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Optimization of Inulinase Production by *Bacillus* sp. B51f Isolated from Rhizosphere Soil of *Agave sisalana*

Aruna K.* and Amita Hati
Department of Microbiology, Wilson College, Mumbai-400007, India
*Corresponding Author E-mail: arunasam2000@yahoo.co.in

ABSTRACT

Extracellular inulinase producing bacterium was isolated from the rhizosphere soil of Agave sisalana and identified as Bacillus sp. B51f by morphological, cultural, biochemical tests and 16S rRNA gene sequence analysis. The maximum inulinase yield was 160.24 U/ml when 2% inoculum (0.8 $OD_{545\ nm}$) of the above isolate was grown in M9 medium containing 1.5% inulin, 0.7% peptone, 0.3% Na_2HPO_4, 0.4% K_2HPO_4, 0.3% NH_4Cl and 0.4% NaCl at pH 7, temperature 30℃ for 24 hours at static conditions. The highest inulinase activity was observed (173.41 U/ml) at pH 8 and 55℃ which indicates it to be thermostable and alkaliphilic. The effect of various metal ions and thermal stabilizers were checked on inulinase activity. Ca^{2+} ions increased the enzyme activity whereas glycerol exhibited a stabilizing effect on the enzyme. HPTLC analysis of hydrolytic prodcuts of inulinase revealed that inulinase hydrolysed inulin exclusively into fructose which confirmed exo inulinase nature of the enzyme.

Keywords: *Bacillus sp. B51f; Inulinase;Agave sisalana.*

INTRODUCTION

Inulinases (2, 1-β-D-fructan fructanohydrolase, EC3.2.1.7) catalyse the hydrolysis of inulin, producing inulo-oligosaccharides, fructose and glucose as main products. Inulin consists of linear chains of β 2, 1-D fructofuranose molecules terminated with a glucose residue at the reducing end. Inulinases can be divided into exo-inulinases and endo-inulinases. The exo-inulinase removes the terminal fructose residues from the non-reducing end of inulin, whereas the endo-inulinase acts on the internal linkages of the inulin molecule but lacks invertase activity[1,2,3].Inulinases have different catalytic properties (molecular weight, optimum pH, optimum temperature, stability) depending especially upon their origin[4].Inulinases can be used in a wide range of industrial applications: ultra-high fructose syrup production which can be used as a prebiotic for improving population of beneficial microorganisms such as *Bifidobacterium* in intestinal flora, bioethanol production,pullulan exopolysaccharide, gluconic inulo-oligosaccharide production, single-cell oil and single-cell protein production, some chemicals production like citric acid, 2,3 butanediol, lactic acid and mannitol[1,2,5,6,7,8].

In the last decades a large number of bacteriaviz. *Bacillus subtilis* 430A[9],*Bacillus polymyxa* 29, *B. polymyxa* 722, *B. subtilis* 68[10], *Bacillus polymyxa* MGL21[11], *Bacillus* sp. LCB41[12], *B. stearothermophilus* KP1289[13], *Bacillus cereus* MU-31[14], *Marinimicrobium* sp. LS-A18[15], *Pseudomonas mucidolens*[16], *Arthrobacter ureafaciens*[17], *Streptococcus salivarius*[18], *Staphylococcus* sp.[5,19], *Clostridium acetobutylicum*[20], *Clostridium thermoautotrophicum*[21], *Streptomyces* sp.[22,23,24,25], yeast (*Kluyveromyces* sp.)[26,27,28] and fungal strains (*Penicillium*, *Fusarium* and *Aspergillus*)[29,30,31,32,33,34] were used for inulinase production. By the use of microbial inulinase, one step enzymatic hydrolysis of inulin yields 95% pure fructose[35].

Inulin is present as a reserve carbohydrate in the underground organs (roots and tubers) of plants such as *Jerusalem artichoke* (*Helianthus tuberosus*), dahlia (*Dahlia pintana*), chicory (*Cichorium intibus*), Agave (*Agave sisalana*), yacon and in smaller amounts in garlic and onion[1,36,37,38,39,40,41,42]. The yields of the

roots and tubers are very high. The dried materials of the tubers contain over 70% inulin[5]. Many inulinolytic microbes such as *Bacillus subtilis*, *Paenibacillus* sp. CDB 003 *Streptomyces* sp. ALKC4, *Penicillium janczewskii* and *Aspergillus niger* were isolated from rhizosphere soil[9,29,43,44,45].

This study focuses on isolation of inulinase producing bacterium from the rhizosphere soil of *Agave sisalana*, which is expected to have higher inulinase producing variants. This strain is identified as *Bacillus sp.* B51f. The culture conditions for maximum inulinase production by this strain were studied and assay conditions for optimum inulinase activity were also investigated.

MATERIALS AND METHODS

Enrichment, isolation, screening and identification

Soil from the rhizosphere area of plant *Agave sisalana* was used as sample for enrichment of inulinase producers. 1gm of the above soil sample was mixed in 10ml distilled water and was allowed to settle for 10mins. The1.5 ml of supernatant collected was inoculated in two 30mlof enrichment broth (M9-Inulin medium- 0.2% NaCl, 0.3% Na_2HPO_4, 0.18% KH_2PO_4, 0.2% NH_4Cl, 1% inulin (as a sole source of carbon and energy) and incubated at 30°C for 48 h under shaker (200 rpm) and static conditions. After 24 h, 1.5 ml of the culture was inoculated into 30 ml of fresh medium. After an additional 24 h, 0.3 ml of the culture was inoculated into 30ml of fresh medium, and 24 h later, single colonies were isolated from M9-inulin agar.

The three isolates obtained from M9-Inulin agar plates were used for spot inoculation on MHI agar plates and incubated at 30°C for 3 days. It was then flooded with 5ml of 1% Lugol's iodine and examined for a zone of clearance around the colony which indicated the presence of the enzyme inulinase[46]. Further, inulinase assay of the promising isolate was carried out and was identified by using, morphological, cultural and biochemical tests as per Bergey's Manual of Determinative Bacteriology, 8th edition[47]. Further confirmation of the strain was done by 16S rRNA sequence analysis by SciGenom Labs Pvt Ltd. Kerala, India.

Extraction and assay of inulinase

Inulinase activity was assayed by method suggested by Miller[48]. The crude enzyme was obtained by centrifuging the growth of the isolate in M9-inulin broth at 3,500 rpm at 30°C for 10 minutes. The supernatant obtained was used as a crude enzyme for inulinase assay. This crude enzyme (0.5 ml) was mixed with 2ml of 0.2% inulin prepared in distilled water and 2ml of 50mM Acetate Buffer (pH 4.6) incubated at 55°C for 20 minutes. After incubation the reaction mixture was kept in ice water bath for 10 minutes to stop the reaction. One ml of DNSA reagent was added to 1 ml of the mixture and kept in boiling water bath for 10 minutes. It was cooled and 6ml of distilled water was added to this mixture. The absorbance of the supernatant was measured at 540 nm using a spectrophotometer. The reaction mixture containing heat inactivated crude enzyme (100^0C for 10 min) instead of the active culture supernatant was used as blank. The absorbance of the test supernatant against blank was obtained and plotted on the standard graph of Fructose (40-4000µg/ml) to obtain the amount of product formed. From the standard graph value, enzyme activity was calculated in U/ml. One unit of enzyme activity is defined as the amount of enzyme liberating 1 µmole of fructose per minute under standard conditions.

Optimization of culture conditions for maximum inulinase production

Five different media were investigated for maximum inulinase production by the selected isolate viz. Medium 1[49], Medium 2[50], Medium 3[51], Medium 4[52] and Medium 5-M9 medium with 1% Inulin. The optimized medium was used for further investigation of various parametersfor maximum inulinase yield by the selected isolate. Optimization of culture conditions for thehighest inulinase production was studied in M9 medium with 1% Inulin. Two percent inoculum (0.8 $OD_{545\ nm}$) was inoculated in 100ml of the above medium and kept for incubation at 30ºC for 24 h at static conditions. After incubation, the medium was centrifuged at 3500rpm for 10mins and the supernatant was used as a crude enzyme for the study of various parameters. One parameter at-a-time approach was used and all the experiments were carried out

in triplicates.The culture conditions (incubation period, aeration, temperature, pH, carbon, nitrogen source and various mineral salt concentrations) were optimized for maximum inulinase production by the selected isolate. Inulinase production was determined at various time intervals such as 12, 24, 36, 48, 60 and 72 h. The effect of aeration on inulinase production was studied by incubating one culture flask on shaker (200rpm) and other under static condition at 30ºC for 24 hours. Optimum culture temperature for inulinase production was determined in the range of 30ºC, 37ºC, 45ºC, 55ºC and 70ºCon static condition for 24 h. Optimum pH for inulinase production was determined in the culture grown in M9 medium with 1% Inulin at different pH range from 4 to 10 (1N NaOH and 1N HCl were used for adjusting pH of the medium). Effect of different carbon sources on inulinase production was checked by adding 1% w/v of Glucose, Fructose and Sucrose and combination 1% inulin plus 1% sugar (Inulin+Glucose, Inulin+Fructose and Inulin+Sucrose) to M9 medium. Optimized sugar (0.5-2% w/vat increments of 0.5 %.) was tested for higher yield of inulinase. Different organic and inorganic nitrogen sources (0.5% concentration) such as Casein, Peptone, Yeast extract, Urea, Sodium nitrate and Potassium nitrate were checked for the maximum inulinase production. In addition optimized nitrogen source was tested (0.5-1% with 0.1% increment) for maximum inulinase yield. The optimal salt concentrations (0.1-0.5%w/v with interval of 0.1%) of K_2HPO_4, Na_2HPO_4, NaCl and NH_4Cl were determined for the highest inulinase production.

Effect of different physicochemical parameters on inulinase activity

Inulinase enzyme from the new isolate was assayed to determine the optimum conditions of temperature and pH. Two ml of inoculum (0.8 $OD_{545\ nm}$) was inoculated in 100ml of M9-Inulin medium containing 1.5% inulin, 0.7% peptone, 0.3% Na_2HPO_4, 0.4% K_2HPO_4, 0.3% NH_4Cl and 0.4% NaCl at pH 7, temperature 30°C for 24 h at static conditions. Cell-free culture supernatent was obtained by centrifugation at 3,500 rpm at 30°C for 10 minutes and used for the assays. Crude inulinase enzyme from the culture supernatant of optimized M9-Inulin medium was assayed in the reaction mixture containing 0.5 ml crude enzyme, 2ml of 0.2% inulin, 2ml of acetate buffer (pH 4.6) and incubated at 55ºC for 20mins. The tubes were then kept in ice bath for the enzyme reaction to stop. One ml of DNSA reagent was added to one ml reaction mixture and was kept in boiling water bath for 10 minutes. It was allowed to cool down and 6ml distilled water was added into it. Absorbance was measured at 540nm.The optimal temperature was determined from 30-70^{0}C with interval of 5^{0}C. The inulinase was assayed at various pH ranging from 4 to 10 in the following buffer systems: 0.1M Acetate buffer (pH range 4-6), 0.1M Phosphate buffer (pHrange 7-8) and 0.1M Glycine-NaOH buffer (pH range 9-12). Effect of metals on enzyme activity was measured with different metals namely (0.1% w/v) $HgCl_2$, $MgCl_2$, $ZnCl_2$, $CaCl_2$, KCl, NaCl, $CdCl_2$ and $MnCl_2$. The control was kept with enzyme without metals (100%). Also, the effects of various thermal stabilizers such as0.1% (v/v) glycerol, mannitol and propanol were examined on the inulinase activity The crude enzyme was pre-incubated with above mentioned respective metal ions and thermal stabilizers (with control) for 30 minutes at 30^{0}C. The residual activity (%) was measured by standard inulinase assay.

Analysis of Hydrolysis products of inulinase by High Performance Thin Layer Chromatography (HPTLC)

Hydrolytic products by inulinase from the isolate were qualitatively identified by HPTLC. Sample of 10µl hydrolysate was spotted on the pre-coated TLC plates (Silica gel 60, Merck, Germany) using CAMAG Linomat 5 band spotterwith the solvent system *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 20 g/l diphenylamine in acetone–20 g/l aniline in acetone–850 g/l phosphoric acid (5:5:1 by volume)[53]. Glucose and Fructose were used as sugar standards (10µl of each).The developed plates were dried in a stream of air. After drying, band scanning was done with CAMAG TLC Scanner using WIN-cats software at 350nm.

RESULTS AND DISCUSSION

Enrichment, isolation, screening and identification

The soil samplefrom rhizosphere area of plant *Agave sisalana* was enriched in M9-Inulin broth containing 1%inulin as a sole source of carbon and energy. Enriched sample was streaked on agar plates containing

the same medium and incubated at 30°C for 24 h. Total 3 isolates were obtained, designated as AS-1, AS-2 and AS-3 and all three of them showed zone of hydrolysis around the colony on MHI agar plates confirming extracellular inulinase activity (Figure 1).All three isolates were assayed for inulinase activitywhich was found to be 123.30U/ml, 103.68U/ml and 109.7U/ml for AS-1, AS-2 and AS-3 respectively. The selected AS-1 isolate was identified as *Bacillus sp* B51f on the basis of morphological, culture and biochemical characteristics according to Bergey's Manual of determinative Bacteriology, 8th edition and by16S rRNA gene sequencing analysis.*Agave sisalana* and many other plants of the Agave genus are considered to be a rich natural source of inulin viz. *Agave americana* contains 7–10% inulin[42,54]. Therefore, inulinase producing organism *Bacillus* sp.B51f was isolated from the rhizosphere soilof *Agave sisalana.*

Optimization of culture conditions for maximum inulinase production

From the results shown in Figure 2, it was found that medium5- M9 medium with 1% inulin facilitated maximum inulinase yield (129.7U/ml) by *Bacillus* sp. B51f, hence this medium was used throughout the study.Presence of inulin in the M9-Inulin medium induced maximum inulinase production by *Bacillus* sp. B51f which supports the fact that inulinase is an inulin inducible enzyme. A variety of media have been used previously by various scientists for the production of inulinase by different microbes viz. Czapex Dox agarfor *Streptomyces* sp.[14,24], Beef extract agar for *Bacillus* sp.[10], yeast extract peptone dextrose (YPD) medium for yeast[35], Potato Dextrose medium for *Aspergillus* sp.[29], Mineral medium for *Bacillus* sp.[12],IB medium[45]and MHI medium for *Marinimicrobium*[15].

Bacillus sp. B51f showed inulinase production starting from 12 h of growth and reached maximum in 24 h and then it declined with further increase in duration of incubation (Figure 3). Incubation periods ranging from few hours to several days have been found to be best suited for maximum inulinase production by bacteria[52].Parallel results were obtained for *Bacillus* sp. strain LCB41 which needed 11 h for maximum inulinase production[12].An incubation period of 24 h was optimum for inulinase production by *Streptomyces* sp. CP01, *Streptomyces* sp. GNDU 1 and *Staphylococcus* sp RRL-M-5[23,24,5]. However, *Staphylococcus* sp.RRL-1and *Streptomyces grisenus* exhibited maximum yield after 48 h[19,22]. It was reported that *Bacillus cereus* MU-31 showed higher yield after 36 h[14] while Zherebtsov *et al.* studied the maximum production of extracellular inulinase by *Bacillus polymyxa* 29, *B. polymyxa* 722 and *B. subtilis* 68 after 72 h of incubation[10]. On the other hand, inulinase production by *Marinimicrobium* sp. LS-A18 was found to be the highest after 96 h[15].

Inulinase production by *Bacillus sp.* B51f was found to be the maximum under static condition 133.69U/ml while areation exhibited slightly lesser yield (113U/ml). Similar results were obtained by Selvakumar and Pandey where aeration showed a decrease in the inulinase production in *Staphylococcus* sp.RRL-1and *Kluyveromyces marxianus*[19]. Interestingly, Gouda also reported that *A. fumigates* was able to produce high inulinase without any agitation[55]. This may be due to the high values of specific oxygen uptake rate by the strain, resulting in the liberation of a proteolytic enzyme and hydrolysis of inulinase[56]. However, in other studies, significant increase in inulinase production has been reported where agitation was essential to produce inulinase compared to static condition viz. *Bacillus* sp. LCB41, *Bacillus cereus* MU-31, *Bacillus polymyxa* 29, *B. polymyxa* 722, B. *subtilis* 68,*Streptomyces grisenus,Streptomyces* sp. CP01 and *Marinimicrobium*sp. LS-A18[12,14,10,22,23,15].

The maximum production of inulinase by *Bacillus sp.* B51fwas obtained at 30°C(Figure 4).The production of inulinase decreased as the temperature increased. Low inulinase production at higher temperature could be due to the reduction of oxygen solubility in the medium, or enzyme denaturation[57]. In the case of extracellular enzymes, temperature may influence their secretion, possibly by changing the physical properties of the cell membrane[58].It has been observed that, in general, inulinases are produced in the temperature range 20-50°C[52]. The results are in accordance with the previous studies where *Bacillus cereus* MU-31,*Staphylococcus* sp,*Streptomyces* sp. CP01,*Streptomyces rochei* E87, *Streptomyces grisenus, A. fumigates* and *Kluyveromyces marxianus* YS-1were found to produce a very active inulinase enzyme at 30°C-32°C [59,19,14,22,23,55,36]. Several past studies demonstrated that the maximum production of

enzyme was at temperatures between 33 and 35°C for *Bacillus polymyxa* 29, *B. polymyxa* 722, and *B. subtilis* 68 while for *Marinimicrobium* sp. LS-A18 it was 37°C[10,15]. The production of extra-cellular inulinase by *Streptomyces* sp. GNDU1 and *Bacillus* sp. LCB41 peaked at 46°C and 50°Crespectively[24,12]. High level of inulin-inducible extracellular inulinase production by a thermophilic soil isolate *B. stearothermophilus* KP1289 in the range of41 to 69°Chas also been demonstrated [13].

Inulinase production by microbial strains depends on pH of the culture medium that affects enzymatic processes and transport of compounds across the cell membrane.The optimum production of inulinase by *Bacillus* sp. B51f was obtained at pH 7 and significant amount of inulinase production was also seen at pH 8 (Figure 5).The microbial sources mainly have a neutral or alkaline pH as optimal for inulinase production[52]. There were reports of the maximum production of extra cellular inulinase enzyme at pH 7-7.5 by *Bacillus polymyxa* 29, *B. polymyxa* 722,*B. subtilis* 68,*Bacillus cereus* MU-31, *Staphylococcus* sp., Streptomyces sp. GNDU1, *Streptomyces grisenus, Marinimicrobium* sp. LS-A18 and *Arthrobacter* sp.[10,15,19,14,24,60]. The maximum yield of inulinase at pH 8 has also been observed by *Flavobacterium multivorum, Bacillus* sp. LCB41 and *Streptomyces* sp.CP01[24,61,23].

When the mineral medium was used, the presence of inulin as the sole carbon source was necessary for the production of the inulinase.A combination of inulin and one sugar had catabolite repression effect on inulinase production by *Bacillus* sp.B51f, the inulinase activity decreased 2.2-fold on glucose (65.9 U/ml), 2.5-fold on sucrose (57.2 U/ml) and 3-fold on fructose (48.1U/ml) as shown in Figure 6. This inhibitory effect on enzyme level remained in the same order when fructose, sucrose, or glucose was used as the sole carbon source The activity decreased 1.3-fold on glucose (104.9 U/ml), 1.4-fold on sucrose (98 U/ml), and 1.9-fold on fructose (76.34U/ml). Fructose was the most suppressive in both cases. *Bacillus* sp. B51f showed maximum production of enzyme in M9-Inulin medium with 1.5% inulin and enzyme production was inhibited at higher concentrations of inulin (Table 1). Above facts support the dual mechanism of regulation exists in bacterial strain *Bacillus* sp. B51f where it might be able to regulate the level of inulinase by a substrate (inulin) induction and product (fructose) repression.Similar duel mechanism of substrate induction and product repression has been observed in *Clostridium acetobutylicum, Aspergillus niger* A42, *A.fumigatus,Streptomyces* sp.GNDU1[62,27,63,64,24]. The production of inulinase by *Staphylococcus* sp. *Bacillus cereus* MU-31, *Bacillus smithii* T7, *Marinimicrobium* sp. LS-A18was found to be higher in the presence 0.5 %, 1.5%, 2% and 4% of inulin in culture medium respectively[65,14,15,5]. In contrast, *B. polymyxa* 722 and *B. polymyxa* 29 displayed highest activity of inulinase on a culture medium containing starch whereas the corresponding maximum in *B. subtilis* 68 was observed in the presence of sucrose[10].Sucrose has also been reported as the best inducer for inulinase production by *Kluyveromyces marxianus* YS-1 and *Aspergillus niger* ATCC20611[62,56]. However, there were reports where the inulinase was not inducible and wasconstitutively expressed in *Xanthomonascampestris* and *Aspergillus niger*[66,43]. On the contrary, inulin has also been noticed as a poor inulinase inducer in *Aspergillus niger* 245[67].

Besides carbon source, the type of nitrogen source in the medium also influences the inulinase yield in production broth.Maximum yield of inulinase (149.64 U/ml) was shown by *Bacillus*sp. B51f when 1% peptone was present in M9-inulin medium (Figure 7). The present finding indicated that the supply of organic nitrogen source like peptone and yeast extract resulted in high inulinase production, whereas inorganic sources had moderate effect on inulinase production.Of the various concentrations of peptone (0.5-1%), maximum inulinase activity (159.52 U/ml)was observed at 0.7% (Table 1).Further increase in peptone concentration had some repressive effect on inulinase production.However, there were reports where the inulinase productionby*B. polymyxa* 722, *B. polymyxa* 29 and *B. subtilis* 68 in the 2% peptone-containingmedium was low, but was high in case of 1% yeast extract followed by malt extract.Similar results were recorded for many bacterial strains which require organic nitrogen source for maximum inulinase yield viz. *Marinimicrobium* sp. LS-A18(1% peptone)[15],*Bacillus cereus*MU-31(1.5% yeast extract)[14],*Staphylococcus* sp. (0.5 % soybean meal)[5], *Streptomyces* sp. CP01 (0.7% tryptone)[24], *Streptomyces* sp.GNDU1(3%yeast extract)[23],*Kluyveromyces marxianus* YS-1 (0.5% peptone)[62]and *Aspergillus niger* ATCC20611(0.5% meat extract)[56].Kim tested a wide range of organic nitrogen sources as to their effectiveness for inulinase production by *Penicillium* sp1 and observed that peptone and corn steep liquor stimulated enzyme production whereas urea and yeast extract had less influence[68].

Among the inorganic sources, urea, sodium nitrate and potassium nitrate were found inhibitory for inulinase synthesis[49]. Gupta et al. who found that maximum inulinase production was observed with $NaNO_3$ for *Fusarium oxysporum*[69]. Nonetheless, *Streptomycesgrisenus* showed higher inulinase production in presence of in organic nitrogen source 0.3% $NaNO_3$ in culture medium whereas *Aspergillus fumigatus* exhibited high yield of inulinase when 0.38% KNO_3 was used as nitrogen source in culture medium[22,55]. Maximum production of inulinase by *Aspergillus niger* ATCC20611 was observed in the control medium without inorganic nitrogen sources[56]in contrast Derycke and Vandamme reported maximum yield of inulinase by *A. niger* strain in presence of $(NH_4)_2SO_4$ as nitrogen source[43]. The inhibitory effects of inorganic nitrogen sources on inuliase production by *K. fragilis*were alsoreported[70].

As indicated in Table 2, when various concentrations of components ofthe medium were used for the production of inulinase by *Bacillus* sp. B51f; there was marginal difference in the amount of inulinase enzyme produced. Optimum concentrations of Na_2HPO_4 (0.3%), K_2HPO_4 (0.4%), NH_4Cl (0.3%) and NaCl (0.4%) exhibited higher inulinase production.

Effect of different physicochemical parameters on inulinase activity

Bacillus sp. B51f when cultivated in M9-Inulin medium containing 1.5% inulin, 0.7% peptone, 0.3% Na_2HPO_4, 0.4% K_2HPO_4, 0.3% NH_4Cl and 0.4% NaCl at pH 7, temperature 30° C for 24 hours at static conditions exhibited maximum inulinase yield. Inulinase enzyme from the culture supernatant of optimized M9-Inulin medium grown with *Bacillus* sp. B51f was assayed in the reaction mixture containing 0.5 ml crude enzyme, 2ml of 0.2% inulin, 2ml of acetate buffer (pH 4.6) and incubated at 55°C for 20 mins.

The inulinase enzyme from *Bacillus sp.* B51f was found to be more active in an alkaline range than acidic range with an optimum of pH 8 (Figure 8) which was similar to optimum pH of inulinase from *Marinimicrobium* sp. LS-A18[15]. However, this optimum pH was higher compared to other bacterial strains such as*Acetobacter diazotropicus* SRT4 (pH5.5)[71], *Clostridium acetobutylicum* (pH5.5)[20], *Streptomyces* sp. ALKC 4 (pH6)[25], *P. mucidolens* (pH6.0)[16], *Streptomyces grisenus* (pH7)[22], *Bacillus polymyxa* MGL21 (pH7)[11],*B. stearothermophilus* KP1289 (pH7)[13], *Streptococcus salivarius* (pH7)[18], *Arthrobacter* sp. (pH7.5)[72] and *C. Thermoautotrophicum* (pH7.5)[21]. Loss of inulinase activity beyond the optimum pH could be as a result of the changes in the state of acidic or basic amino acids in the protein. Changes in pH may also change the shape or charge properties of the substrate so that neither the substrate can bind to the active site nor it can undergo catalysis[73].

Bacterial inulinases generally have temperature optima in the range 30–60°C[24]. The Inulinase enzyme from *Bacillus* sp. B51f demonstrated optimum activity at 55°C but it also retained enzyme activity between 30°C to 70°C indicating its thermostable nature (Figure 9). Parallel results were also observed for exoinulinase from the bacterium *Geobacillus stearothermophilus* KP1289 which was active between 30°C and 75°C with an optimum at 60°C[13] whereas the inulinase from *Bacillus subtilis* 430A was stable at an optimal temperature of45 to 50°C[9].In contrast, the exoinulinase activity produced by B. *polymyxa* MGL21 is optimal at 35°C[11]. The past research work showed that the inulinases from *Staphylococcus* sp., *Arthrobacter* sp., *Streptomyces* sp. CP01, *Marinimicrobium* sp. LS-A18 and *Pseudomonas mucidolens* were optimallyactive at 50°C-55°C[19,16,15,17,23] whereas inulinases from *C. thermoautotrophicum, Streptomyces* sp. GNDU1[24] have optimum temperature of 60°C. The inuliases from *Streptomyces* sp. ALKC4 and *Thermotoga maritima* were reported to be optimally active at70°C and 90° Crespectively indicating the optimal temperatures of the exoinulinase from different species of bacteria are significantly different[25,74].

In an attempt to further characterize the inulinase enzyme from *Bacillus* sp. B51f; various metal ions were tested on inulinase enzyme activity. Residual activity of the inulinase enzyme was positively enhanced by Ca^{2+} andwasmarginally inhibited by Mg^{2+}, Cd^{2+}, $Na^{+}$and K^{+} whereas Zn^{2+} and Hg^{2+} inhibited it by 30% and 10% respectively (Figure 10). Similar results were reported for pure inulinase enzyme from *Streptomyces grisenus*; itwas stable in the presence of $CaCl_2$ and was inhibited bycompounds such as

$ZnSo_4$, $MgSo_4$, $MnSo_4$, $FeSo_4$, $CuSo_4$ and $MnCl_2$[22] while Zn^{2+} and Hg^{2+} were inhibitors for inulinase from *P. mucidolens*[16]. The past research work of inulinase from *Bacillus cereus* MU-31 wasstimulated by Mg^{2+} and was strongly inhibited by $Hg^{2+}$and Pb^{2+}at 1mM concentration[14] whereas inulinase from *Streptomyces* sp. ALKC4 and *Streptomyces* CP01 tolerated several metal salts except $HgCl_2$[25,23]. Garuba and Onilude reported a total loss of activity of inulinase from *Saccharomyces* sp. inthe presence of Hg^{2+} while in the presence of K^+ and Ca^{2+} the inulinase activity was increased[73]. A number of inulinases produced from other microorganisms were found to be Ca^{2+} dependent[75]. Cofactors are generally not required for inulinase activity but divalent cations such as Ca^{2+} often stimulate the enzyme activity. The negative effect of ions on the inulinase is generally the result from direct inhibition of the catalytic site like many other enzymes. The strong inhibitory effect observed with Hg^{2+} suggested that some –SH- group in the protein might be essential for the activity of inulinase. This has been also observed for other microbial inulinases[76,77,78]. This effect may be due to the formation of complex with ionized inulinase resulting in changing solubility and behavior at the substrate interfaces.

The effect of various thermal stabilizers on inulinase activity from *Bacillus* sp. B51fwas analyzed. Mannitol and propanol retained 73% and 87% of the enzyme activity respectively whereas Glycerol enhanced the activity by 105% (Figure 11). Similar results were observed with *Streptomyces* sp. ALKC4 where glycerol and mannitol exhibited a protective effect on inulinase enzyme activity[25]. Garuba and Onilude also reported stabilizing effect of glycerol on inulinase from *Saccharomyces* sp[73]. Amongst the thermal stabilizers used, glycerol exhibited a significant stabilizing effect on the enzyme suggesting that the thermostability of the enzyme can be further enhanced, thus making it suitable for commercial application[15]. Glycerol having the best stabilizing effect could be as a result of preferential exclusion of the polyols with proteins, which increases with an increasing polyol size[6], resulting in an indirect interaction that prevent the protein from thermal unfolding[79].Similar results have been previously reported by Öngen-Baysal et al.[63] and Taylor et al.[80].

Analysis of Hydrolysis products of inulinase by HPTLC

Inorder to confirm the exo- or endo- nature of the crude inulinase produced by *Bacillus* sp. B51f, HPTLC analysis was carried out. The HPTLC analysis of the hydrolysis product of inulunase demonstrated that fructose was the major sugar produced during hydrolysis (Figure 12). This supports the view that inulinase is an end group cleaving enzyme[4]. It was concluded that the nature of inulinase produced by *Bacillus* sp. B51f is of exoinulinase type suggesting its potential application for the production of ultra-high fructose syrup. In this respect, it resembles the exoinulinases of *Marinimicrobium* sp. LS-A18[15], *Bacillus polymyxa*[11], *Streptomyces* sp. ALKC4[25], *Streptomyces* sp. GNDU1[23], *Geobacillus stearothermophilus*[13], *A. awamori*[81], *Kluyveromyces marxianus* sp.[27,82], *Saccharomyces* sp.[73] and *Erwinia* sp.[83].

Fig.1: Screening of inulin degrading organism on MHI agar plate stained with Lugol's iodine solution. Zone of clearance of inulinase enzyme produced by AS-1 isolate

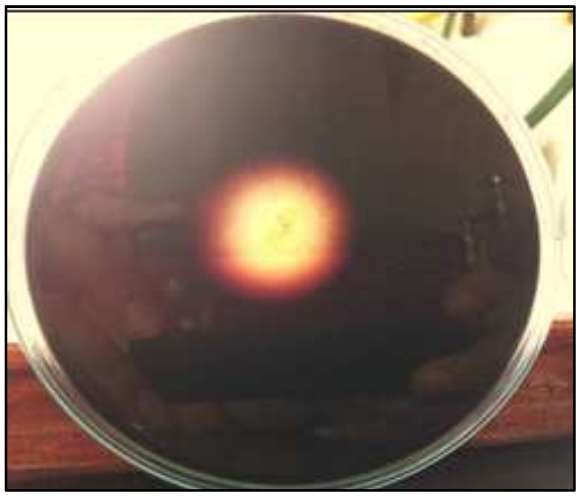


Fig.2: Effect of different media on inulinase production by*Bacillus* sp.B51f

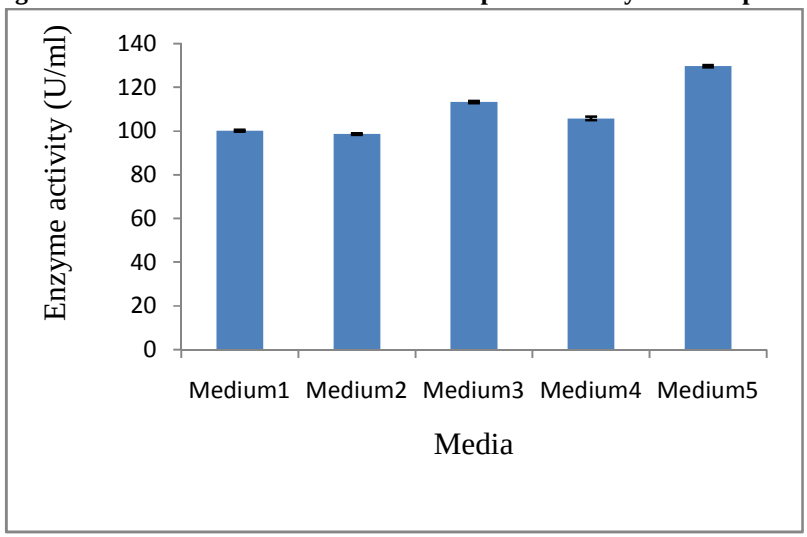


Fig.3: Effect of incubation period on inulinase production by*Bacillus* sp.B51f

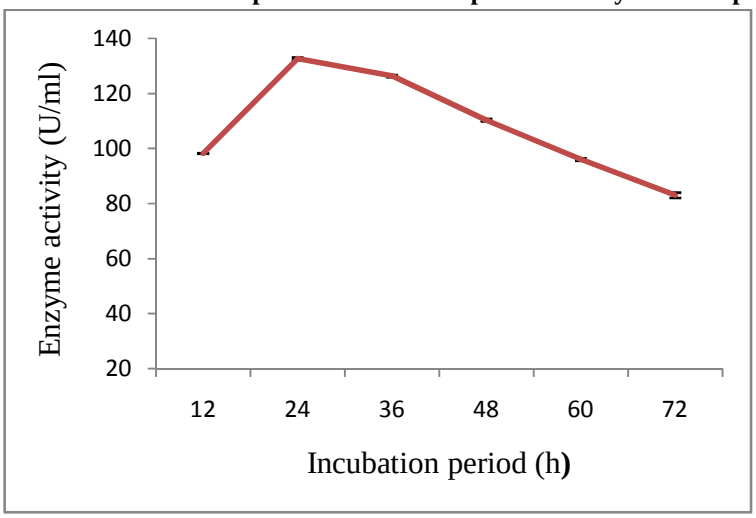


Fig.4: Effect of incubation temperature on inulinase production by*Bacillus* sp.B51f

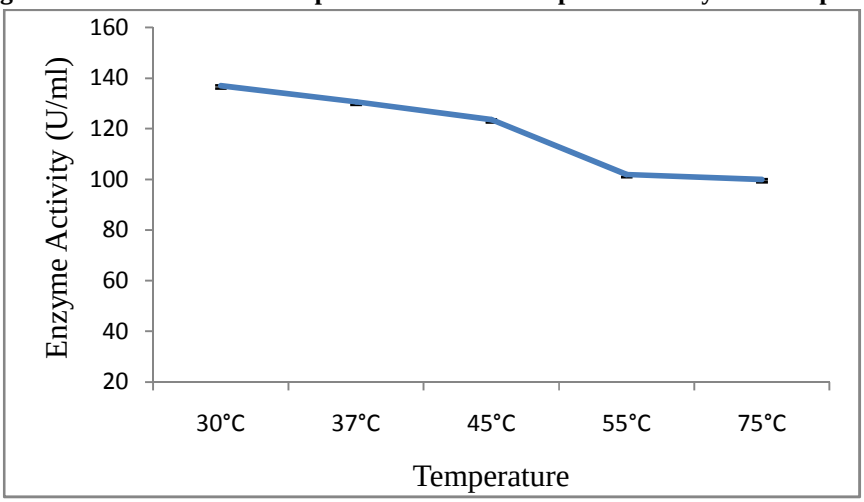


Fig.5: Effect of different pH on inulinase production by*Bacillus* sp.B51f

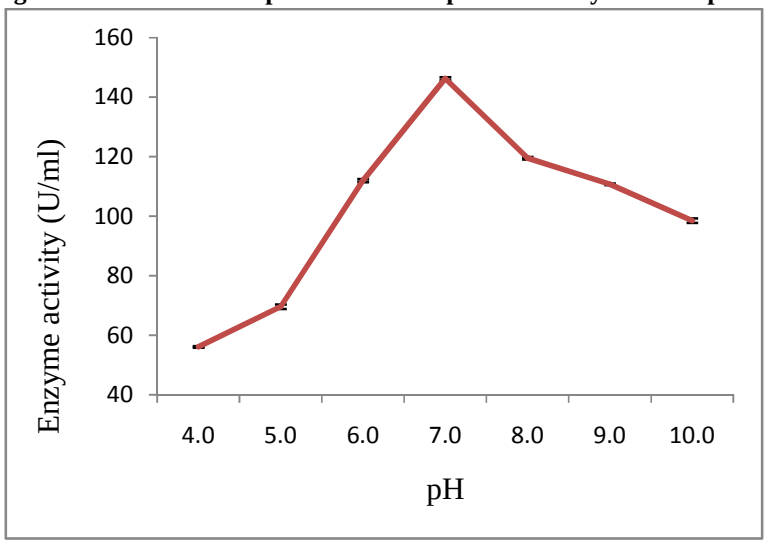


Fig.6: Effect of different carbon sources on inulinase production by*Bacillus* sp.B51f

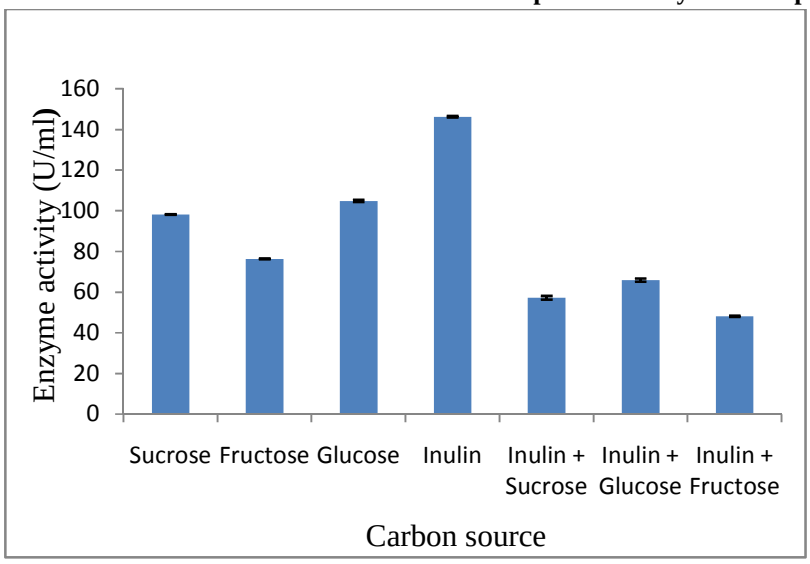


Fig.7: Effect of different nitrogen sources on inulinase production by*Bacillus* sp.B51f

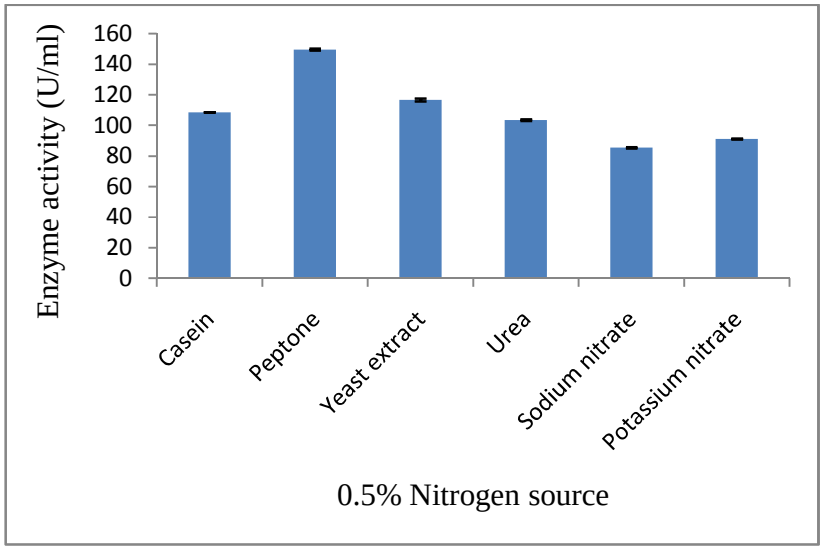


Fig.8: Effect of different pH on activity of inulinase from*Bacillus* sp.B51f

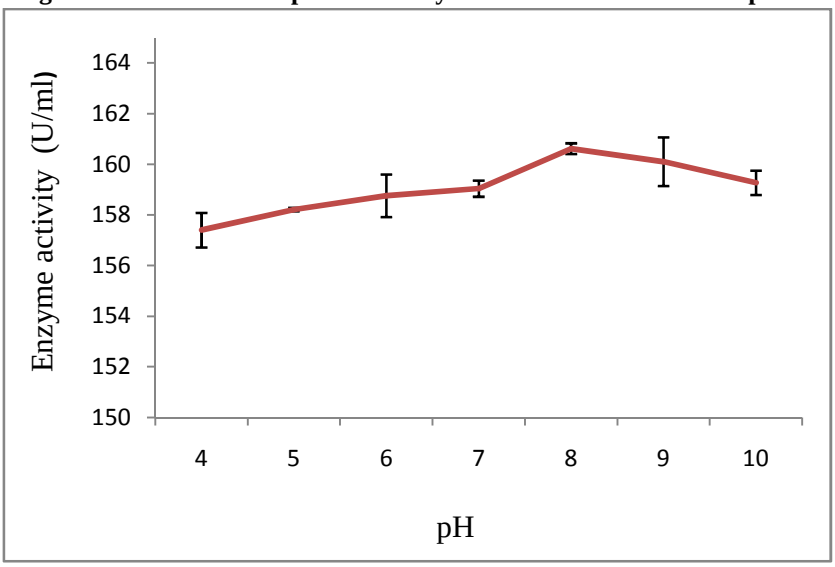


Fig.9: Effect of different temperature on activity of inulinase from*Bacillus* sp.B51f

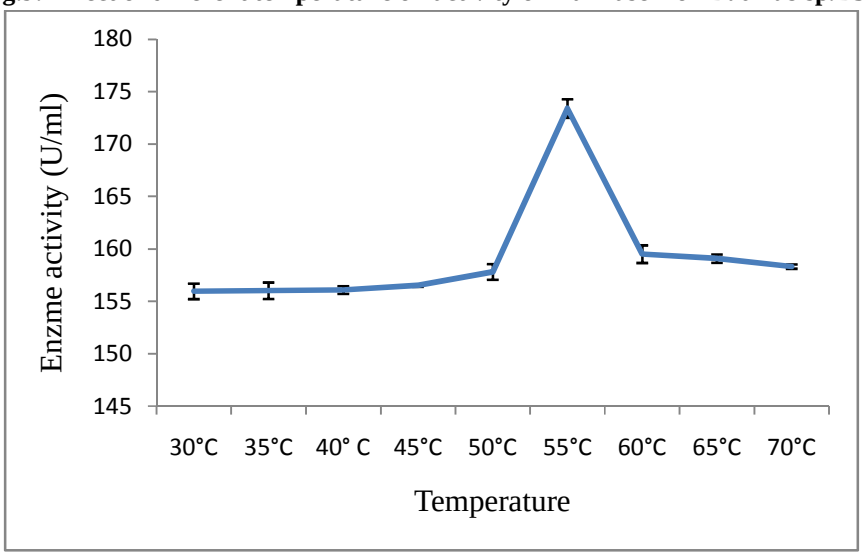


Fig.10: Effect of different metal ions on activity of inulinase from*Bacillus* sp.B51f

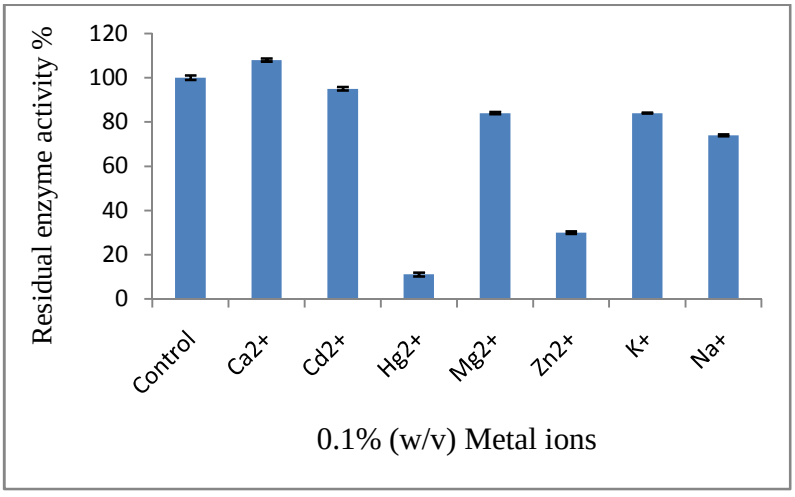


Fig.11: Effect of different stabilizers on activity of inulinase from*Bacillus* sp.B51f

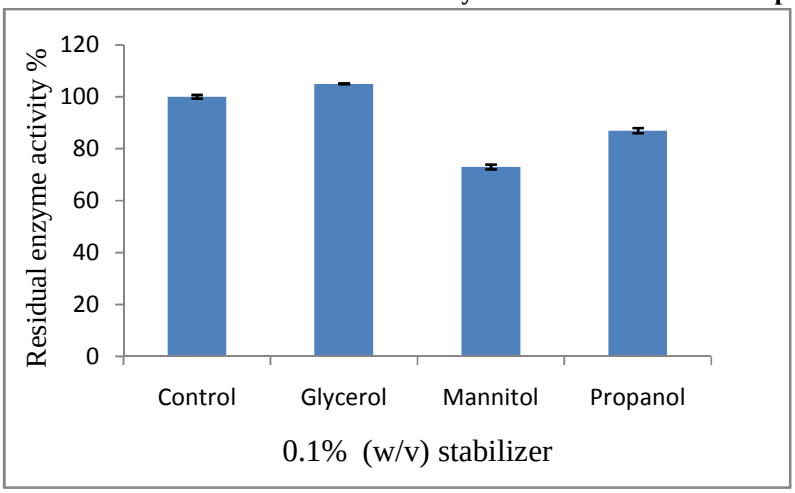
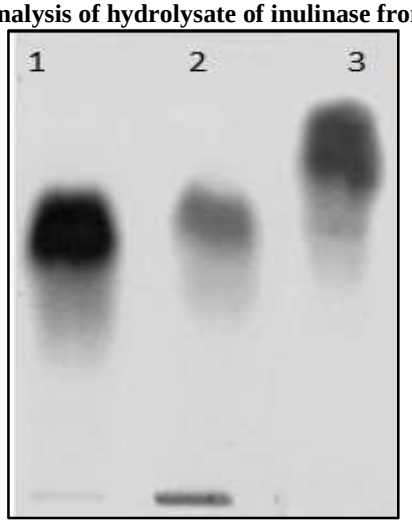


Fig.12: HPTLC analysis of hydrolysate of inulinase from*Bacillus* sp.B51



Lane1- 10µl of Fructose, Lane 2- 10µl of Sample hydrolysate, Lane 3- 10µl of Glucose

Table 1: Effect of different concentration of Inulin and peptone on production of inulinase by *Bacillus* sp. B51f

Inulin conc. (%)	Enzyme activity (U/ml) Mean±SD	Peptone conc.(%)	Enzyme activity (U/ml) Mean±SD
0.5	129 ± 0.92	0.5	149.64 ± 0.72
1.0	146.22 ± 0.33	0.6	151.2 ± 0.04
1.5	150.9 ± 0.01	**0.7**	159.52 ± 0.83
2.0	113.63 ± 0.56	0.8	147 ± 0.77
		0.9	139.12 ± 0.79
		1.0	136.4 ± 0.12

Table 2: Effect of different concentrations of salts on production of inulinase by *Bacillus* sp. B51f

Conc. (%)	Enzyme activity (U/ml) (Mean±SD)			
	Na_2HPO_4	K_2HPO_4	NH_4Cl	NaCl
0.1	149.2 ± 0.93	154.3 ± 0.24	158.1 ± 0.7	154.02 ± 0.4
0.2	151.25 ± 0.04	156.4 ± 0.41	158.7 ± 0.08	154.7 ± 0.69
0.3	158.6 ± 0.51	157.8 ± 0.84	159.3 ± 0.61	155.56 ± 0.92
0.4	156.1 ± 1.01	160.24 ± 0.53	158.4 ± 0.65	156.4 ± 0.65
0.5	154.32 ± 0.32	156.5 ± 0.99	157.2 ± 0.71	154.9 ± 0.75

CONCLUSION

The present work reveals that *Bacillus sp.* B51f isolated from rhizosphere soil of *Agave sisalana* is a promising organism for industrial production of thermostable exoinulinase. Inulinase is one of the key enzymes in the food industry which can be used for production of fructose from inulin.The results of this study warrant further characterization of the strain for commercial exploitation for food and pharmaceutical industries.

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REFERENCES

1. Chi, Z.M. Zhang, T. Cao, T.S. Liu, X.Y. Cui, W. Zhao, C.H., Biotechnological potential of inulin for bioprocesses. *Bioresource Technology*. **102**: 4295-4303 (2011)
2. Chi, Z. Chi, Z. Zhang, T. Liu, G. Li, J. Wang, X., Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications. *Biotechnology Advances*. **27**: 236- 255 (2009).
3. Ertan, F. Aktac, T. Kaboglu, C. Ekinci, F. Bakar, E., Determination of optimum cultivation conditions on the production of inulinase from *Rhizoctonia solani*. *Pakistan Journal of Biological Sciences*. **6(16)**:1386-1388 (2003)
4. Camelia Neagu (bonciu), Gabriela Bahrim Inulinases–A versatile tool for biotechnology. *Innovative Romanian Food Biotechnology*. **9**: 1-11(2011).
5. Pandey, A. Soccol, C.R. Selvakumar, P. Soccol, V.T. Krieger, N. Fontana, J.D., Recent developments in microbial inulinases. *Applied Biochemistry and Biotechnology.* **81**: 35-52 (1999)
6. Liu, X.Y. Chi, Z. Liu, G.L. Wang, F. Madzak, C. Chi, Z.M., Inulin hydrolysis and citric acid production from inulin using the surfaceengineered *Yarrowia lipolytica* displaying Inulinase. *Metabolic Engineering*. **12**: 469-476 (2010)
7. Rowland, I.R. Rumney, C.J. Coutts, J.T. Lievense, L.C., Effect of *Bifidobacteriumlongum* and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogensis,* **19**: 281–285 (1998)
8. Saha, B.C., Production of mannitol from inulin by simultaneous enzymatic saccharification and fermentation with *Lactobacillus intermedius* NRRL B-3693, *Enzyme and Microbial Technology*, **39**: 991-995 (2006)
9. Vullo, D. Coto, C. Sineriz, F., Characteristics of an Inulinase produced by *Bacillus subtilis* 430A isolated from the rhizosphere of Vernonia herbacae(Vell Rusby). *Applied and Environmental Microbiology*. **579(8)**: 2392-2394 (1991)
10. Zherebtsov, N. Shelamova, S.A. Abramova, IN. Biosynthesis of inulinases by *Bacillus* bacteria. *Applied Biochemistry and Microbiology*. **38(6)**: 634-638 (2002)
11. Kwon, H.J. Jeon, S.J. You, D.J. Kim, K.H. Jeong, Y.K. Kim, Y.H. Kim, Y.M. Kim, B.W., Cloning and characterization of an exoinulinase from *Bacillus polymyxa*. *Biotechnol Lett*., **25**:155–159 (2003)
12. Allais, J.J. Hoyos-Lopez, G. Baratti, J.C., Characterization and properties of an inulinase from a thermophilic bacteria, *Carbohydr. Polym.* **7**: 277–290 (1987)
13. Kato K., T. Araki, T. Kitamura, N. Morita, M. Moori, Y. Suzuki, Purification and properties of a thermostable inulinase (β-D-fructan fructohydrolase) from *Bacillus stearothermophilus* KP1289, *Starch*, **51**:253–258 (1999)
14. Meenakshi, S.S. Umayaparvathi, P. Manivasagan, M. Arumugam & Balasubramanian, T., Purification and characterization of Inulinase from marine bacterium, *Bacillus cereus* MU-31 *Indian Journal of Geo-Marine Sciences.* **42(4)**: 510-515 (2013)
15. Li, A.X. Guo, L.Z. Lu, W.D., Alkaline Inulinase production by a newly isolated bacterium *Marinimicrobium* sp. LS-A18 and inulin hydrolysis by the enzyme. World *J Microbiol Biotechnol*. **28**: 81-89 (2012)

16. Kwon, Y.M. Kim, H.Y. Choi, Y.J., Cloning and characterization of Pseudomonas mucidolens exoinulinase. *J Microbiol Biotechnol*.**, 10(2**): 238–243 (2000)
17. Tanaka, K. Uchiyama, T. and Ito, A., Formation of di-D-fructofuranose 1,2': 2,3'-dianhydride form by an extracellular inulase of *Arthrobacter ureafaciens*. *Biochim. Biophys.Acta,* **284**: 248-256 (1972)
18. Takahashi, N. Mizuno, F. Takamori, K., Purification and preliminary characterization of exo-beta-D-fructosidase in *Streptococcus salivarius* KTA-19, *Infect. Immun.* **47**: 271–276 (1985)
19. Selvakumar, P. Pandey, A., Solis state fermentation for the synthesis of Inulinase from *Staphylococcus* sp. and *Kluyveromyces marxianus*. *Process Biochemistry*. **34**: 851-855(1999)
20. Looten, P. Blanchet, P.D. Vandecasteele, J.P., The β-fructofuranosidase activities of a strain of *Clostridium acetobutylicum* grown on inulin, *Appl. Microbiol. Biotechnol.* **25**: 419–425 (1987)
21. Drent, W.J. Gottschal, J.C., Fermentation of inulin by a new strain of *Clostridium thermoautotrophicum* isolated from *Dahlia* tubers, *FEMS Microbiol. Lett.* **78**: 285–292 (1991)
22. Tohamy Eman, Y. Purification and Characterization of Exoinulinase Enzyme from *Streptomyces grisenus*. *Pakistan Journal of Biological Sciences,* **9**: 911-916 (2006)
23. Nirobol Laowklom, Rungtrakarn Chantanaphan, Pairoh Pinphanichakarn Production, Purification and Characterization of Inulinase from a Newly Isolated *Streptomyces* sp. CP01. *Natural Resources,* **3**: 137-144 (2012)
24. Gill, P.K. Sharma, A.D. Harchand, R.K. Singh, P., Effect of media supplements and culture conditions on inulinase production by an actinomycete strain. *Bioresour. Technol.* **87**: 359–362 (2003)
25. Sharma, A.D. Gill, P.K., Purification and characterization of heat-stable exo-inulinase from Streptomyces sp. *Journal of food Food engineering*. **79**: 1172-1178 (2007)
26. Singh, R.S. Dhaliwal R. and Puri, M., "Production of In- ulinase from *Kluyveromyces marxianus* YS-1 Using Root Extract of *Asparagus racemosus*," *Process Biochemistry*, **41(7)**: 1703-1707 (2006)
27. Singh, R.S. Bhermi, H.K., Production of extracellular exoinulinase from Kluyveromyces marxianus YS-1 using root tubers of Asparagus officinalis. *Bioresour. Technol*., **99**: 7418-7423 (2008)
28. Kushi, R.T. Monti, R. Contiero, J., Production, purification and characterization of an extracelular inulinase from *Kluyveromyces marxianus* var. bulgaricus. *J. Ind. Microbiol. Biotechnol*., **25**: 63-69(2000)
29. Pessoni, R.A.B. Fgueiredo, R. Braga, M.R., Extracellular inulinases from *Penicillium janczewskii. J. Appl. Microbiol.* **87**: 141-147 (1999)
30. Nakamura, T. Shitara, A. Matsuda, S. Matsuo, T. Suiko, M. Ohta, K., Production, purification and properties of an en- do-inulinase from Penicillium sp. TN-88, *J. Ferment. Bioeng*., **84**: 313–318 (1997)
31. Uhm, T.B. Chung, M.S. Lee, S.H. Gourronc, F. Housen, I. Kim, J.H. Van Beeumen, J. Haye, B. Vandenhaute, J., Puri- fication and characterization of *Aspergillus ficuum* endoinulinase, *Biosci. Biotechnol. Biochem*. **63**: 146–151(1999)
32. Ettalibi, M. Baratti, J.C., Purification, properties and comparison of invertase, exoinulinases and endoinulinases of *Aspergillus ficuum*, *Appl. Microbiol. Biotechnol.* **26**: 13–20 (1987)
33. Gupta, A.K. Rathore, P. Kaur, N. Singh, R., Production, thermal stability and immobilisation of inulinase from *Fusarium oxysporum*, *J. Chem. Technol. Biotechnol*., **47**: 245–257 (1990)
34. Kaur, N. Kaur, M. Gupta, A.K. Singh, R., Properties of β-fructosidases (invertases and inulinases) of *Fusarium oxysporum* grown on aqueous extract of Cichorium intybus roots, *J. Chem. Technol. Biotechnol*., **53**: 279–284 (1992)
35. Bharathi, S. Saravanan, D. Radhakrishnan, M. Balagurunathan, R., Bioprospecting of marine yeast with special reference to Inulinase production. *International Journal of Chemtech Research.* **3(3)**: 1514-1519 (2011)
36. Singh, P. and P.K. Gill: Production of Inulinases, Recent Advances. *Food Technol. Biotechnol*., **44 (2)**: 151–162 (2006)

37. Nagem, R.A.P. Rojas, A., Golubev, A.M. Korneeva, O.S. Eneyskaya, E.V. Kulminskaya, K.N. Neustroev, K.N. Polikarpov, I., Crystal structure of exo-inulinase from *Aspergillus awamori*: the enzyme fold and structural determinants of substrate recognition. *J. Mol. Biol*., **344**: 471-480 (2004)
38. Molina, D.L. Martinez, M.D.N. Melgarejo, F.R. Hiner, A.N.P. Chazarra, S. Lopez, J.N.R., Molecular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.).*Phytochemistry*. **66**:1476-1484 (2005)
39. Cazetta, M. L., Martins, P. M. M., Monti, R., & Contiero, J. Yacon (Polymnia sanchifolia) extract as a substrate to produce inulinase by *Kluyveromyces marxianus* var. bulgaricus. *Journal of Food Engineering*, **66**: 301–305 (2005)
40. Trojanova, I. V. Rada, L. Kokoska and Vlkova E. The Bifidogenic Effect of *Taraxacum officinale* Root," *Fi- toterapia*, **75 (7-8)**: 760-763 (2004)
41. Gupta A K, Kaur N Fructan storing plants—a potential source of high fructose syrups. *J Sci Ind Res* ,**56**: 447–452 (1997)
42. Lopez M, Norma A, Margalli M, Diaz GM**.**Molecular Structures of Fructans from *Agave tequilana* Webervar. *azul*. *Journal of Agricultural and Food Chemistry*. **51**:7835-7840 (2003)
43. Vandamme, E. J., and D. G. Derycke. Microbial inulinases: fermentation process, properties and applications. *Adv. Appl. Microbiol*. **29**:139-176 (1983)
44. Sharma, A.D. Kainth, S. Gill, P.K., Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. *Journal of Food Engineering*. **77**: 486-491(2006)
45. Regina M.M. Gern Sandra A. Furlan Jorge L. Ninow Rainer Jonas Screening for microorganisms that produce only endoinulinase. *Appl Microbiol Biotechnol*., **55**: 632–635 (2001)
46. Li Ai-Xia, Li-Zhong Guo, Qiang Fu and Wei-Dong Lu A simple and rapid plate assay for screening of inulin degrading microorganisms using Lugol's iodine solution. *African Journal of Biotechnology*, **46(10)**: 9518-9521(2011)
47. Buchanan R.E. and Gibbons N.E., Bergey's Manual of Determinative Bacteriology, Eighth Edition. The Williams and Wilkins Company/ Baltimore. pp 529-550(1974)
48. Miller, G. L. Use of dinitrosalicylic acid for determination of reducing sugar. *Anal. Chem.*, **31**:426-428 (1959)
49. Mazutti, M.A. Skrowonski, A. Boni, G. Zabot, G.L. Silva, M.F. Oliveira, D. Luccio, M. Filho, F.M. Rodrigues, M.I. Treichel, H., Partial characterization of inulinases obtained by submerged and solid-state fermentation using agroindustrial residues as substrates: a comparative study. *Appl. Biochem. Biotechnol*., **160**: 682-693 (2010)
50. Kalil, S.J. Barboza, M., Studies on the Adsorption of Inulinase from Kluyveromyces marxianus ATCC 16045 onto an Ion Exchange Resin. *Braz. J. Food Technol*., **9(3)**: 223-228 (2006)
51. Dilipkumar, M. Rajasimman, M. Rajamohan, N., Application of statistical design for the production of inulinase by *Streptomyces* sp. using pressmud. *Front. Chem. Sci. Eng*., **5**: 463–470 (2011)
52. Gill, P.K. Manhas, R.K. Singh, P., Comparative analysis of thermostability of extracellular inulinase activity from *Aspergillus fumigates* with commercially available (Novozyme) Inulinase. *Bioresource Technology*. **97**: 355-358 (2006)
53. Gao, L. Chi, Z. Sheng, J. Wang, L, Li, J. Gong, F., Inulinase-producing marine yeasts: evaluation of their diversity and inulin hydrolysis by their crude enzymes. *Microbial Ecology*. **54**: 722-729 (2007)
54. Partida, Z. Lopez, A.C. Gomez, A.J.M., Method of producing fructose syrup from agave plants. *US patent 5846333* (1998)
55. Gouda, M. Some properties of inulinase from *Aspergillus fumigatus*. *Pakistan Journal of Biological Sciences*, **5(5)**: 589-593(2002)
56. Dinarvand Mojdeh, Arbakariya B. Ariff, Hassan Moeini, Malihe Masomian' Seyed Sadegh Mousavi, Reza Nahavandi, Shuhaimi Mustafa. Effect of extrinsic and intrinsic parameters on inulinase production by *Aspergillus niger* ATCC 20611, *Elecronic Journal of Biotechnology*,**15(4)**: fulltext-9, (2012)

57. Masomian, M. Rahman, R.N.Z.R.A. Salleh, A. and Basri, M., A unique thermostable and organic solvent tolerant lipase from newly isolated *Aneurinibacillus thermoaerophilus* strain HZ: Physical factor studies. *World Journal of Microbiology and Biotechnology*, **26(9)**:1693-1701(2010)
58. Ebrahimpour, A. Rahman, R.N.Z.R.A. Kamarudin, N.H.A. Basri, M. And Salleh, A.B., Lipase production and growth modeling of a novel thermophilic bacterium: *Aneurinibacillus thermoaerophilus* strain AFNA. *Electronic Journal of Biotechnology*,**14(4)**: full text 8 (2011)
59. Yokota, A. Hirayama, S. Enomoto, K. Miura, Y. Takao, S. Tomita, F., Production of Inulin Fructotransferase (depolymerizing) by *Artrobacter* sp. H65-7 and Preparation of DFA III from Inulin by the Enzyme, *Journal of Fermentation and Bioengineering*,**72(4)**: 258-261 (1991)
60. Elyachioui, M. Horeiz, J.P. Taillez, R., General properties of extracellular bacterial inulinase, *J. Appl. Bacteriol.*, **73**: 514–519(1992)
61. Allais, J.J. Kammoun, S. Blanc, P. Girard, C. Baratti, J.C., Isolation and characterization of bacterial strains with inulinase activity. *Appl. Environ. Microbiol.*, **52**: 1086-1090 (1986)
62. Singh RS, Sooch BS, Puri M Optimization of medium and process parameters for the production of inulinase from a newly isolated *Kluyveromyces marxianus* YS-1. *Biores Technol.*, **98**: 2518–2525 (2007)
63. Ongen-Baysal, G., Sukan, S.S., Vassiler, N. Production and properties from *Aspergillus niger*. *Biotechnol. Lett.*, **16**: 275–280 (1994)
64. Kaur, A., Sharma, D., Harchand, R.K., Singh, P., Bhullar, S.S., Kaur, A., Production of a thermostable extracellular inulinase by Aspergillus fumigatus. *Indian J. Microbiol.*, **39**: 99–103 (1999)
65. Gao, W., Y. Bo, Y. Liu, X. Zhang, J. Wang and L. An, "Characterization of Thermo-Stable Endoinulinase from a New Strain *Bacillus smithii* T7," *Applied Biochemistry and Biotechnology*, **157(3)**: 498-506 (2009)
66. Ayyachamy, M.K. Khelawan, D. Pillay, K.P. Singh, S., Production of Inulinase by *Xanthomonas campestris* using onion and garlic peels. *Letters in Applied Microbiology*. **45**: 439-444 (2007)
67. Cruz VD, Belote JG, Belline MZ, Cruz R. Production and action pattern of inulinase from *Aspergillus niger*-245: Hydrolysis of inulin from several sources. *Rev. Microbiol.*,**29(4)**: 257-261 (1998)
68. Kim, K. C., Studies on the hydrolysis of inulin in Jerusalem artichoke by fungal inulinase. *J. Korean Agric. Chem. Soc*. **18**: 177-182 (1975)
69. Gupta, A.K., Rathore, P., Kaur, N. & Singh, R. Production, thermal stability and immobilization of Inulinase from *Fusarium oxysporum*. *Journal of Chemical Technology and Biotechnology***47**:245–257(1990)
70. Choi, W.S. Choe, Y.K. Kim, S.I. Byon, S.M., Production of inulinase using Jerusalem artichoke tuber extract. J. *Korean Agric. Chem. Soc.* **27**: 238–244 (1984)
71. Tambara, Y. Hormaza, J.V. Perez, C. Leon, A. Arrieta, J. Hernandez, L., Structural analysis and optimized production of fructo-olgiosaccharides by levansucrase from *Acetobacter diazotrophicus* SRT4. *Biotechnol. Lett.*, **21**: 117–121(1999)
72. Kang, S.I. Chang, Y.J. Oh, S.J. Kim, S.I., Purification and properties of an endo-inulinase from an *Arthrobacter* sp., *Biotechnology Letters.*, **20(10)**: 983-986 (1998)
73. Garuba, Emmanuel Oluwaseun and Onilude Anthony Abiodun Characterization of Crude Thermostable Exoinulinase Produced by *Saccharomyces* sp. *AU J.T.* **16(2)**: 81-88 (2012)
74. Liebl, W. Brem, D. Gotschlich, A., Analysis of the gene for βfructosidase (invertase, inulinase) of the hyperthermophilic bacterium *Thermotoga maritima* and characterization of the enzyme expressed in *Escherichia coli*, *Appl. Microbiol.Biotechnol.* **50**: 55–64 (1998)
75. Kim, Y.H. Nam, S.W. Chung, B.H., Simultaneous saccharification of inulin and ethanol fermentation by recombinant *Saccharomyces cerevisiae* secreting Inulinase. *Biotechnol. Bioprocess.* **3**: 55-60 (1998)
76. Ettalibi, M. Baratti, J.C., Molecular and kinetic properties of *Aspergillus ficuum* inulinases. *Agric. Biol. Chem.*, **54**: 61-68 (1990)

77. Kim, K.Y. Nascimento, A.S. Golubev, A.M. Polikarpov, I. Kim, C.S. Kang, S.I. Kim, S.I., Catalytic mechanism of inulinase from *Arthrobacter* sp. S37. *Biochemical andBiophysical Research Communications.* **371**: 600- 605 (2000)
78. Kocchar A, Kaur N, Gupta AK. Inulinase as a potent enzyme for producing fructose from inulin. *Journal of Scientific and Industrial Research.* **56**:721-726 (1997)
79. Taravati, A.; Shokrzadeh, M.; Ebadi, A.G.; Valipour, P.; Hassan, A.T.M.; and Farrakhi F. Various effects of sugar and polyols on the protein structure and function: role as osmolyte on protein stability. World Applied Sciences Journal **2(4)**: 353-62. (2007)
80. Taylor, L.S. York, P. Williams, A.C., of protein denaturation by a chaotropic agent. *Biochimica et Biophysica Acta 1*, **253(1)**: 39-46 (1995)
81. Arand, M. Golubev, A.M. Neto, J.R.B. Polikarpov, I. Wattiez, R. Korneeva, O.S. *et al.* Purification, characterization, gene cloning and preliminary X-ray data of the exo-inulinase from *Aspergillus awamori*. *Biochem J.*, **362**: 131–5 (2002)
82. Jain Sumat Chand, P.C. Jain, Naveen Kango. Production of inulinase from *Kluyveromyces marxianus* using dahlia tuber extract, *Brazilian Journal of Microbiology* 62-69 (2012)
83. Abraham Amith and Jayachandran, K., Fermentative Production of the Extracellular Exo-acting Inulinase from a Novel Strain of Erwinia sp. *Res. J. Biotech*, **2(2)**: 45-49 (2007)