Title:

"ISOLATION AND CHARACTERIZATION OF INULINASE ENZYME FROM SOIL BACTERIA"

Project report

Submitted for the fulfillment of the requirement for the award of degree of

Master of Technology in Biotechnology

Submitted by

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DECLARATION BY CANDIDATE

I hereby declare that the project entitled "Isolation and Characterization of inulinase enzyme from soil bacteria" is an authentic record of our own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfillment of the award of Master of Technology in Biotechnology under the guidance of **Mr. Sanjeev Singh (**Assistant Professor, Domain of Biotechnology, and Lovely Professional University).

This work is our original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

Place: **TaranjitKaurRai(11205115)**

Date:

CERTIFICATE

This is to certify that Taranjit Kaur Rai(11205115) have completed her project, entitled "Isolation and Characterization of inulinase enzyme from soil bacteria"under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study.

No part of the Dissertation has ever been submitted for any other degree or diploma at any University. The Report is fit for the submission and the partial fulfillment of the conditions for the award of B.Tech-M.Tech dual degree in Biotechnology.

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TaranjitKaurRai

ABBREVIATIONS

S. no.	Abbreviations	Full Form
1	*A. fumigates*	*Aspergillus fumigates*
2	*A. Niger*	*Aspergillus niger*
3	*Aspergillus spp.*	*Aspergillus species*
4	*C. cladosporioide.*	*Cladosporiumcladosporioide.*
5	CTAB	Cetyltrimethylammonium bromide
6	DEAE	Diethylaminoethyl
7	DNA	Deoxyribonucleic acid
8	DNS	Dinitrosalicylic acid
9	dNTPs	Deoxynucleoside triphosphate
10	EDTA	Etylenediaminetetraacetic acid
11	GFR	Glomerular filtration rate
12	HFCS	High fructose corn syrup
13	INU	Inulin
14	*K. fragilis*	*Kluyveromy cesfragilis*
15	*K. marxianus*	*Kluyveromy cesmarxianus*
16	MTCC	Microbial Type culture collection
17	NCYC	National collection of yeast culture
18	O.D.	Optical density
19	PCR	Polymerase chain reaction
20	RNA	Ribonucleic acid
21	*S. Acidophilum*	*Sulfobacillus acidophilus*
22	TAE	Trisbase,acetic acid and EDTA
23	TLC	Thin-layer chromatography
24	UV	Ultraviolet radiations

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Abstract

This report highlights the study work examined the invention of inulinases from a variety of inulin substrates using strains of fungi. *Aspergillus niger* isolated through soil samples that showed huge ability to produce extracellular inulinase. Maximum inulinase activity was being observed in Dahlia around 25.3 nkat mL^{-1} as carbon source. Inulin is one of the various polysaccharides of plant source containing glucose or fructose. It is also used as a substrate in industrial fermentation processes, food industries attributably cheap and rich in source for the microbiological making of high-fructose syrups, ethanol as well as acetone-butanol. There are different oligosaccharides obtained from inulin having uses in the medical and dietary sector. The inulinase acts on the beta-(2,1)-D-fructoside attached to inulin and release D-fructose. Therefore, this report illustrates the ability of microbes hydrolyze the carbon at its finest nutrient absorption.

CHAPTER-1

INTRODUCTION

INTRODUCTION

Inulin a polyfructosan is wide spread in the flora. Like starch, it is a reservoir of carbohydrates for many plants like dahlia, and chicory.Inulinase present in small concentration in many monocotyledonic plants such as liliaceae, iridaceae and grasses. Because of high content of fructose, inulin serves as starting substance for processing of this sugar. These single fructose molecules are connected by β (2→1) linkages in inulin. Glucose molecules are often attached by 1-5 linkages at the reducing end of the inulin molecule.

(2→1)-β-D-fructofuranan

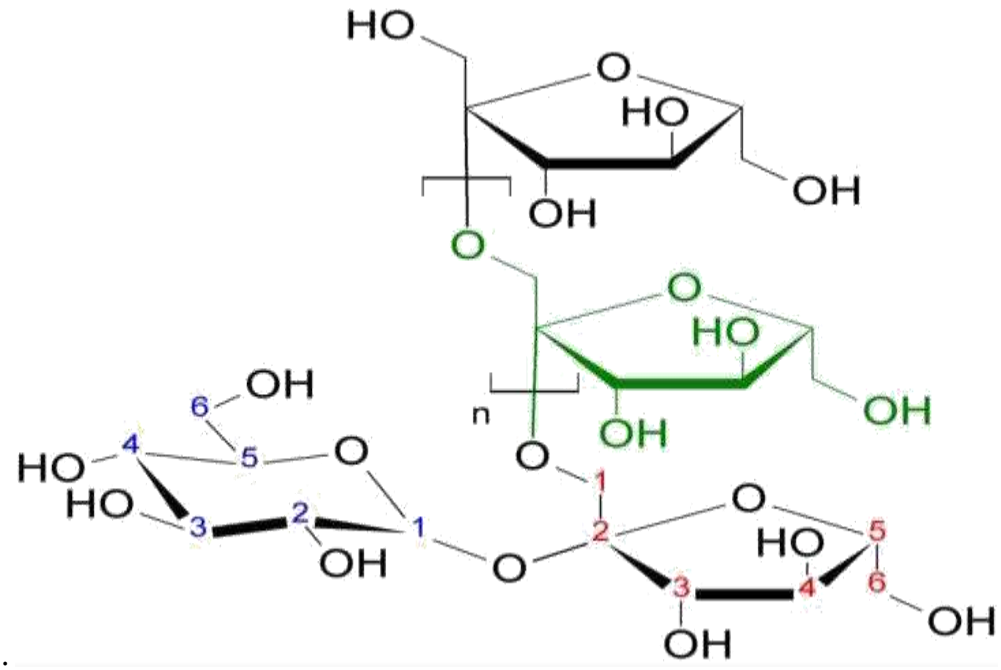


Fig1: Chemical structure of inulinase enzyme (Ref.8)

Fructose and fructo-oligosaccharides plays an important role in the food and pharmaceutical industry. Fructose known to be as sweetener to sucrose as it helps to decrease diabetes and also helps in absorption of iron. Fructose more soluble than sucrose and has low viscous. So therefore used in confectionary and in other various dairy industries. Syrup of fructose was earlier obtained from corn starch but now a days fructose and fructo-oligosaccharides isolated from inulin Inulinases are the enzymes that

act on inulin. These are found in bacteria, molds, plants. These inulinases rarely show activity with sucrose. A few endo-enzymes have been reported to liberate oligofructosides as primary products of hydrolysis. These fructose syrups can compete as sweeteners with the so called high-fructose corn syrups and ultra-high-fructose glucose syrups prepared from starch by enzymatic hydrolysis and further separation of fructose on an ion exchange resin.Maximum content of glucose, sucrose and fructose were found in roots as well as in stressed leaves but inulin was found in roots only rather than in leaves.

Ethanol has been produced by many fungal species and has been described in many works. Many of the metabolic engineering works also report the production of ethanol in certain researches through modification of metabolic pathways of many of the fungal and yeast strains. Fructose syrup was firstly obtained from corn starch..Another application of inulin is in fermentation of biofuel, food, pharmaceutical, feed and ultra- high- fructose syrup. Other than this, it is use in production of citric acid, single-cell protein, lactic acid. Moreover; there are two important properties of inulin that is degree of polymerization and presence of branches. There are some difference between plant origin and bacterial origin .the degree of polymerization of plant origin is lower (<200) than the bacterial origin range from 10,000 to 100,000.

Fructansplays an important role as a cryoprotectors, quantity of hexoses and sucrose increases and then decrease in freezing point depending fructan's depolymerisation. Here inulin directly attaches to membrane lipids and membrane which is present in liquid-crystalline phase prevents transition phase and leakage of solute. This process occurs only under rehydration (Hinchey et al., 2000). Most of the inulinases are exo-enzymes that split units of fructose from the molecule of inuline. A few endoenzymes have been reported to liberate oligofructosides as primary products of hydrolysis.

Importance of high fructose corn syrup (HFSC)(Garuba et al., 2015) HFCS has better solubility than sucrose. It is in liquid form and therefore transportation get easy other application in soft drinks.

Uses of Inulin (Fawzi, 2011): Inprocessed Foods, Inulin is progressively utilized since it has abnormally versatile qualities. Its flavor ranges from tasteless to quietly sweet. While inulin also an adaptable fixing, it likewise has medical advantages. Inulin builds calcium retention and conceivably magnesium assimilation, while advancing the development of intestinal microorganisms.

CHAPTER-2

TERMINOLOGY

Terminology:

- **Chromatography:** A method for the parting of a mixture by transient it in solution or holdup through a medium in which the constituents move at different rates.
- **Denaturation :** The distraction and damage of both the secondary and tertiary structures.
- **Extraction :** Method to split a preferred substance while it is varied with others.
- **Fermentation:** The biochemical analysis of a substance by yeast, bacteria or other microorganisms, typically relating foaminess and the giving off of heat.
- **Filtration:** The action or process of filtering something like solid part mixed with liquid and separated out.
- **Gel electrophoresis**: Method used to divide DNA and RNA as well as proteins , according to the size of molecules that is separated by electrical field throughout the small pores known to be as gel.
- **Isolation :** Partition of a strain from a miscellaneous population of living microbes.
- **Optimization :** The act of making the best or most useful situation or resource.
- **PCR: Polymerase chain reaction,** method that make numerous copies of a fragment of DNA. It is used to copy a exact DNA target through a fusion of DNA molecules.
- **Pouring :** Method of counting the number of colonies forming bacteria that is present in a liquid sample.
- **Precipitation :** Process of precipitating a substance through a solution.
- **Stabilization :** It is the method of completion of stable state.
- **Sterilization:** Method of making somewhat free from bacteria or from living microorganisms.

CHAPTER-3

LITERATURE REVIEW

LITERATURE REVIEW

(Zhang et al., 2010) analyzed and studied on inulinasegene from Pichiaguilliermondii .It affirmed that Saccharomyces sp. W0 can deliver high grouping of ethanol. In this study, the INU1 quality cloned from the marine-determined Pichiaguilliermondii was changed into uracil mutant of Saccharomyces sp. W0. The positive transformant Inu-66 acquired could deliver 34.2 U ml-1 of extracellular inulinase inside 72 h of development. It was found that 15.2 U of inulinase action per one gram of inulin was appropriate for inulin hydrolysis what's more, ethanol generation by the transformant Inu-66. Amid the little scale aging, 13.7 ml of ethanol in 100 ml of medium was created and 99.1% of the additional inulin was used by the transformant. Amid the 2 l aging, 14.9% (v/v) of ethanol was created from inulin and 99.5% of the additional inulin was changed over into ethanol, CO2 and cell mass (Szambelan 2004).

(Fawzi, 2011) worked on comparive of two disinfected inulinases by thermophile *Thielaviaterrestris*. He selected 30 parasitic types established on *Cichoriumintybus L*.Separate root as a only carbon source, were partitioned for the generation of exoinulinase. *Thielaviaterrestris* NRRL 8126 and mesophile*Aspergillusfoetidus* NRRL 337 provided the most elevated creation stages of inulinases I and II. Yeast concentrate and peptone the best nitrogen hotspots most noteworthy creation of inulinases I and II at five and seven days of hatching separately. The two inulinases I and II were filtered by gel-filtration and particle trade chromatography 66.0 and 42.0 double of cleansing individually. Temperatures of purged inulinas I and II observed 75 and 50 ºC individually. An ideal pH actions were observed in between 4.5 and 5.5 inulinases I and II distinctly. Nearly bring down Michaelis–Menten steady and greater most extreme .All abovediscoveries had beendangerous for its possible modern usage.(Vandamme 1983)

(Jain et al., 2012) another studied on creation of inulinase from *Kluyveromycesmarxianus* using dahlia tuber extract.Different carbon causes were assessed intended for generation of inulinase by yeast, Kluyveromycesmarxianus MTCC 3995. Most elevated inulin movement had seen in Dahlia separate (25.3 nkat mL-1). The

protein movement was 1.4 folds higher than that saw in media containing immaculate chicory inulin (17.8 nkat mL-1). Yeast demonstrated great development that is straightforward medium having dahlia remove 20 percent and yeast separate 2 percent as carbon and nitrogen source individually, after 96 hours. at 28°C and120 rpm. Most minimal

Inulin yield that was found in the medium having glucose as carbon. Albeit differed inulinase stages had been seen on various sources of carbon, Inulinase: Sucrase proportions had been seen to be comparable. Between different sources of protein tried, yeast concentrate had been observed considerd as finest source took (17.9 nkat mL-1) as well as peptone (13.8 nkat mL-1). The chemical had been ideally dynamic around pH (4.0) and 50°C. TLC investigation finished result uncovered that inulinase enzyme hydrolyzed inulin solely converted into fructose. Dahlia separate incited exoinulinase union in Kluyveromycesmarxianus and can be used as a potential substrate for inulinase creation.

Various works have been done in the field of sweeteners and their sweetening capacity, of them the works on fructose and fructose based high graded syrups form an important niche. Fructose and fructo-oligosaccharides have become an important area of work pertaining to their beneficial properties such as positive health effects in diabetic patients, increase in the iron absorption in children, and having a higher sweetening capacity as compared to sucrose (Pawan*et al.* 1973). Sucrose can also cause certain ill effects associated to corpulence, cariogenecity and atherosclerosis (Vandamme 1983). Due to low viscosity and metabolization fructose has greater solubility than sucrose without a need for inulin (Fleming 1979). Fructo-oligosaccharides have good functional and nutritional properties such as low calorie diet, *Bifidus*stimulating factor, and source of dietary fibre in food preparations. These oligosaccharides, therefore, are now widely used to replace sugars in many food applications such as in confectionery, chocolate and dairy products (Vandamme 1983). Both fructose and fructo-oligosaccharides can be produced

from inulin, which consists of a linear β 2→1-linked polyfructose chain, terminated by a glucose residue through a sucrose-type linkage at the reducing end. Hydrolysation of Inulindone throughacid. Degradation of fructose happens due to low pH. Theproduction of fructose by conventional procedure depends on amylolysis of starch with the help of two enzymes that are α-amylase and amyloglucosidase followed by the production of glucose isomerase, which is responsible for catalysing the alteration of glucose to fructose. This process leads to the production fructose around 45 %, the remaining is glucose (50 %) and oligosaccharides (8 %). However chromatography techniques i.e. ion exchange has been developed for enhancement of fructose, these techniques add to the cost of production (*Vandamme 1983*). Thus the use of microbial inulinases has been proposed as the most promising approach to obtain pure fructose syrups from inulin. Inulin is degraded by inulinase, which cleaves glycoside bonds to form largely (95 %) D-fructose by a single-step process and is attractive for the industrial production of high fructose inulin syrups.

Sources of Inulin

Hydrolysationof inulin reported as strains of microbes (Vandamme 1983), yeasts (*Kluyveromyces*spp.) composed with *Aspergillus*species that shown to be useful. The primary action of inulinase found in *K. marxianus*. The incompletelysterile inulinase has been found in *K. fragilis,* The optimum temperature at which. The inulinase (2, 1-β-D-fructanfructanhydrolase,) of *K. fragilis*is purified to similarity and arrested on 2-aminoethyl cellulose, good operational stability was found to be in the presence of inulin or the tuber removed of Jerusalem artichoke (Kim *et al.* 1982).Assorted cultures of *K. fragilis*and *Saccharomyces cerevisiae*and*Zymomonasmobilis* in 2–12 percentgreatermaking of ethanol in tubers, souses of inulinase that produce yeast (Szambelan 2004). Its activity was found in cell wall as well as extracellular, so formed in two isoforms (Kim *et al*. 1982).Characterization it was validated intracellular as well as extracellular forms have same subunit .Having differentsize because of its diverse subunit, firstly dimer, and secondly a tetramer. Other inulinase-hyperproducing *K. marxianus*CDBB-L-278,grown on a medium containing inulin as the main carbon source

in the occurrence of 2-deoxyglucose, made up to 3.3 times of the control activity strain *K. marxianus*NCYC-1429, and 3.6 times in a medium with glycerol as the main carbon source (Rouwenhorst *et al.* 1998).Various works have been done in the field of sweeteners and their sweetening capacity, of them the works on fructose and fructose based high graded syrups form an important niche. Fructose and fructo-oligosaccharides have become an important area of work pertaining to their beneficial properties such as positive health effects in diabetic patients, increase in the iron absorption in children, and having a higher sweetening capacity as compared to sucrose (Pawan*et al.* 1973).

Filamentous fungi : The activity of inulinase stated as well as characterized various causes. The first inulinase purified is basidiomycete form *Panaeoluspapillonaceus*(Mukherjee and Sengupta 1987.*Chrysosporiumpannorum*AHU 9700 is a mould that is extracted through soil and was originate to yield a inulin-hydrolyzing enzyme ,found to be made by inulin (Parekh *et al.* 1986). On the other hand, its activity of *C. cladosporioide*. Rough enzyme prepared by *C. pannorum* containe xoinulinase as well as endoinulinase (Parekh *et al.* 1986). The action of exoinulinase *C. pannorum* AHU 9700 related to two glycoproteins having molecular wt. of 84 -70 kDa (Kim *et al.* 1994). The highest enzyme action obtained by *S. acidophilum*has been identified.Moreover the inulinase from *S. acidophilum*is found to be thermostable (Kaur*et al.* 1992), itfound to have highest inulinase activity was observed after 9 days of growth and at a temperature of 25 °C. Entrapment of the enzyme on different supports resulted temperature as associated to the permitted enzyme. The *Aspergillus* spp. greatest producers. Various studies that has been done on action of inulin of *A. ficuum* discovered is forms. Its ratio ranged 0.34-1.16 and 2.75 -6.38 (Ettalibi*et al.* 1987).

Thermostability: Optimum temperature of inulinases is an important factor for commercial production and application of fructose from inulin. High temperatures (60°C) confirm solubility of inulin and prevent microbial contamination. Higher is the thermostability lesser cost of manufacture because lesser quantity of enzyme is needed to yield the final item for consumption.Yeasts and *Aspergillus* spp. play an important sources of inulinases. Its lesser production may be a restrictive factor for hydrolysation of inuline. Thermostabaizationof inulin improved by restriction. Moreover (~70 % up to 48

h) entrapped into*A. fumigatus* 60 degree Celsius greater than restrained inulinases. By adding stabilizers (polyethylene glycol 6000 and glycerol,ethylene glycol), improve life time, and enhance the thermostability of inulinas (Wei *et al.* 1997).

CHAPTER-4

SCOPE OF THE STUDY

4. Scope of study

Inulin used for the production of sweet contents. The vital point of using inulin for production is that is sweetening reagents that are used for different purposes. Inulin content found in dahlia plant from its roots and soil. In recent era, diabetes, heart attack, high cholesterol level, obesity, lung infection increasing day by day, therefore, by replacing sugar content by inulin powder can be more beneficial and also be useful in medical field to prevent any disorders.

CHAPTER-5

AIM AND OBJECTIVE

Aim and Objective

1. Isolation of inulinase secreting microorganism.

2. Characterization of inulinase for specificity for different substrate and optimization of inulin.

3. Characterization of inulinase gene function.

CHAPTER-6

MATERIALS AND METHODS

Material and Methods:

4.1 List of chemicals

All the chemicals and reagents are used in experimentation procedure were of analytical grade and are obtained from commercial manufacturers .

Name of chemicals

➢ Sodium hydroxide	➢ Ferros sulphate
➢ Sodium sulphite	➢ Acetate buffer
➢ EDTA	➢ 3,5 dinitro salicyclic acid
➢ Inulin	➢ Bromophenol blue
➢ Ammonium sulphate	➢ Chloroform
➢ Copper sulphate	➢ DEAE sephrose
➢ Sodium chloride	➢ Glycerol
➢ Magnesium sulfate Heptahydrate	➢ Glycin
➢ Calcium chloride	➢ n-butanol
➢ Potassium sodium tartrate	➢ Potassium chloride
➢ Folin reagent	➢ Potassium phosphate dibasic anhydrous
➢ Agar	➢ silica gel
➢ Mono Potassium Phosphate	➢ Sodium acetate
➢ Sodium carbonate	➢ Sodium nitrate
➢ Tris HCL	

Reagents

- Ammonium acetate
- B- Mercaptoethanol
- Chloroform
- CTAB
- Isoamyl alchohol
- Isopropyl alchohol
- Nacl
- Proteinase K
- Rnase(100mg/ml Dnase free)

4.2 METHODS

COLLECTION OF SAMPLE

(a) Sample containers

Samples were collected in glass conical flasks and test tubes that were properly washed with acid, detergent and finally rinsed with deionized water 3 times. Sterilization is done in autoclave at 121°C for 15 min.

(b) Sample collection

Dahlia plant was collected from nursery near Jalandhar. The tubers and soil surrounding the plant roots were utilized for isolation of inulin degrading organism. The roots of size (5cm) were cut and put inside the sterilized conical flask. The rhizosphere soil was collected in test tubes. The containers and tubes were labelled for further analysis.

4.2.1 ISOLATION OF INULINASE SECRETING MICROORGANISMS

Firstly inulin containing minimal medium agar plates was prepared for fungal species. All the preparation was done under highly sterilized conditions.

Infected portions in dahlia tubers were removed out and were finally sliced with a sterilized razor. These were transferred to inulin medium and enrichment technique was followed. The method for the enrichment cultures was as follows. Soil (0.5 g) was dispersed in 30 ml of medium and incubated at 30 or 37°C. After 24 h, 1.5 ml of the culture was inoculated into 30 ml of fresh medium. After 24 h, 0.3 ml of the culture was inoculated into 30 ml of fresh medium, and 24 h later, single colonies were isolated from inulin agar. Colonies were picked up and grown in liquid medium.

Fig 2:-Dahlia plant from Patiala

Fig 3:-Dahlia plant from sangrur

Fig 4:- Dahlia plant from Andhra Pradesh

Fig 5:- Dahlia Plant from Dehradun

Fig 6:- Dahlia plant from Kolkata

Fig 7:- samples

4.2.2 Enzyme production

The fermentation broth (200 ml) was prepared with minimal media containing inulin as described before. It was done by transferring a loop full of culture (approx. 107 spores and incubating for 6 days at 32°C.

4.2.3 Extraction of Crude Inulinase

Crude Inulinase was extracted from culture broth by centrifugation at 5000 x g for 20 minutes. It was stored at 2°C in refrigerator.Extraction process is done to increase the yield of inulin.tubers od dahlia sliced and hot water added. Inulin extract evaporated as well as precipitated around 95% ethanol.then inulin separated ,dried at 50°C for 1 day.

4.2.4 Estimation of sugar (Fructose)

Inulinase activity was measured in the form of fructose units liberated by enzyme when it acts on substrate inulin. The fructose units were measured as reducing sugar by Dinitrosalicylic acid reagent as described by Miller (1959).

Miller Method for Estimation of Sugar

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, the ketone functional group in fructose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions:

- aldehyde group converted into carboxyl group by oxidation.
- 3, 5-dinitrosalicylic acid converted into 3-amino, 5-nitrosalicylic acid by reduction.

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.
The above reaction scheme shows that one mole of sugar will react with one mole of 3, 5-dinitrosalicylic acid.

A. List of Reagents and Instruments

i. Equipment

- Test tubes
- Pipettes
- Spectrophotometer

ii. Reagents

- Dinitrosalicylic Acid Reagent Solution, 1%
- Sodium sulfite: 0.5 g
- Sodium hydroxide: 10 g
- Add water to: 1 liter
- Potassium sodium tartrate solution, 40%

B. Procedure

Standard curve preparation (Fructose)

Standard fructose curve was prepared by taking altering concentration of fructose (50-1000 μg /ml) and adding 3ml of DNS reagent. The solution boiled for 5 minutes dark orange colour was produced. Sodium potassium tartrate (40%) also added for stabalization of color. The O.D. was occupied at 575nm aligned with reagent blank. Through reading standard curve was primed.

i) **Measurement of Sugar**

1. 0.1 ml of crude Inulinase was added to 0.9 ml of 1.1% inulin solution, incubation was done at 60°C

2. Add 3 ml of DNS reagent to sample in a tightly capped test tube.

3. Mixture was heated at 90°C for 5-15 minutes to develop the red-brown color.

4. 1ml of a 40% potassium sodium tartrate (Rochelle salt) solution added to stabilize the color.

5. After cooling in a cold water bath, the absorbance with a spectrophotometer at 575 nm was recorded.

ii) **Control reaction**

0.1ml distilled water was added to 0.9 ml 1.1% inulin solution. Incubation was done at 60°C for 30 minutes and 1 ml distilled water was added. To this solution 3 ml DNS reagent was added and boiled for 5 minutes. Absorbance was measured at 575nM.

4.2.5 TOTAL PROTEIN ESTIMATION

Total protein was projected by the method of Lowry et al. (1951). The color urbanized was measured at 750 nm. The color arrangement occurs caused by reaction of protein with copper and finally drop of phosphomolybdic-phosphotungstic reagent by copper treated protein.

(A) Reagents

I. Preparations of folin ciocalteau and other reagents

a. Reagent A= 1% of copper sulphate W/V

b. Reagent B= 2% of sodium potassium tartrate W/V

c. Reagent C= 0.2 M of sodium hydroxide

d. Reagent D= 4% of sodium carbonate W/V

These reagents are remain steady at room temperature. All through enzyme assay reagent E was prepared as under

e. E = C:D:A:B = 49:49:1:1

f. F = Folin ciocalteau reagent : water = 1:1

(B) Procedure

i. Standard curve preparation

Standard fructose curve was arranged by taking unstable concentration of protein from (50-500 µg/ml) and adding up 4ml of E reagent to it. This solution was incubated at room temperature for 10 minutes then 0.4 ml reagent F and incubation at room temperature for 30 minutes. The colour was developed and O.D. was taken at 750nm next to reagent blank. The Standard curve was plotted.

ii. Total protein estimation

Total protein was predictable from the crude enzyme solution, 1ml of enzyme solution was then added to reagent E, by incubation at room temperature for 10 minutes, with further accumulation of reagent F, 0.4 ml and incubation at room temperature for 30 minutes was followed. The colour was obtained and O.D. was taken at 750nm against reagent blank.

4.2.6. CHARACTERIZATION OF INULINASE SPECIFICITY FOR DIFFERENT SUBSTRATE

Fungal Strain was cultured on the mineral medium with diverse carbon based inducers or repressors for the enzyme biosynthesis. Growth on sucrose, inulin, glucose and other carbon sources were examined. The consequence of inulinase enzyme on different carbon sources were resoluted by incubating the purified inulinase enzyme with inulin, sucrose, fructose, glucose, xylose and lactose with changeable time intervals.

4.2.7 OPTIMIZATION OF INULINASE ENZYME ACTIVITY

i. Effect of Temperature

Enzyme activity of the crude inulinase was calculated at different temperature i.e. (10-85°C) at constant pH (5.5) by D.N.S method.

ii. Effect of pH

Enzyme activity of the crude Inulinase was precised at different pH i.e. (4.0-8.0), keeping temperature steady at 55°C.Then adjusting pH, sodium acetate buffer was taken in which substrate inulin solution was arranged.

iii. Effect of Incubation period

The activity of inulinase enzyme for fungal strain was being assessed for the effect of diverse incubation period. The crude inulinase activity were assessed at standard time period intervals (10 minutes to 40 minutes) observing temperature (60°C) and pH (5.5) constant.

4.2.8 BIOMASS DETERMINATION

The mycelial mass of fungus was being collected by filteration (whatman paper). The biomass was resoluted after washing mycelial mass with distilled water and dried at 105ºC overnight until stable weight.

4.2.9 CHARACTERIZATION OF INULINASE GENE FUNCTION

1. **Isolation of DNA from Fungus**

CTAB Extraction buffer

• 2% CTAB (hexadecyltrimethylammonium bromide)

• 100 mM TrisHCl [pH=8]

• 20 mM EDTA,

• 1.4 M NaCl

• 0.2% _-mercaptoethanol

• 0.1 mg/mL proteinase K

Procedure

Cells were resuspended in 0.8 mL of pre-warmed (60°C) CTAB extraction buffer and incubated at 60°C for 1 hour. Gently mix up by inverting the microtube. After 1hr, add 0.8mL of chloroform/isoamylalcohol (24:1) solution. Gently mix for 2 min by inverting the tube. Spin 10 min at maximum speed (14000 x *g*) at 4°C. Suspiciously transfer the aqueous phase (above the white interface layer) to a fresh microtube (then discard the rest). To do so fast (before the interface declines), prepare a p1000 with tip on and a p200 with tip on take the larger volume off with the p1000 and then you can more gently get near the edge with the p200. Add 1 µL RNase (DNase-free) and incubate for 30 min at 37°C. Add 0.6 Ml of isopropanol. Lightly invert the microtube to be sure mixing was completed. Depart to precipitate for 2 hr to overnight at room temperature to permit the structure of the "DNA jellyfish". Spin 15 min at 14000 x *g* at 4°C to pellet the DNA. Remove the supernatant suspiciously, then wash the pellet once or twice with cold EtOH, Spin 15 min at max speed, 4°C. Take out supernatant and dry the pellet by leaving tube open at room temperature. Suspend the pellet in TE buffer (pH 8.0), aliquot and store at -20°C (worden, 2009).

2. Agarose Gel Electrophoresis

Gel electrophoresis is a technique used in clinical chemistry to split proteins by charge and size and in biochemistry and molecular biology to detach a mixed population of DNA and

RNA fragments by length, to estimate the size of DNA and RNA fragments or to split proteins by charge. Nucleic acid molecules are divided by applying an electric field to progress the negatively charged molecules through an agarose matrix. Shorter molecules move faster and then migrate beyond longer ones as shorter molecules travel more easily during the pores of the gel. This observable fact is called sieving. Proteins are alienated by charge in agarose as the pores of the gel are too huge to sieve proteins. Gel electrophoresis can also be used for the division of nano particles.

3. Gel Preparation

A. Preparation of 500 ml 1X TAE Buffer

- Tris base – 2.42g
- Acetic acid – 0.57ml
- EDTA – 0.37g

B. Preparation of 0.8% Agarose gel

- 0.8 g agarose assorted in 100ml ditilled water
- Bring agarose solution and then boiled in 100ml erlenmeyer flask. Boil until the entire particles
  dissolved and then solution becomes clear.
- Let allowed to be cooled at room temp.
- Add Ethidium bromide in liquid gel (1-5µl of a 10mg/ml stock solution per 100 of agarose gel).

4. Pouring the Gel

1. Seal the ends of the gel tray strongly with strips of colored lab tape. Press the tape tightly to the edges of the gel tray to outline a fluid-tight seal.
2. Place the comb above the gel tray. For flexible combs, adjust the altitude with the aid of the thumb screws so that it remains higher than the tray.
3. Mix up agarose and melt in microwave oven.
4. When agarose was totally dissolved, allow to cool gradually.
5. After agarose has been cooled to about 60°C, dispense the agarose into the gel tray.

6. Allow the gel to set at room temperature for about 1 hour.
7. Carefully remove the comb from the solidify gel. For very low percent gel, try refrigerating the gel first to avoid damage.
8. Eliminate the tape from the boundaries of the gel tray.
9. Put the tray into the gel box containing buffer.
10. Immerse the gel under 2 to 6 mm buffer. Use greater intensity overlay with rising voltages to keep away from pH and heat effects.
11. Cut a piece of parafilm and place a 5 µl drop of glycerol loading dye on top of the waxy side for all sample to be loaded.
12. Keeping all samples on ice, pipette up 5 µl of a sample, insert the sample to one of the drops of loading dye.
13. Control the pipette tip to another pipette set for 10 µl. Mixup the sample and loading dye by filling and Emptying the pipette a few times then load that mixture into the well.
14. Keep on loading the rest of the samples, placing 5 µl of 1 Kb ladder at both ends of the sequence of samples.
15. Set the cover on the gel rig and run the samples towards the anode (red) end. Switch off the power, eliminate the gel. Examine with U.V. light and photograph with a Polaroid Photo camera.

5. PCR

The polymerase chain reaction (PCR) is a scientific procedure in molecular biology to enlarge a single or a few copies of a piece of DNA across several orders of extent, generating thousands to millions of copies of a particular DNA sequence. PCR is now a common and often very important method used in medical and biological research labs for a variety of applications. All these contain DNA cloning for sequencing, DNA-based phylogeny, and functional analysis of genes; the identification of hereditary diseases; the identification of genetic fingerprints; and the recognition and diagnosis of contagious diseases.

a)PCR Reaction

• 0.25 µl Taq Polymerase

• 5 µl Mgcl2 buffer

• 4 µl dNTPs

• 0.5 µl F1 Primer and 0.5 µl R1 Primer

• 2 µl template DNA

• Nuclease free water make up to volume of 37.75 µl

b)Denaturation 94°C for 1 minute

35 cycles of 98°C for 15 seconds

c)Primer Extension 55°C for 30 seconds.

d) Annealing 72°C for 2 minutes.

CHAPTER-7
RESULTS

5. RESULTS

1. ISOLATION OF INULINASE SECRETING MICROORGANISMS

One strain of fungus was isolated from root material and rhizospore soil collected as sample. It was screened for inulinase production and its enzyme action was checked by D.N.S. method. The following result viewing the isolated fungi growing in vegetative state.

2. CHARACTERIZATION OF INULINASE SPECIFICITY FOR DIFFERENT SUBSTRATE

Inulinase secreting microorganism was being isolated and grown on agar medium containing inulin as sole carbon source. Inulinase enzyme was obtained from inulin containing culture broth. The following results showing the growth of fungi on inulin that contain agar medium.

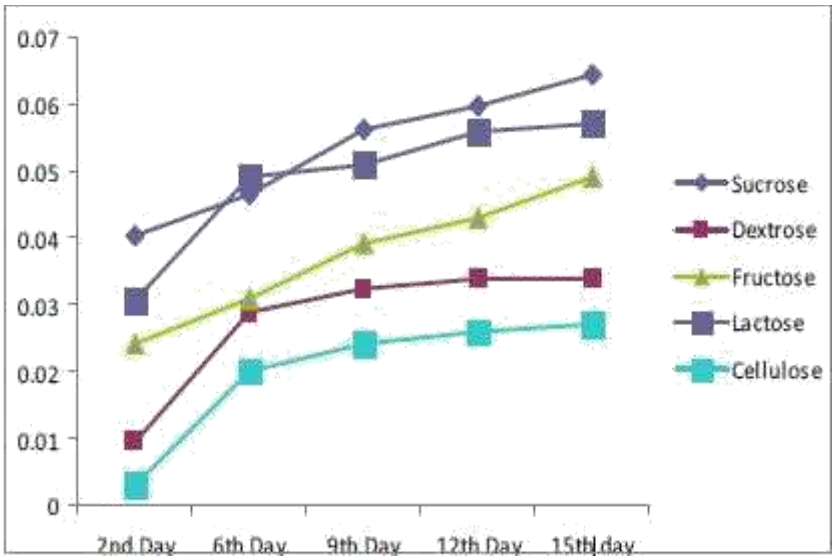
Fig:8 Showing fungi growth on inulin as sole carbon source

Fig:9 Showing the growth fungus on inulin containing medium

3. EFFECT OF OTHER CARBON SOURCES ON INULINASE ACTIVITY

The following experiment shows the activity of inulinase at differnt carbon sources. The result was obtained and that shows sucrose has maximum enzyme activity than other carbon sources as when fungal culture is cultured on mineral media with sucrose as carbon source. Cellulose shows least enzyme activity by means of varying time intervals (i.e 2-15 days).

Fig :10 Showing of inulinase activity with different carbon sources

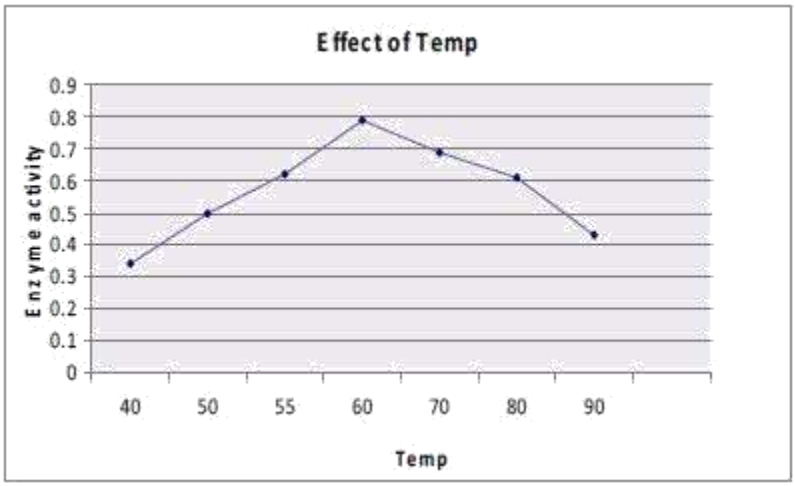


Graph:1 Graph showing the activity of inulinase enzyme on different carbon sources (excluding inulin).

4. OPTIMIZATION OF INULINASE ENZYME ACTIVITY

i. Effect of Temperature

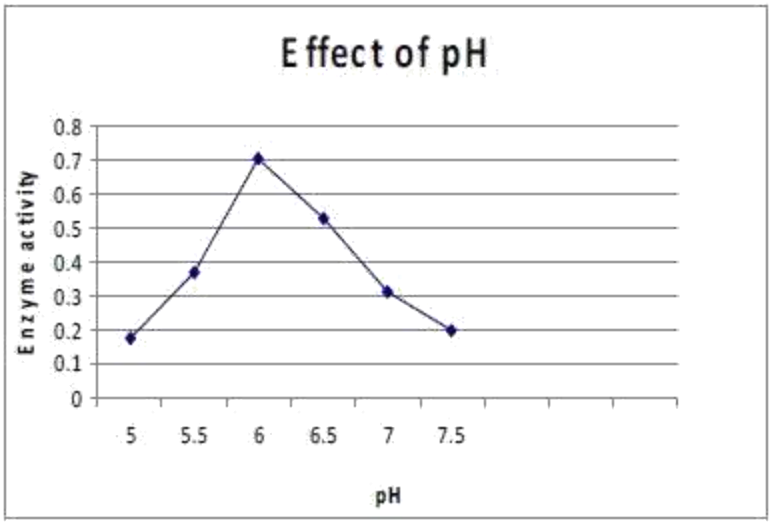
The optimum temperature of crude inulinase extract obtain from fungal strain was measured in the temperature range of 40°C to 90°C by DNS method having constant pH. The result obtained shows the effect of temperature on inulinase enzyme activity. Inulinase enzyme showed maximum activity at the temperature of 60°C. After 60°C temperature, enzyme starts degraded.



Graph:2 Graph showing the optimum temperature at 60□C for fungal strain

ii. Effect of pH

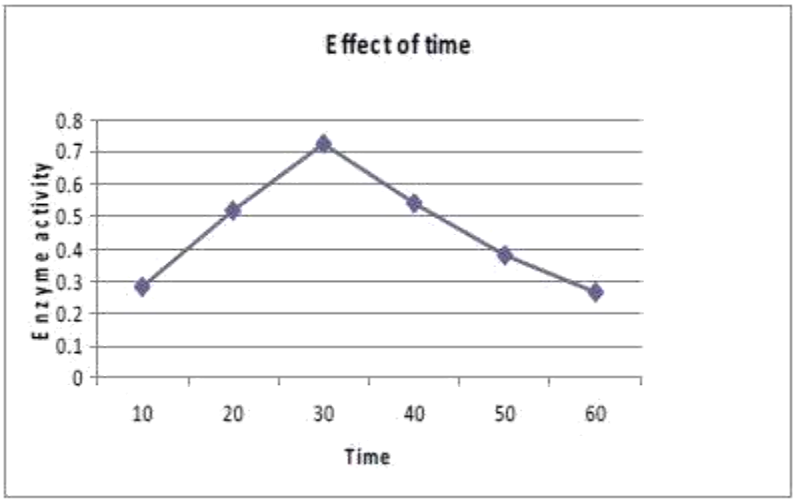
The crude inulinase activity for the fungal strain were analysed at pH 4,6,8,10 maintaining temperature constant at 60°C. The result obtained shows the effect of pH on inulinase enzyme activity. Inulinase showed maximum enzyme activity at pH of 6.



Graph :3 Graph showing optimum pH of 6 for fungus strain

iii. Effect of Incubation time

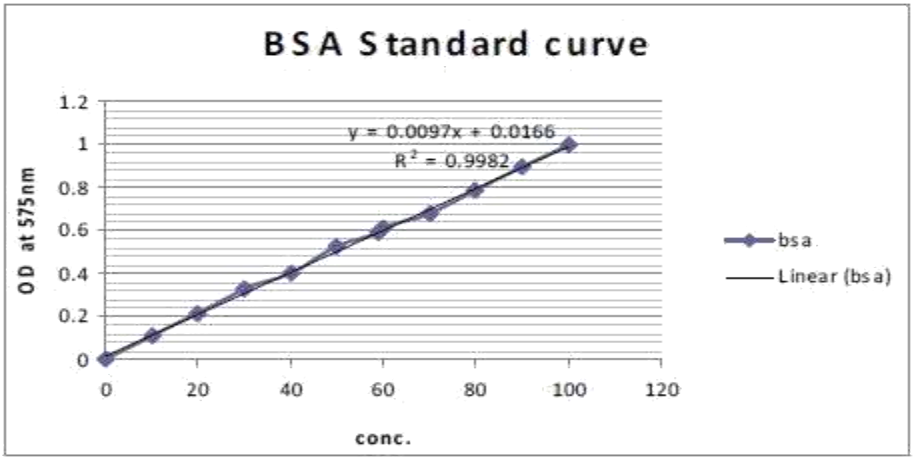
In order to assess the effect of different incubation period on the inulinase activity of fungal strain. The crude inulinase extract were assessed at different time period (10 minutes to 60 minutes) keeping temperature (60°C) and pH (6) constant. The results showing the effect of incubation time on inulinase enzyme activity. Inulinase showed maximum enzyme activity at the interval of 30 minutes.



Graph :4 Graph showing the optimum incubation time at the interval of 30 minutes for fungus strain

5. PROTEIN ESTIMATION

Protein content of crude inulinase extract of fungal strain was estimated by the method of Lowry et al (1951) using BSA as standard. Detection was monitored at 750 nm. The result obtained shows the total protein concentration of crude inulinase solution was 58.8mg/mL.

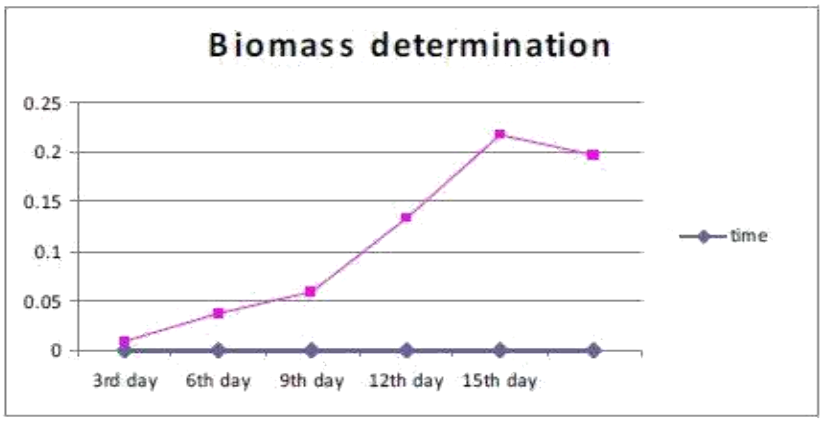


Graph :5 Graph showing the total protein concentration on BSA standard curve

Total Protein of enzyme solution = 58.8mg/mL

6. BIOMASS DETERMINATION

Fungus was grown in culture broth. The mycelial mass of fungus in culture broth was filtered through filter paper (whatman paper). Biomass was determined after washing mycelial mass of fungus with distilled water and dried at 105ºC overnight. The results obtained shows biomass of fungus was increasing from 3rd day of growth to 15th day. After 15th day, fungus biomass starts decreasing due to the consumption of all substrates by fungus in broth.



Graph 6 : Graph showing growth of fungus with varying time intervals (i.e. 3rd day to 15thday)

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a) Agarose Gel Electrophoresis

Genomic DNA was isolated from fungus grown on inulin containing medium. Agarose gel electrophoresis technique was utilized to separate the isolated DNA fragments of fungus. The following results showing the DNA bands on gel electrophoresis in comparison with 1 kb ladder.

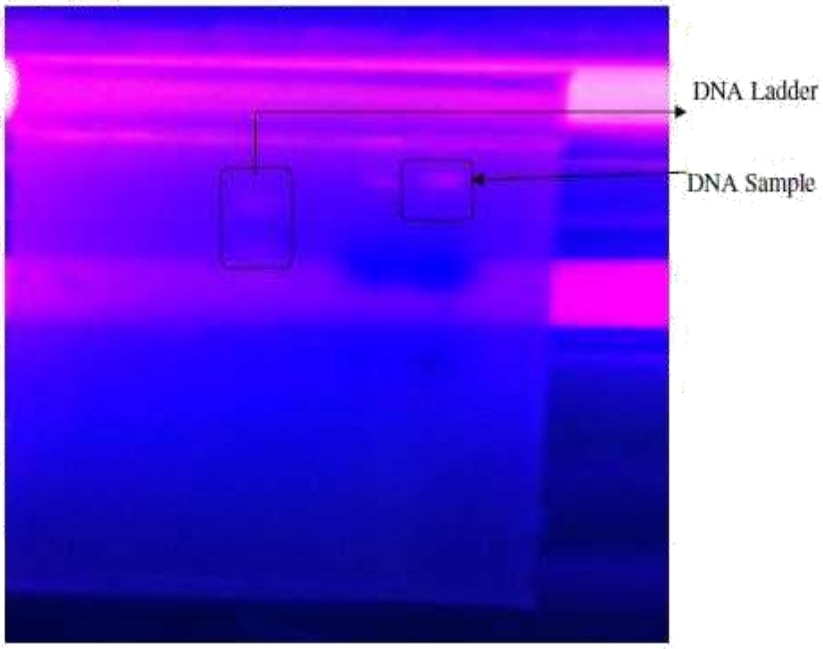

Fig :11 Showing Isolated DNA bands on gel electrophoresis under U.V

b) PCR

PCR technique was utilized to amplify the DNA sample to generate multiple copies of single DNA. Results were observed on agarose gel electrophoresis. The following results showing the PCR product on gel electrophoresis.

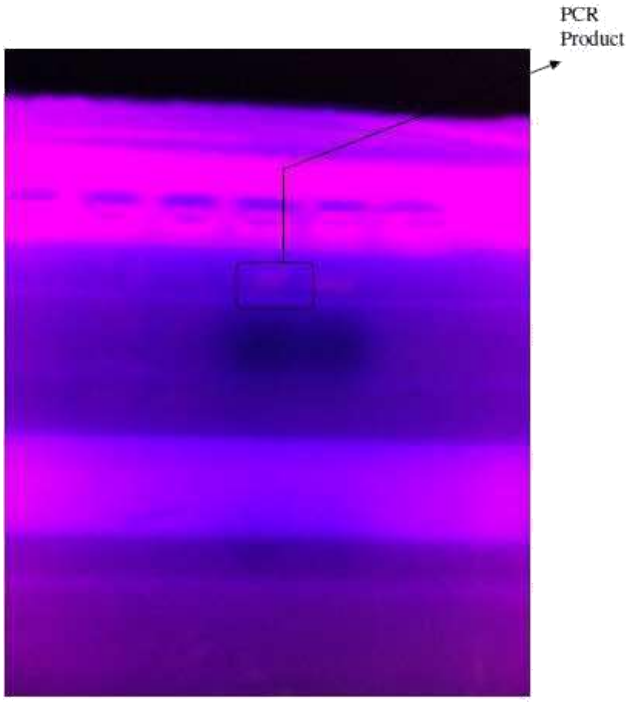

Fig :12 Showing PCR product on gel electrophoresis under U.V

CHAPTER-8

DISCUSSION AND CONCLUSION

6. DISCUSSION AND CONCLUSION

Inulin is one of the various polysaccharides of plant origin that have fructose as monomer unit and used in food industry and in fermentation industry as a substrate. Inulin provides carbohydrate for many plants artichoke, dandelion, dahlia, and several other members of the family composite**.** Inulin is a linear chain of fructose residues united by (β2-1) osidic bonds. Glucose is associated with a β2-1 bond to the terminal fructose.Length of chain is25 to 35 residues.

Hydrolysation of inulin liberate fructose but also glucose. These fructose syrups can compete as sweeteners with the so called high-fructose corn syrups and ultra-high-fructose glucose syrups prepared from starch by enzymatic hydrolysis and separation of fructose on an ion exchange resin. Hydrolysation of inulinproducesyrups with great fructose content and useful for further purposes. Chemical hydrolysationof inulin was verifiedcostly and produce more unwanted degradations. The enzymes used were, inulinases, which are active on inulin and on sucrose. These enzymes, which were derived from plants, fungi, and yeasts, have been intensively studied.

There has been a large amount of research data produced in this context. There has been a continuous search for an inulinase producing strain that produces the enzyme with higher thermostability and has higher level of bond breaking capability for inulin so as to yield fructose syrup of higher grade of purity without any difructose units. The current research was carried out in the same context successful in the context of above search. They are initially having high level of thermostability above 55 °C and very high level of fructose producing capability from inulin. Further the temperature and pH standardisations have also been completed along with some of the protein purification protocols. The morphological observation revealed that the fungus selected was Aspergillus species. The enzyme units observed in the present study are less than those reported by Gupta (1994), Derycke (1984) Kocher et al. (1997) and Gupta (1997). Perhaps earlier researchers used commercial mutated strain while in the present study the microbes have been isolated

from nature.More work is needed for complete analysis of the overall characteristics of this strain.

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CHAPTER-9

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CHAPTER 10

APPENDIX

Chemical composition

Sno.	**Chemicals/reagents/elements**	**Amount**
1	DNS reagent	3ml
2	Distilled water	0.1ml
3	Folin reagent	0.4ml
4	Tris HCL	100mM
5	EDTA	20mM
6	NaCl	1.4M
7	Proteinase k	0.1mg/ml
8	Chloroform	0.8ml
9	trisbase	242g
10	Acetic acid	0.57ml
11	Ethidium bromide	10mg/ml
12	Glycerol	5µl
13	Polymerase	0.25µl
14	Mgcl2	5µl
15	DNTPs	4µl
16	Fermentation broth	200ml