization and successive recycling of inulinase will be facilitates its reuse and ensures continuous production of high fructose syrup.
Optimization of *Aspergillus niger*

Purification and characterization of inulinase was done from *Aspergillus niger* NFCCI and *Penicillium* sp. NFCCI 2768.

B9. Benefits that will accrue to M.P. on the completion of your research project

The project outcome will be useful for pharmaceutical, fermentation and food industries of Madhya Pradesh. Based on the findings of the project the native isolates obtained from Sagar MP may be utilized for production of inulinase. Inulinase thus obtained may be immobilized as demonstrated in the report and a process of continuous generation of FOS or Fructose may be developed.

B10. Agencies which can utilize the findings/ results/ outcome of the project

Production of high fructose syrup can be exploited in food, pharmaceutical and nutraceutical industries or corporations of Madhya Pradesh.

B11. Further research potential and support required from the council, if any

Financial support has been submitted for agencies for further research.

Section C: Details of grant utilization

C1. Equipment acquired or place order with actual cost

Non-Recurring:

Amount Sanctioned & List of approved Equipment	**List of procured equipment**
Rs. 55,000=00 Equipment (Microscopic Image Projection System, CCD Camera)	01-MIPS Catcam, Microscope Eyepiece Digital Camera (CC130): CatCam 130 1.3 Mega Pixel Microscope Camera Date of invoice: 8.10.2013

C2. Manpower staffing and expenditure details

Please see enclosed Utilization certificate

C3. Details of recurring expenditure

Please see enclosed Utilization certificate

List of Papers presented in conferences/seminars

I. **Production, properties and immobilization of *Penicillium* sp. NFCCI 2768 inulinase grown on inulin containing raw materials.** Organized by (Madhya Pradesh Biotechnology Council, Bhopal) M.P. Young Scientist Congress-28 Feb. to 1 March 2014.
Paper Presented in New Biology Stream

II. **Screening, production and application of inulinase and fructosyltransferase (FTase) producing microorganisms. FDEOLS-2014 (international Conference)** Department of Biotechnology, Dr. H.S. Gour University, Sagar (M.P.) during 13-15 February 2014.
Best Poster Award

III. Development of Bioprocess for generation of high fructose syrup (HFS) held at Department of Microbiology, Dr. H.S. Gour University, Sagar (M.P.) National Science Day during 28 February 2013.
Best Poster Award

IV. **Production and characterization of *Aspergillus fumigatus* inulinase and its application for the generation of high fructose syrup (HFS) (ISCA chapter, Symposium)** Department of Zoology, Dr. H.S. Gour University, Sagar (M.P.) during 27-28 February 2014.
Presented Poster

V. **Enzymes (Endo-inulinase from *Fusarium oxysporum)* Are Playing Role in Green Chemistry (National Seminar)** Organized by (Govt. Auto. Excellence Girls College, Sagar)
Delivered Lecture

Research Articles (Under Review)

5. Rawat H. K., Jain S.C., Soni H., Ahirwar S. and **Kango N.** (2015) Optimized production and characterization of inulinase from newly isolated Hirsutella sp. and its potential for use in the generation of inulooligosaccharide and fructose. Journal of Food Science and Technology, JFST-S-15-02626-R1.

Honors and Awards received

1. Best Poster Presentation (First Prize) award in National Conference (MRSC-2015) Organized by Maharaja Ranjit Singh College of Professional Sciences, Indore 'Optimization and inulinase production from *Aspergillus fumigatus* for the generation of inulooligosaccharides and fructose' (16th October, 2015)

2. Best Oral Presentation award in National Conference (RACBS-2015) Organized by Dept. of Zoology, Dr. H.S. Gour Central University, Sagar. 'Biotransformation of sucrose into fructooligosaccharide using *A. fumigatus* GNCC 1351' (17 March 2015)

3. Best Poster Award in International Conference (FDEOLS-2014) in Microbial Technology Discipline. Organized by Dept. of Biotechnology, Dr. H.S. Gour Central University, Sagar 'Screening, production and application of inulinase and fructosyltrasferase (FTase) producing microorganisms' (13-15 February-2014).

4. Fellowship for Training of Young Scientist. MPCST, Young Scientist Congress-2014 Presented Research paper in New Biology Discipline during 28 Feb to March 1-2015.

5. Fellowship for Training of Young Scientist. MPCST, Young Scientist Congress-2014 Presented Research paper in New Biology Discipline during 28 Feb to March 1-2014.

6. Best Poster award in National Science Day- 2013 Sponsored by MPCST-Bhopal organized by Dept of Applied Microbiology and Biotechnology Dr. H. S. Gour Central University, Sagar 'Bioprocess development for high fructose syrup generation' (15 March 2013).

Paper presented in Conferences and Seminars

1. National conference on Bioremediation and our Environment Organized by Noble College, Sagar Presented paper Continuous production of high fructose syrup using *Aspergillus fumigatus* thermostable inulinase. 16-17th May 2015.

2. National conference on evolving trends in biotechnology ISCA, Sagar Chapter Presented paper-Production and properties of inulinase producing Penicillium for the fructose generation. 28-30 Mar 2015.

3. National conference Indian Science Congress Association (ISCA), Kolkata Presented poster- 'Isolation and screening of inulinase producing fungi isolated from decomposing vegetal sources' In New Biology (3-7 Jan 2013).

4. National seminar on Role of Green Technology Delivered lecture on 'Enzymes (Endoinulinase from Fusarium oxysporum) are playing role in green chemistry'. Organized by Govt. Auto Girls P.G. College of Excellence, Sagar, M.P. (26-27th Feb 2014)

5. National symposium on Frontiers Modern Biology. Presented paper- Production and characterization of Aspergillus fumigatus inulinase for generation of fructose syrup. ISCA Sagar Chapter Central University, Sagar (March 24-25th, 2014)

6. National Seminar of ISCA, Sagar Chapter Presented paper/abstract entitled-Isolation and screening of inulin hydrolyzing Fungi from different vegetal sources' 30 Sep-Oct 1 2012.

UTILIZATION CERTIFICATE

(for the financial year ending 31st March 2016)

1	Title of the project:	**Optimization and scale up of process parameters for high fructose syrup production using *A. niger* OP-3 and *Penicillium* sp. NFCCI 2768 inulinase**
2	Name of the Organization:	**Madhya Pradesh Biotechnology Council MPBC, Bhopal**
3	Principal Investigator:	**Dr. Naveen Kango** Associate Professor and Head Department of Applied Microbiology Dr. Harisingh Gour University Sagar (M.P.) 470003
4	Madhya Pradesh Biotechnology Council Sanction Order No. & date of sanctioning the project:	(PA-23/656 Dated 22.11.2012)
5	Amount received from Council during the financial year (Please give No. and date of sanction order showing the amount paid):	**Rs. 2, 81,000**
6	Amount brought forward from the previous financial year quoting Council letter No. & date in which the authority to carry forward the said amount was given:	**Rs. 70,969**
7	Total amount that was available for expenditure during the financial year (Sl. nos. 5 & 6):	**Rs. 3,51,969**
8	Actual expenditure incurred during the financial year (statement of expenditure is enclosed):	**Rs. 2,80,969**
9	Unspent balance refunded, if any (Please give details of Checque No. etc.)	**Rs. 14000**
10	Balance amount available at the end of the financial year	**Rs. 14000**
11	Amount allowed to be carried forward to the next financial year vide letter No. & date:	**Nil**

6/5/16

Statement of Expenditure

Referred to in para 8 of the Utilization Certificate

Showing grants received from the Madhya Pradesh Biotechnology Council and the expenditure incurred during the period from 1st Aril 2015 to 31st March 2016

Heads	Grants received from the Council during the year	Unspent balance carried forward from previous year	Total (2+3)	Expenditure incurred during the year	Balance (4-5)	Remarks (if any)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Non-Recurring Eqipment	Nil	-	-	-	-	
Recurring HR	1,44,000	-	1,44,000	144000	Nil	
Consumables	50,000	-	50,000	46000	4000	
Travel	10,000	-	10,000	Nil	10000	
Contingency	20,000	-	20,000	20000	Nil	
Overheads	-	-	-	-	-	
Carried forward		70969	70969	70969		
Total	**2,24,000**	**70969**	**294969**	**280969**	**14000**	

(Project Investigator) (Head of the Department) (Finance / Accounts Officer) (Head of the Institute)

Dr. Naveen Kango
Project Investigator
M.P. Biotechnology
Council (MPBC) Bhopal

अध्यक्ष
Head
सूक्ष्मजीव विज्ञान विभाग
Dept. of Microbiology
डॉ. हरीसिंह गौर विश्वविद्यालय
Dr. Harisingh Gour Univers...

Finance Officer
Dr. H.S. Gour Vishwavidyalaya
SAGAR (M. P.)

REGISTRAR
Dr. H.S. Gour Vishwavidyalaya,
SAGAR (M. P.)

(Auditor)

Internal Audit Officer
Dr. H.S.Gour V.V.Sagar (M.P.)

Note: Signatures of all the competent authorities with seal are mandatory

1. Certified that the amount of Rs. **2,80,969** mentioned against col. 8 has been utilized on the project for the purpose for which it was sanctioned and that the balance of **Rs. 14000** remaining unutilized at the end of the year has been surrendered to MPBTC (vide No. _____ dated _____)/ will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled.

6/5/16

(Project Investigator) (Head of the Department) (Finance / Accounts Officer) (Head Of the Institute)

Dr. Naveen Kango
Project Investigator
M.P. Biotechnology
Council (MPBC) Bhopal

Head
सूक्ष्मजीव विज्ञान विभाग
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Dr. Harisingh Gour University, Sagar

Finance Officer
H. S. Gour Vishwavidyalaya
SAGAR (M. P.)

REGISTRAR
H. S. Gour Vishwavidyalay
SAGAR (M. P.)

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Antonie van Leeuwenhoek (2015) 107:799–811
DOI 10.1007/s10482-014-0373-3

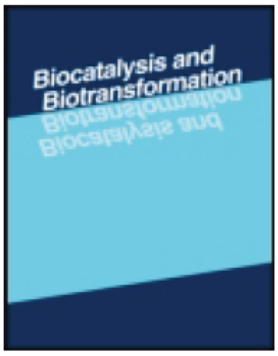
ORIGINAL PAPER

Production of inulinase, fructosyltransferase and sucrase from fungi on low-value inulin-rich substrates and their use in generation of fructose and fructo-oligosaccharides

Hemant Kumar Rawat · Mohd Anis Ganaie · Naveen Kango

Received: 18 October 2014 / Accepted: 29 December 2014 / Published online: 6 January 2015

Acknowledgments Authors are thankful to Madhya Pradesh Biotechnology Council (MPBC), Bhopal, India major research project-(*PA-23/656*) for financial assistance.



Biocatalysis and Biotransformation
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ibab20

Production and properties of inulinase from Penicillium sp. NFCC 2768 grown on inulin-rich vegetal infusions

Hemant Kumar Rawat[a], Sumat Chand Jain[a] & Naveen Kango[a]
[a] Department of Applied Microbiology and Biotechnology, Dr. Harisingh Gour University, Sagar, Madhya Pradesh, India
Published online: 30 Jun 2015.

study emphasizes the value of low-value inulin-rich infusions for the production of inulinase.

Acknowledgements

The authors acknowledge the financial support received from M.P. Biotechnology Council, Bhopal (Grant No. PA-23/656).

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.
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Critical Reviews in Food Science and Nutrition, 57: (2016)

ISSN: 1040-8398
DOI: 10.1080/10408398.2016.1147419

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Biotechnological potential of microbial inulinases: Recent perspective

HEMANT KUMAR RAWAT[a], HEMANT SONI[a], HELEN TREICHEL[b] and NAVEEN KANGO[a]*
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Among microbial enzymes, inulinases or fructofuranosylhydrolases have received considerable attention in past decade and as a result a variety of applications based on enzymatic hydrolysis of inulin have been documented. Inulinases are employed for generation of fructose and inulooligosaccharides (IOS) in a single step reaction with specificity. The high fructose syrup can be biotransformed into value added products such as ethanol, single cell protein while IOS are indicated in nutraceutical industry as prebiotic. Myriad microorganisms produce inulinases and a number of exo and endoinulinases have been characterized and expressed in heterologous hosts. Initially predominated by Aspergilli, Penicillia and some yeasts (Kluyveromyces spp.), the list of prominent inulinase producers has gradually expanded and now includes extremophilic prokaryotes and marine derived microorganisms producing robust inulinases. The present article summarizes important developments about microbial inulinases and their applications made in the last decade.