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Purification and characterization of Inulinase from marine bacterium, *Bacillus cereus* MU-31

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Marine bacterium MU-31 strain isolated from sediment of Vellar estuary Parangipettai (Southeast coast of India) was found to secrete a large amount of inulinase into the medium. It was identified as this marine bacterium potent isolated strain was identified as *Bacillus cereus* by various biochemical studies. Optimum reaction conditions were as follows: temperature, 30°C; *p*H, 7.0; NaCl, 2%; substrate concentration, 1.5%; carbon sources, inulin and nitrogen sources, yeast extract. Purified enzyme gave a single band on gel electrophoresis and its molecular weight was estimated to be 66 kDa. Among the different chemicals used, only SDS enhanced the enzyme production and 1,10-phenanthroline was strongly inhibited the inulinase production. Among metal ions, Mg^{2+} stimulated inulinase activity and strongly inhibited by Hg^{2+} and Pb^{2+} at 1mM concentration. All these conditions make *Bacillus cereus* MU-31 a potential candidate for industrial enzymatic production of high fructose syrup and in other large-scale biotechnological processes.

[**Keywords:** *Bacillus cereus*, Extracellular inulinase, Fructose, Inulin]

Introduction

Inulin is a linear β-2, 1 linked fructan terminated by a sucrose residue, which is usually found as reserve carbohydrate in various plants such as chicory, dahlia, and Jerusalem artichoke. Inulinases are fructofuranosyl hydrolases, which are produced by bacteria and plants as well as moulds[1,2,3]. Commercially inulin can be converted into fructose by chemical approach (acid hydrolysis) and it has some drawbacks[4,5]. Fructose can also be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase and glucose isomerase[5]. Conventional fructose production from starch needs at least three enzymatic steps and yields only 45% fructose. By the use of microbial inulinase, one step enzymatic hydrolysis of inulin, yields 95% pure fructose. Inulinase is produced by many microorganisms, such as *Streptomyces* sp. GNDU 1[6], *Bacillus* sp[7], *Aspergillus*, *Staphylococcus*, *Xanthomonas* and *Pseudomonas*. Yeasts such as, *Kluyveromyces fragilis*, *Kluyveromyces marxianus*, *Candida kefyr* and *Debaryomyces cantarelli* species are the common inulinase producers[4].

From the marine samples, yeast *Cryptococcus aureus* was isolated and characterized for its inulinase production[8]. However, the reports from marine *Bacillus* sp especially *Bacillus cereus* are very scandy. Therefore, marine samples were screened for inulinase producing strains with specific media and isolated and characterized *B. cereus* with ability to produce extracellular inulinase. Inulinase production media was optimized by different parameters, enzymatic assay and its molecular weight.

Materials and Methods

All the chemicals purchased from analytical grade. Inulin was purchased from Sigma Chemical, USA, Sediment samples were aseptically collected from Vellar estuary, Parangipettai (Lat; 11°46' Long; 79°46') (Southeast coast of India). Samples were serially diluted (10^{-1} to 10^{-7}) with sterile 50% sea water plated on Czapex-Dox Agar (CDA) agar medium for isolation of inulinase producing bacteria[9]. After 24-48 hrs incubation the pure colonies were sub cultured on CDA agar slant (50% sea water) for further analysis.

Morphologically different colony MU-31 were performed for physiological and biochemical tests like gram staining, motility, spore formation, hydrolysis of starch, gelatin, indole, urea, arabinose, maltose, sucrose, mannitol, inositol and nitrate reduction. All the results were compared with *Bergey's Manual of Systematic Bacteriology*[10].

Medium (CDA) used for maintenance[9]. The Erlenmeyer flasks (250 mL) containing 50 mL aliquots of enzyme production medium contained (g/l): $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$. $7H_2O$, 0.01; inulin 10 and *p*H 7.0 were autoclaved (15 min, 121°C). After inoculated with 2.0% of inoculum containing $3.1\text{-}4.7 \times 10^4$ CFU mL^{-1} on production medium, flasks were incubated at 30°C on a rotary shaker (150 rpm). Flasks were withdrawn at regular interval of 6 h and assayed for enzyme activity, *p*H and biomass. All the experiments were carried out in triplicate. Culture broth was centrifuged at 10,000 rpm for 20 min at 4°C and stored at −20°C till further analysis.

The selected strain MU-31 was subjected to different culture conditions to derive the optimum conditions for inulinase production. Biomass and inulinase production were estimated at various temperatures (20, 25, 30, 35, 40 and 45°C), *p*H (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%), substrates (0.5, 1, 1.5, 2.0, 2.5 and 3.0), carbon sources (glucose, starch, inulin, lactose, maltose, sucrose and raffinose) and nitrogen sources (peptone, yeast extract, beef extract, soybean powder and sodium nitrate). All the experiments were carried out in triplicate. Culture broth was centrifuged at 10,000 rpm for 20 min at 4°C and they are stored for further analysis. Average values have been reported. Standardization of culture condition was carried out one parameter at one time and the standardized values were used for subsequent experiments.

Inulinase activity was assayed by measuring the amount of reducing sugars (fructose) released from inulin and sucrose, respectively, using Nelson's method[11]. Assay mixture for inulinase contained 0.1 mL of enzyme extract and 0.9 mL of 1.1% (w/v) inulin in 0.1 M citrate-phosphate buffer (*p*H 7.0). Mixture was incubated at 35°C for 1 h. After incubating for 1 h at 35°C, the reaction was stopped by adding 1 mL of Nelson's reagent and amount of reducing sugars released was measured by reading the absorbance at 575 nm on a Shimadzu 160A, spectrophotometer, Japan. The reducing sugars already present in the enzyme samples were also estimated and an appropriate correction made while calculating the actual amount of sugar released. Calibration curve was drawn with fructose (10-100 mg). One unit of inulinase was defined as the amount of enzyme that released one 1 µmole of fructose per minute from inulin at 35°C.

In order to analyze the product(s) of inulin hydrolysis, the purified enzyme was incubated at 35°C for 24 h with 1.1% inulin in 0.1 M citrate-phosphate buffer *p*H 7.0. The reaction was terminated by adding a double volume of absolute ethanol[12]. The different fractions were analyzed in thin layer chromatography.

Thin layer chromatography (TLC) was used for qualitative analysis of the reaction products. Pre-coated TLC plates (Silica gel 60 F_{254} sheet, Merck, Germany) spotted with samples, were developed with the solvent systems using n-butanol: acetic acid: water (2:1:1 v/v/v) as an irrigating solvent and the sugars were visualized by heating the plates for 30 min at 80°C after spraying with orcinolsulphuric acid reagent prepared by adding 0.2% (w/v) orcinol prepared in ethanol–water–sulphuric acid (40:50:10).

Protein concentration of the inulinase in supernatant was determined by the literature method[13], using bovine serum albumin as the standard. Chemicals like SDS, Triton X-100, β-mercaptoethanol, 1: 10-phenanthroline and EDTA were added to the enzyme preparations and incubated for 1 h at room temperature before being tested for inulinase activity.

The effect of metal ions on inulinase activity was studied using concentrations of 1 mM. The enzyme was mixed with different metals concentrations and pre-incubated for 1 h at ambient temperature before assay. Effect of metals on enzyme activity was measured with different metals namely $FeCl_2$, $HgCl_2$, $MgCl_2$, $AgNO_3$, $CuCl_2$, $PbCl_2$, $CaCl_2$, $NiCl_2$ and $MnCl_2$. The control was kept with enzyme without metals (100%).

The crude enzyme sample (100 mL) was fractionated by precipitation with 80% saturated $(NH_4)_2SO_4$. The precipitation was dialyzed against 20 mM potassium phosphate buffer for 12 h at 4°C. Proteins in the dialyzed enzyme preparation were separated with an anion exchange column chromatography (DEAE-Cellulose). The dialyzed protein was applied to a DEAE- Cellulose A-50 column (20 mm diameter×60 mm long), pre-equilibrated with 20 mM potassium phosphate buffer (*p*H 7.0). The fractions showing high inulinase activity were pooled and concentrated in lyophilizer.

The molecular weight of the inulinase enzyme was determined by 15% Sodium dodecylsulfate polyacrylamide gel electrophoresis[14] in LKB Bromma 2050 Midget electrophoresis units (Pharmacia

Amersham Co). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Range molecular markers (29-200 kDa) with five polypeptides were used as a marker.

Results and Discussion

Inulin and fructo-oligosaccharides (FOS) are known as non-digestible food ingredients that beneficially affect the health status of a human body[15]. This is because of carbohydrates with small molecular size are able to pass through a cell wall easily and long chain inulins (10-65 monomers) are poorly pass through and soluble in water. Therefore, the application of inulinase is necessary for food and beverages industry.

Bacillus cereus MU-31 is the first report from marine sediments with the capability of inulinase production. Based on morphological and biochemical characteristics, it is a gram positive non motile spore formers. It can utilize sucrose, mannitol, xylose and not able to use arabinose and maltose. It hydrolysis starch, gelatin, indole and urea and it is negative for catalase.

Maximum biomass of *B. cereus* MU-31 was observed on 36 h and similarly, maximum inulinase production (3783 U/mL) was observed on 36 h. After 36 h, growth and enzyme production became stationary (Fig. 1). *Bacillus* produced large amounts of extracellular inulinase when grown on carbon sources[7]. Extracellular inulinase from *Bacillus subtilis* has also been reported earlier[16]. The inulinase synthesis from *K. marxianus* was growth associated and reached in the optima near the stationary phase was reported by Al-Dagal and Bazaraa (1998)[17].

For optimization of inulinase production, different *p*H, temperature, sodium chloride, substrates, carbon sources and nitrogen sources were used in the production medium. Maximum enzyme production (94 U/mL) was observed at 30°C (Fig. 2) and the enzyme activity was significantly reduced at higher temperatures. Generally, temperature influences the metabolic activity of cells. Various workers have used 30°C as fermentation temperature for the production of inulinase from *K.* marxianus[18-22].

The results showed an increase in enzyme production (90 U/mL) with the increase of initial *p*H of medium up to 7.0 (Fig. 3) and thereafter, it declined. Maximum enzyme production was 90 U/mL at *p*H 7.0. At *p*H 4.0 very less inulinase production of 46 U/mL was observed, respectively. A *p*H 7.0 has been recommended for the optimal production of inulinase from *Xanthomonas oryzae* No. 5[1]. Thus, a *p*H 7.0 in the fermentation medium was selected for further studies.

Many results have shown that many enzyme activities in marine bacteria were enhanced in the presence of NaCl[23,24]. Maximum enzyme production (89 U/mL) was recorded at 2% of NaCl concentration (Fig. 4). Because the bacterial strain used in this study was isolated from marine environment, it is very important to examine effects of different concentrations of NaCl in the natural seawater on inulinase production and cell growth by the marine bacteria. The concentration of NaCl at 4.0% for inulinase production by *Cryptococcus aureus* have been reported by Sheng *et al*. (2007)[8].

Maximum enzyme production (94 U/mL) was observed at 1.5% of substrate (Fig. 5). A substrate concentration (1.0%) have been reported that optimum production of inulinase from actinomycetes strain[6].

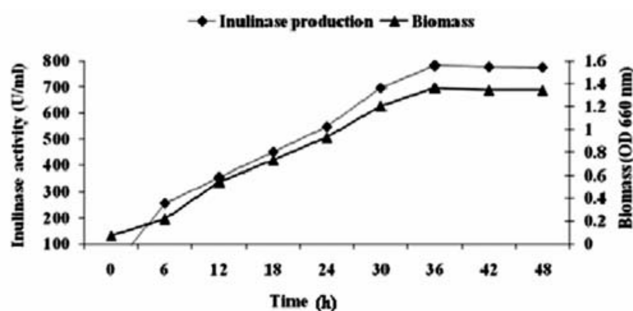

Fig. 1—Biomass and inulinase production

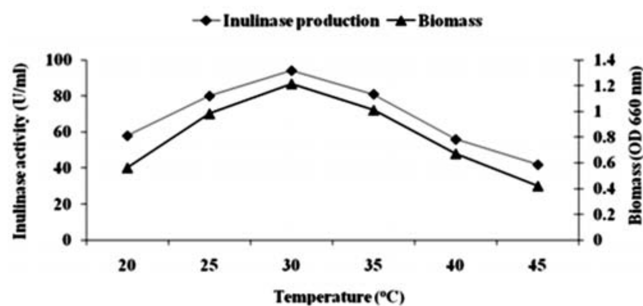

Fig. 2—Effect of temperature on inulinase activity

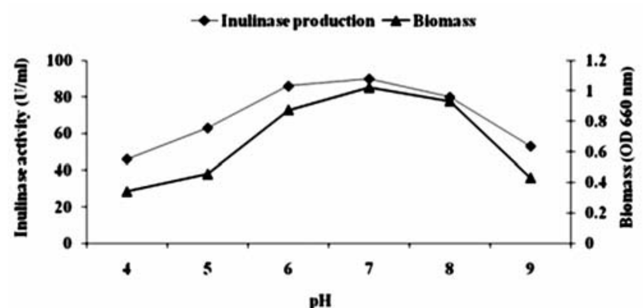

Fig. 3—Effect of *p*H on inulinase activity

The enzyme production from various carbon sources was glucose, strach, inulin, lactose, maltose, sucrose and raffinose (Fig. 6). Inulin was the best carbon source for the production of inulinase from *B. cereus* MU-31. Inulinase production was maximum (94 U/mL) in inulin and minimum (52 U/mL) in raffinose. There was a significant increase in enzyme activity when inulin was used in combination with each carbon source and increase in enzyme production was recorded at higher concentration of inducer. Thus, it was concluded that the enzyme was inducible. Inulin has been reported to be the best carbon source for inulinase production by *Xanthomonas oryzae* No. 5[1].

The production of inulinase under the influence of various nitrogen sources was peptone, yeast extract, beef extract, soybean powder and sodium nitrate (Fig. 7). Maximum inulinase production (96 U/mL) was shown by yeast extract. Yeast extract has been reported as the best nitrogen source for the production of inulinase from *Aspergillus niger*[25].

Among the different chemicals used, only SDS enhanced the enzyme production (Table 1). 1,10-phenanthroline was strongly inhibited the inulinase production. The use of SDS has been reported in the medium for the production of inulinase from *Kluyveromyces marxianus* YS-1[26]. Table 2 shows the effect of metal ions on inulinase activity.

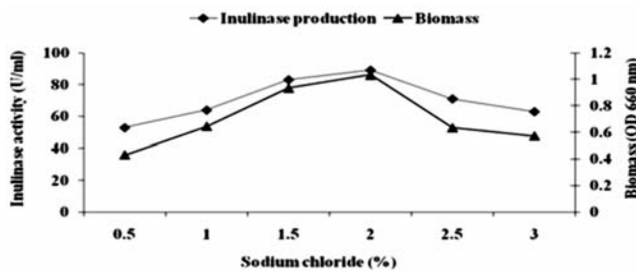

Fig. 4—Effect of sodium chloride on inulinase activity

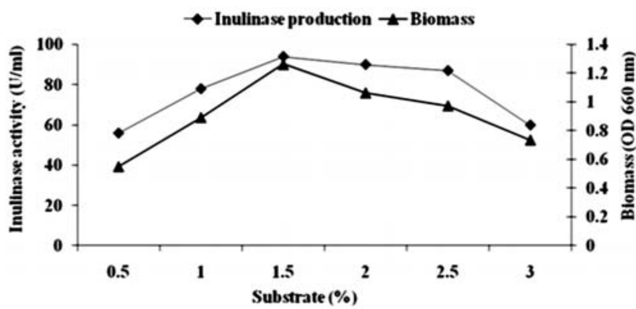

Fig. 5—Effect of substrate on inulinase activity

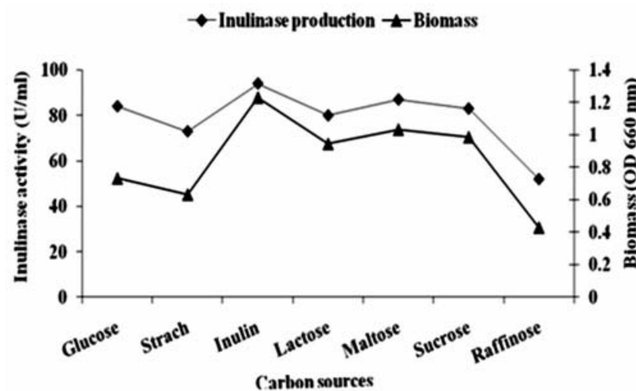

Fig. 6—Effect of carbon sources on inulinase activity

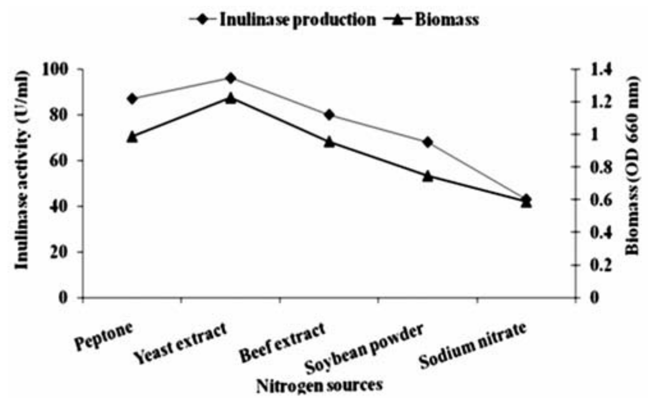

Fig. 7—Effect of nitrogen sources on inulinase activity

Table 1—Effect of various chemicals on inulinase activity

Chemicals	Concentration	Inulinase activity (%)
Control*	-	100
SDS	0.1w/v	116
Triton X-100	0.5v/v	90
β-mercaptoethanol	0.1v/v	90
1,10-phenanthroline	5 mM	0
EDTA	5 mM	86

*Without addition of chemicals

Table 2—Effect of metal ions on inulinase activity

Chemicals	Concentration	Inulinase activity (%)
Control*	-	100
FeCl2	1 mM	91
HgCl2	1 mM	0
MgCl2	1 mM	110
AgNO3	1 mM	71
CuCl2	1 mM	75
PbCl2	1 mM	0
CaCl2	1 mM	75
NiCl2	1 mM	50
MnCl2	1 mM	15

*Without addition of metals.

Table 3—Purification of inulinase from *Bacillus cereus* MU-31

Purification step	Volume (mL)	Total activity (U/mL)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	100	3533.90	120.4	29.35	-	100
Ammonium sulphate precipitation (80% saturation) and dialysis	25	1921.90	29.4	65.37	2.23	54.39
DEAE-Cellulose chromatography	5	523.61	0.32	1636.28	25.03	27.26

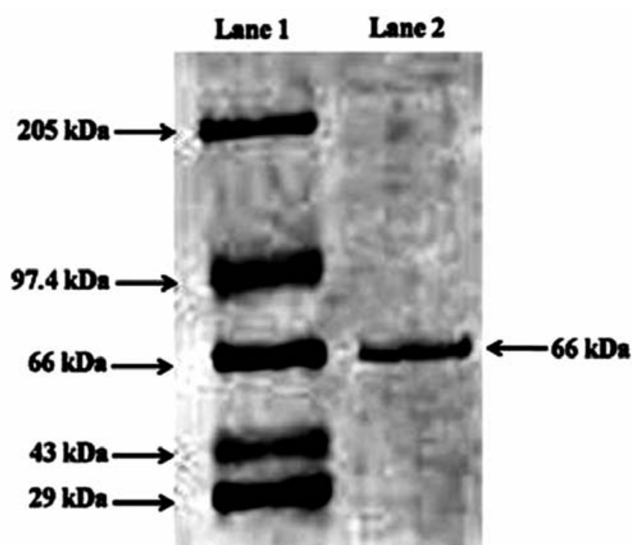

Fig. 8—SDS-PAGE of the purified inulinase: Lane 1, molecular weight markers; Lane 2, inulinase enzyme

Among the different metals used, only Mg^{2+} enhanced the inulinase production. Hg^{2+} and Pb^{2+} showed strongly inhibited the inulinase activity. Hg^{2+} strongly inhibited the inulinase production by *Streptomyces* sp also reported by Sharma and Gill (2007)[27].

The variations of the specific activity of inulinase on inulin during the purification procedure are shown in Table 3. The specific activity of purified inulinase was 1636.28 U/mg, the purification factor was 25.03, and the activity yields were 27.26%, which was purified with an anion exchange column chromatography on a protein preparation machine. A single band was obtained by denaturing polyacrylamide gel electrophoresis of inulinase, with an apparent molecular weight of 66 kDa (Fig. 8). The purified inulinase molecular weight of 83 kDa by *Aspergillus niger* AF10 have been reported by Zhang *et al*. (2004)[28].

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