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# Purification and Characterization of Exoinulinase Enzyme from *Streptomyces grisenus*

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**Abstract:** *Streptomyces grisenus* isolated from soil, was found to produce a very active inulinase enzyme. The optimum growth and inulinase activity occurred in the presence of 0.5% inuline, 0.3% $NaNO_3$ and 0.5% $CaCl_2$ in the production broth medium. Maximum growth was observed after 2 days at 30°C and pH 7 under shaking condition. Ammonium sulphate (70%) precipitation of cell free filtrate of *Streptomyces grisenus* increase the specific enzyme activity by 2.3 folds, further purification by gel filtration using Sephadex $G_{100}$ increased the specific enzyme activity to 6.2 folds, respectively. Analysis of purified enzyme using gel electrophoresis revealed an apparent molecular weight of 140 KDa. Characterization of purified inulinase enzyme showed the maximum activity at pH 7, increased activity up to 40°C and incubated for 24 h. It was showed that inulinase activity was inhibited by all compounds ($ZnSo_4$, $MgSo_4$, $MnSo_4$, $FeSo_4$, $CuSo_4$ and $MnCl_2$) and the loss of activity reached to about 100, 93, 80, 76, 42 and 20%, respectively. However pure enzyme was stable in the presence of $CaCl_2$. The purified enzyme was immobilized on (DEAE cellulose with 6% glutaraldehyde) gave better yield of activity, conc. of enzyme constant between 1.4 μM of inuline. The purified inulinase enzyme was subjected to N-terminal sequence analysis.

**Key words:** *Streptomyces grisenius,* purification, extracellular inulinase, N-Terminal sequence analysis

## INTRODUCTION

Microbial enzyme inulinase (EC 3.2.1.7) hydrolyze plant polymer inuline into fructooligo sacharides and pure fructose with some glucose Theoretically, inulin showed contain 30 sugar units at aminimum. Oligosaccharides are compounds with great potential of use in food industry particularly interesting are fructooligosaccharids (FOS), because of their favorable functional properties such as low calorie and noncariogenic sweeteners, improvement of the intestinal microbial flora, relief of constipation, decrease of total cholesterol and lipid in the serum and promotion of animal growth (Vandamme and Derycke, 1983). Inulin can be considered as dietary fibre, a substitute for fat and low calorie sweetener (Roberfroid, 1993). Prebiotics exert their beneficial effects through direct and selective stimulation of healthy bacterial species in the colon flora, Ingestion of the prebiotic inulin leads to increased content of beneficial bacteria from genera *Bifidobacterium* and *Lactobacillus* in the colon there by promoting gut health. The fermentation products from these groups of bacteria give rise to local and systemic health effects. Those effects are: lowered colonic pH. Increased bioavailability of minerals, lowering of serum lipids levels (relevant for cardiovascular disease) and stimulation immune response (Coudray *et al*.,1997). Brand names of inulin are raftilin or raftilose (ORAFTT Active food ingredients, Belgium) and frutafit (SENSUS, Netherlands). Fructose formation from complete hydrolysis of inulin is a single step inulinase reaction and yields up to 95% of frutose. Conventional fructose production from starch needs at least three enzymatic steps, yielding only 45% of fructose. Inulinase can be used for production of pure fructose syrups and Ultra High Fructose "Glucose syrups" (UHFGS), not from starch but from inulin (Pandey *et al*., 1999). Inulinase can be found in plants and microorganisms. It is difficult to isolate plant inulinases in sufficient quantity. Therefore, microbial inulinases which can be induced by growing microorganisms, have a potential for industrial use in the production of fructose frminulin (Edelman and Jefford, 1964). Many micro organisms, including filamentous fungi, yeast, bacteria and *streptomyces* sp. are able to produce inulinase (Wei *et al*., 1999). *Streptomyces* sp. produced high levels of extracellular inulinase and a potential candidate for industrial enzymatic production of fructose from inuline.

## MATERIALS AND METHODS

**Organism:** Highly active isolate of *Streptomyces grisenus* isolated from soil sample and identified according to Cause *et al*. (1957), Waksman (1961), Shirling and Gottlieb (1966, 1968a, b, 1969 and 1972) and Williams *et al*. (1984).

**Screening medium:** The medium used for the production of inulinase contained (8 $L^{-1}$) according to Lim *et al.* (1998): Inulin 5.0, yeast extract 3.0, $NaNO_3$, 3.0, $CaCl_2$ 5.0 adjust at pH 7 and distilled water up to 1000 mL.

**Enzyme assay:** Enzyme solution (0.1 mL) was mixed with 0.1% inulin (1 mL) in 0.1 m sodium acetate buffer pH 5.0 and the mixture was incubated at 35°C for 15 min. As a result of reaction, reducing sugar was determined by the 3.5 dinitrosalisilic acid method, (Miller, 1959). One unit of inulinase activity was defined as the amount of enzyme required to liberate 1 μmol of fructose per min. The activity units of the enzyme were determined from calibration curve of different concentrations of fructose solution (inulin assay). Blanks were run simultaneously with the enzyme and substrate solutions.

**Protein determination:** Protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard and by absorbance measurements at 750 μm.

**Optimization of culture condition for inulinase production:** The production broth medium was optimized for maximum inulinase production by addition of 0.5% sucrose, 0.5% inulin, 0.5% $CaCl_2$ , 0.3% yeast extract, 0.3% $NaNO_3$ and 0.001% $NaCl_2$ , pH adjusted at 7.0 incubated for 2 days at 30°C.

**Purification of the extra cellular inulinase:** At the end of incubation period, the cells were separated by centrifugation at 20000 rpm for 20 min (under cooling) and the clear supernatant (crude extract) was used for enzyme purification. The inulinase obtained was partially purified by salting out with ammonium sulphate (enzyme grade) at concentration of 70% saturations and the mixture was left for 2 h. The precipitate formed was obtained by centrifugation in an ultracentrifuge at 20.000 rpm. Then, the enzyme precipitate was dissolved in 0.05 m phosphate buffer at pH 7.0 and dialyzed against the same buffer for 18 h.

**Gel filtration using sephadex $G_{100}$:** The gel permeation chromatography on Sephadex $G_{100}$ column provided large additional increase in the purity of the enzyme according to Ettalibi and Baratti (1987). Elution was carried out by 0.05 m phosphate buffer at pH 7.0. The fractions were collected at flow rate (mL/min) in the form of 10 mL aliquots separately in dry and clean tubes. Assay of inulinase activity and protein content of each fraction were examined.

Determination of molecular weight of purified enzyme by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

**Factors affecting purified inulinase enzyme:** Inulinase activity was assayed in the reaction mixture inoculated with different enzyme concentration (0.25-3.0 mM) under different incubation temp. (20-70°C), when the mixture was adjusted at different pH values (4-10) for different time durations (0.5-1.5 h), affect of metalions ($CaCl_2$, $MnCl_2$, $CuSO_4$, $ZnSO_4$, $FeSO_4$, $MgSO_4$ and $MnSO_4$) and Immobilized on DEAE cellulose with 6% glutaraldehyde.

**N-Terminal amino acid sequencing:** In order to determine the N-terminal amino acid sequence, the protein was extracted from *streptomyces grisenus* cells by 8 M urea treatment and then separated in an SDS-PAGE gel as already described (Laemmli, 1970). After separation, the proteins were transferred to a polyvinylidene difluoride membrane, and after staining with ponceau S solution, the inulinase protein band was cut from the membrane and subjected to amino acid sequence analysis.

## RESULTS AND DISCUSSION

The bacterial culture used in this study was the strongest inulinalytic strain among 50 isolates: actinomycetes (40) and bacterial cultures (10), isolated from soil sample. It was identified and confirmed according to description of ISP reported by Shirling and Gottlieb (1966, 1968a,b, 1969 and (1972) and key of Bergey's Manual of Williams *et al.* (1984) depending on chemical properties, colony, morphological, physiological characters and microscopic examination. The most potent isolate was identified as *Streptomyces grisenus*.

**Culture condition for inulinase production:** As shown in Table 1 the maximum activity was found on the 2nd day of incubation period. These results are similar to those obtained by yokota *et al.* (1995) , Baron *et al.* (1996) and Lim *et al.* (1998).

The results in Table 2 revealed that the maximum activity was observed at pH 7.0. These results coincide with (Allais *et al.*, 1988) who obtained the highest inulinase on a medium adjusted at pH 7.5 and Vullo *et al.* (1990, 1991) and Abeer (2004) which obtained the highest inulinase yield on a medium adjusted at pH 7.0 .

The maximum inulinase was measured at 30°C Table 3 these results agreement with Vranesic *et al.* (2002) found the optimum temperature between 30 and 33°C for inulinase from *Aspergillus ficum* and (Park and Yun, 2001) verified for *pseudomonas* sp. was 42°C .

The effect of carbon source on inulinase production was shown in Table 4. Inulin was the best carbon source for inulinase production, while starch, maltose and lactose were inadequate. Similar results were obtained (YoKota *et al.*, 1991) with *Arthrobacter* sp. and

Table 1: Effect of incubation period on the inulinase activity of *Streptomyces grisenus*

| Incubation period No. | Inulinase activity ($mL^{-1}$) |
|---|---|
| 24 | 400 |
| 48 | 600 |
| 72 | 450 |
| 96 | 300 |
| t-test 3.873** | 7.00** |

**significant at level 0.01

Table 2: Effect of temperature on the inulinase activity of *Streptomyces grisenus*

| Temperature°C | Inulinase activity ($mL^{-1}$) |
|---|---|
| 10 | 100.0 |
| 20 | 450.0 |
| 30 | 600.0 |
| 40 | 250.0 |
| 50 | 000.0 |
| T-test 9.820** | 2.668** |

**significant at level 0.01

Table 3: Effect of different pH values on the inulinase activity of *Streptomyces grisenus*

| pH values | Inulinase activity ($mL^{-1}$) |
|---|---|
| 5 | 000.0 |
| 6 | 065.0 |
| 7 | 600.0 |
| 8 | 500.0 |
| 9 | 350.0 |
| 10 | 000.0 |
| T-test 4.243** | 2.540** |

**significant at level 0.01

Table 4: Effect of carbon source on the inulinase activity of *Streptomyces grisenus*

| Carbon source | Inulinase activity ($mL^{-1}$) |
|---|---|
| Starch | 350 |
| Sucrose | 550 |
| Glucose | 250 |
| Xylose | 150 |
| Glycerol | 065 |
| Inulin | 600 |
| Maltose | 300 |
| Lactose | 250 |

(Xiao *et al*., 1989) for *C. pannarum* inulinase. These authors concluded that inulin induces inulinase production, where as cultivation using starch, maltose and lactose as carbon sources restrains the production of this enzyme by optimizing culture conditions for maximum inulinase production, purification of streptomyces grisenius crude inulinase in cell free extracts was the aim of further research in order to increase its specific enzyme activity.

Results presented Fig. 1 and Table 5 revealed that the partial purification using 70% saturated ammonium sulphate solution at 4°C for an over night increased specific activity from 1.062 to 2.83 μ $mg^{-1}$ protein and its concentration increased up to 20.7 folds . Further purification by gel permeation sephadex $G_{100}$ increased the specific activity to 60.4 folds. Representing the results of fraction (50 fraction) of inuline lyase enzyme using the chromatograph resulted in one sharp peak and the molecular weight was obtained 140 KDa. Similar results were obtained by Burne *et al*. (1987) who purified fractosidase produced by Streptococcus mutans using HPLC. Inulinase was purified wit a recovery of 2.3 from crude culture super natant, the molecular weight of inulinase was 140 KDa in sodium dodecy/salphate polyacrylaminde gel electrophoresis and Abeer (2004) showed that inulinase produced by *streptomyces griseus* was purified with a recovery 2.4% and its molecular weight was obtained 104 KDa.

The results in Table 6 and 7 revealed the effect of some physical conditions on purified enzyme produced by *streptomyces grisenus* viz., incubation temperature incubation period, different concentrations of pure enzyme our results proved that the maximum activity was recorded at pH 7 and 40°C . Present findings are in agreement with Lim *et al*. (1998) they stated that *streptomyces* sp. 366L produced a levanase of an optimum pH 7 and temperature of 40°C (Cho and Yun, 2002) stated that *Xanthomonas oryzae* No. 5 produced an endoinulinase of optimum pH at 7.5 and temperature of 50°C. (Abeer, 2004) indicated that *streptomyces griseus* produced an inulinase of optimum pH at 8 and temperature of 40°C. *Streptomyces* sp. GNDuI produced high levels of extra cellular inlinase after 24 h at pH 7.5 and 46°C.

Results represented in Table 8 Indicated that the inulinase activity increased with increasing the substrate concentration until 1 mu and the activity was constant between 1- muinulin. These results agreement with Abeer (2004) indicated that the activity of inulinase by *streptomyces griseus* increased with increasing the sub-state concentration until 1 mu.

Table 5: The purification steps of extracellular inulinase from *Streptomyces grisenus*

| Purification steps | Volume (mL) | Total protein (mg) | Enzyme activity | Total activity | Specific inulinase activity ($mg^{-1}$ protein) | Purif. fold |
|---|---|---|---|---|---|---|
| Crud enzyme | 100 | 1470.5 | 1562.5 | 156260 | 1.06 | 1.0 |
| Amm. Sulphate ppt. | 30 | 291.1 | 812.6 | 24375 | 2.82 | 20.6 |
| Gel permeation Sephadex $G_{100}$ | 1.5 | 357.2 | 2500.0 | 3752 | 6.88 | 60.4 |
| t-test | 1.498 | 1.846 | 3.329 | 1.287 | 2.081 | 1.564 |

**significant at level 0.01

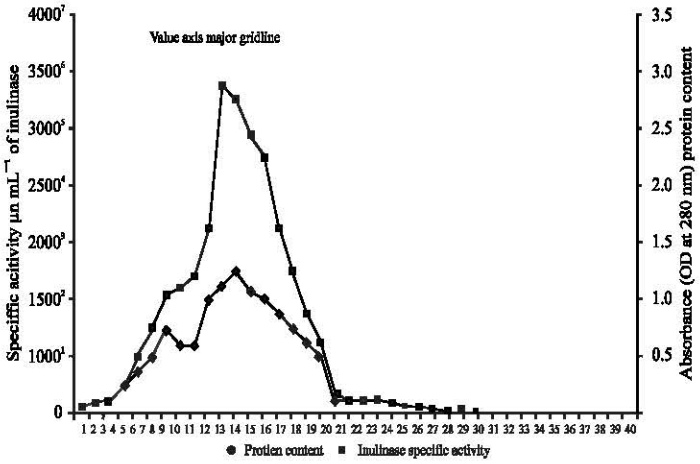


Fig. 1: Elution profile of the enzyme (produced streptomyces grisenus) after sephadex $G_{100}$ filtration. The eluate was assayed for protein content and inulinase activity

Table 6: Effect of pH value on the specific activity of purified inulinase from *Streptomyces grisenus*

| pH value | Specific activity ($mg^{-1}$) |
|---|---|
| 4 | 00.00 |
| 5 | 00.61 |
| 6 | 40.20 |
| 7 | 70.30 |
| 8 | 70.80 |
| 9 | 50.00 |
| 10 | 00.52 |

Table 7: Effect of temperature on the activity of purified inulinase from *Streptomyces grisenus*

| Temperature°C | Specific activity ($mg^{-1}$) |
|---|---|
| 30 | 70.40 |
| 40 | 70.80 |
| 50 | 30.50 |
| 60 | 00.85 |
| 70 | 00.00 |

Table 8: Effect of different substrate concentration on the purified inulinase produced by *Streptomyces grisenus*

| Inulinase conc. (mM) | Specific activity ($mg^{-1}$) |
|---|---|
| 0.25 | 00.82 |
| 0.30 | 10.60 |
| 0.50 | 60.30 |
| 1.00 | 70.80 |
| 1.50 | 70.80 |
| 2.00 | 70.80 |
| 2.50 | 70.40 |
| 3.00 | 70.00 |
| 3.50 | 60.00 |
| 4.0 | 50.00 |

Table 9: Effect of metal ions on the purified inulinase produced by *Streptomyces grisenius*

| Metal ions (5.3 mM) | Specific activity of inulinase | Relation activity (%) |
|---|---|---|
| Control | 70.80 | 100.00 |
| $CuSO_4$ | 40.30 | 056.70 |
| $ZnSO_4$ | 00.00 | 000.00 |
| $FeSO_4$ | 10.40 | 022.70 |
| $MgSO_4$ | 00.51 | 006.80 |
| $MnSO_4$ | 10.30 | 018.00 |
| $CaCl_2$ | 70.80 | 100.00 |
| $MnCl_2$ | 60.10 | 079.50 |
| T-test | 3.071** | 3.274** |

**significant at level 0.01

Table 10: The purified inulinase immobilized on DEAE cellulose with or without glulardehyde

| Matrix | specific Inulinase activity unit / (mg protein) | Relation activity (%) |
|---|---|---|
| Control | 70.71 | 00.0 |
| DEAE-cellulose | 70.61 | 00.0 |
| DEAE-cellulose + glulardehyde | 76.01 | 02.3 |
| T-test | 40.612** | 131.435** |

**significant at level 0.01

Table 11: Thermal stability of inulinase immobilized on DEAE cellulose with or without glulardehyde

| | Residual inulinase activity % | | | |
|---|---|---|---|---|
| Matrix | 30°C | 40°C | 50°C | 60°C |
| Control | 98.0 | 95.01 | 2.00 | 00.00 |
| DEAE cellulose | 100.0 | 100.0 | 80.0 | 65.0 |
| DEAE cellulose + 6% glulardehyde | 100.0 | 100.0 | 86.0 | 77.0 |
| t-test | 149.0** | 59.0** | 3.280* | 1.979** |

**significant at level 0.01*significant at level 0.05

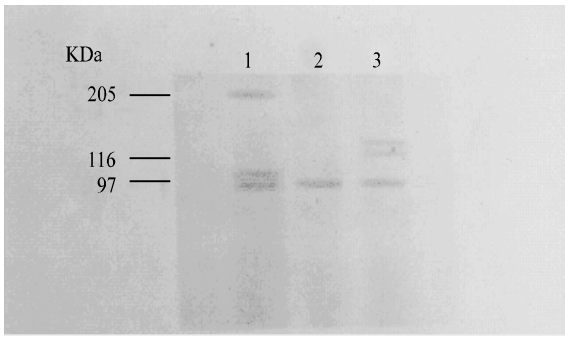


Fig. 2: SDS-PAG electrophoresis stained with coomassie brilliant blue and showing in lane (1), the molecular weight of the marker proteins, in lane (2), fraction with the highest inulinase activity of peak A, in lane (3), supernatant protein precipitated with 67% saturated ammonium sulphate

Data in Table 9 revealed the effect of metal ions on the activity of purified inulinase in the enzyme mixture. the metal ions $Zn^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Fe^{+3}$ and $Cu^{+2}$ were inhibited its activity by about 100, 93, 82, 7 and 43, respectively , However the pure enzyme was stable in the presence of $CaCl^2$ . This supposition also is supported by Abeer (2004) concluded that the inulinase enzyme produced by *streptomyces griseus* was inhibited by $Zn^{+2}$, $Mg^{+2}$, $Fe^{+3}$, $Cu^{+2}$ and $MnCl^2$ and stable in the presence of $CaCl^2$.

Results in Table 10 indicated the maximum activity of inulinase immobilized on DEAE - cellulose as a function of glutaraldehyde concentration, DEAE cellulose with 6% glutaraldehyde gave better yield of activity and stability of immobilized enzyme, therefore, inulinase was simply immobilized on DEAE. cellulose with glutaraldehyde treatment, which has little diffusional limitation to inulin, a high molecular weight substrate. Similar result was obtained by Kim and Byum (1982) who stated that the immobilization of inulinase on various matrices such as, nylon and 2-aminoethyl - cellulose (AE-cellulose), showed that immobilization on AE-cellulose with glutaraldehyde treatment gives good results in terms of activity and stability.

The data in Table 11 showed that the immobilized inulinase become more stable when exposed to heating at 60°C for 90 min. recording a residual activity of 78%, while the free enzyme completely lost its activity under these conditions. These results agreement with Gupta *et al.* (1992) stated that inulinase immobilized on DEAE-cellulose and on protein supports from mung bean and Soya bean lost 50% of its activity on heating the enzyme preparation for 45 18 and 15 min, respectively at 50°C and Abeer (2004) recorded that the immobilized inulinase become mor stable when exposed to heating at 60°C.

The analysis of the deduced inulinase amino acid sequence revealed the presence of a putative gram-positive signal peptide and a cleavage site following amino acid 39 was predicted with the program designed by Nielsen *et al.* (1997). To confirm the cleavage site, the *streptomyces griseus* inulinase enzyme was purified by SDS-PAGE (Fig. 2) and subjected to N-terminal sequence analysis. The first 10 amino acids were identified as DVNQPLLAQK, except for the 10th residue, this sequence is identical to that of the deduced amino acid sequence after the predicted signal peptide cleavage site.

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