Optimization of extraction parameters of Inulin from *Catharanthus roseus*.

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CERTIFICATE

This is to certify that the thesis entitled **"Optimization of extraction parameters of Inulin from *Catharanthus roseus*"** submitted by **Ankush Goyal** and **Ashish K. Verma** to the Jaypee University of Information Technology, Waknaghat in fulfillment of the requirement for the award of the degree of **Bachelor in technology in Biotechnology** is a record of bona fide research work carried out by them under my guidance and supervision and no part of this work has been submitted for any other degree or diploma.

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Date-

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of **Mrs. Runni Mukherjee**, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for any degree or diploma to any other university. All assistance and help received during the course of the investigation has been duly acknowledged.

By

Ankush Goyal

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We express our heartfelt gratitude to all those who have contributed directly or indirectly towards obtaining our baccalaureate degree and at the same time, we cherish the years spent in the department of Bioinformatics and Biotechnology. We are highly indebted to our esteemed supervisor, Mrs. Runni Mukherjee, who has guided us through thick and thin. This project would not have been possible without his guidance and active support. His positive attitude towards research and zest for high quality research work has prompted us for its timely completion. We are indebted to (Vice Chancellor, JUIT), Brig. (Retd.) Balbir Singh (Registrar, JUIT) and Prof. R. S. Chauhan (Head of the department) for having provided all kinds of facilities to carry out our project. The help rendered by all our teachers, in one way or the other, is thankfully acknowledged.

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(AnkushGoyal)

(AshishVerma)

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LIST OF ABBREVIATIONS

gm	Gram
mm	Millimeter
cm	Centimeter
nm	Nanometer
°C	Celsius
ml	Milliliter
min	Minutes
n	No of atoms
w/v	Weight by volume
KHz	Kilo Hertz
RPM	Rounds per minute
TLC	Thin Layer Chromatography
R_f	Retention factor
GFR	Glomerular Filtration Rate
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
DNS	Dinitrosalicylic

SUMMARY

In this study an attempt was made to analyze the extraction of inulin and optimizing the extraction parameters of inulin. The method comprises of cutting and chopping of *Catharanthus* rosesus roots. Sonication is done for extracting the carbohydrates present in the samples. Partial purification is achieved by centrifugation Spectrophotometer helps in determining the control or calibration, what substances are present in a target and exactly how much through calculations of observed absorbance of samples at 510 nm.

An attempt for Thin Layer Chromatography was done for ensuring the presence of inulin in the samples. Stationary phase comprises of gypsum and silica gel while mobile phase comprises of 2-butanol, isopropanol, glacial acetic acid and methanol. Developing reagent is used to identify the spot which comprises of ethanol, p-methoxy-benzaldehyde, glacial acetic acid and conc. Sulphuric acid. Strip of paper on which tlc was run shows the presence if inulin.

CHAPTER 1

INTRODUCTION

Today, the industrialized countries are facing, among others, three major challenges. Firstly, to control the cost of health care, secondly, to offer to their aging population a real opportunity to live, not only longer, but also better and thirdly, to provide to more and more. Jobs, consumers, a choice of healthy processed or ready to eat foods Roberfroid, 1999). Busy life styles and the increasing demand from consumers for meals and snacks that are quick sources of good nutrition have prompted the food industry to develop foods like nutrition bars that combine convenience and nutrition (Izzo and Niness, 2001).Inulin is a storage polysaccharide consisting of a chain of fructose molecules with a terminal glucose molecule. Silva (1996), inulin is water soluble, the solubility being temperature dependent. At 10°C its solubility is about 6% whereas at 90°C it is about 35%. Inulin type fructans, are the best documented oligosaccharides for their effect on intestinal bifidobacteria and are considered important prebiotic substrates (Vos*et al.,* 2006). *Catharanthus roseus* tubers with 14–19% inulin can be a valuable source of inulin (Vanloo, et al., 1995).Several methods for inulin extraction from Jerusalem artichoke have been developed. A pretreatment step involving boiling water-extracting for 10. 15 min of the ground tubers had been used (Laurenzo*et al.,* 1999). Initially, the application of inulin in the food industry was restricted to the production of a drink similar to coffee, due to its bitter taste. However, it was found that inulin could substitute sugar and fat with the advantage of exhibiting low caloric value (Applied Technology, 1993). The application of inulin as a fat substitute is associated with its capacity of producing a cream-like substance, similar to fat dissolved in water, which can act as a rheological modifier (Cândido and Campos, 1995 and Silva, 1996). Inulin shows interesting technological properties, as a low-calorie sweetener, as a fat substitute, or it can be used to modify texture (Tungland and Meyer, 2002). These properties are linked to the degree of chain polymerization. Inulin is added during cheese processing to decrease its fat percentage without losing its organoleptic characteristics, such as texture and flavor.

One of the interesting functions of inulin and oligo fructose in human nutrition is related to their prebiotic effect, i.e. the specific stimulation of growth and/or activity of a limited number of colonic bacteria beneficial to the host, as well as the growth inhibition of pathogens and harmful microorganisms (Roberfroid, 2007). The combination of prebiotics and probiotics has given rise to the so-called 'synbiotics', with promising healthy properties (Buritiet al., 2007; Pool- Zobel and Sauer, 2007).The functional effects of inulin on humans and experimental animals include relief of constipation lower blood glucose levels, improved absorption of calcium, reduced fasting triglycerides, LDL cholesterol, and inhibition of the growth of various kinds of tumors (Kaur*and Gupta* 2002). Marchetti (1993) reported that, inulin is a natural polymer that not hydrolysable by the intestinal enzymes, because it has β (2-1) link which is not be hydrolyzed. So it could be considered a calorie free fiber, although some calories may occur due to the digestible

Fermentation of these by products in the colon. Inulin is such carbohydrates have a high potential nutritional advantage as low energy dietary supplements. It can be used as a source of carbohydrates for diabetic patients and more generally as dietary fiber. During 4 to 6 weeks improves glucose tolerance, decreases glycemia, and partially restores insulin secretion (Cani*et al.,* 2005) Moreover, an improvement of glucose/insulin ratio has also been observed in rats receiving Oligo fructose added in a high fructose diet (inulin) (Busserolles*et al.,* 2003). In the case of low-calorie chocolates and derivatives, fibre compounds such as inulin and oligo fructose are used as sugar substitutes (Gonze and Van der Schueren, 1997).

This study was carried out to investigate studying the best conditions to obtain inulin from *Catharanthus Roseus* for production of new foods and beverages with high biological value.

CHAPTER 2

REVIEW OF LITERATURE

PLANT MATERIAL

Catharanthus roseus

Catharanthus (**Madagascar Periwinkle**) is a genus of eight species of herbaceous perennial plants, seven endemic to the island of Madagascar, the eighth native to the Indian subcontinent in southern Asia . *Catharanthus roseus* goes by its common name "sadaphuli" (perennially flowering) in parts of Western India.

Fig.1 *Catharanthus roseus* (Genus: Catharanthus)

Catharanthus roseus

Catharanthus roseus (Madagascar Periwinkle) is a species of Catharanthus native and endemic to Madagascar. Synonyms include Vinca rosea (the basionym), Ammocallisrosea, and Lochnerarosea; other English names occasionally used include Cape Periwinkle, Rose Periwinkle, Rosy Periwinkle, and "Old-maid".

In the wild, it is an endangered plant; the main cause of decline is habitat destruction by slash and burnagriculture. It is also however widely cultivated and is naturalised in subtropical and tropical areas of the world.

It is an evergreen subshrub or herbaceous plant growing to 1 m tall. The leaves are oval to oblong, 2.5–9 cm long and 1–3.5 cm broad, glossy green, hairless, with a pale midrib and a short petiole 1–1.8 cm long; they are arranged in opposite pairs. The flowers are white to dark pink with a darker red centre, with a basal tube 2.5-3 cm long and a corolla 2–5 cm diameter with five petal-like lobes. The fruit is a pair of follicles 2–4 cm long and 3 mm broad.

Cultivation and uses

The species has long been cultivated for herbal medicine and as an ornamental plant. In traditional Chinese medicine, extracts from it have been used to treat numerous diseases, including diabetes, malaria, and Hodgkin's disease. The substances vinblastine and vincristine extracted from the plant are used in the treatment of leukemia.

This conflict between historical indigenous use, and recent patents on *Catharanthus roseus* derived drugs by westernpharma ceutical companies, without compensation, has led to accusations of biopiracy.

It can be dangerous if consumed orally. It can be hallucinogenic, and is cited (under its synonym *Vincarosea*) in Louisiana State Act 159.

As an ornamental plant, it is appreciated for its hardiness in dry and nutritionally deficient conditions, popular in subtropical gardens where temperatures never fall below 5 °C to 7 °C, and as a warm-season bedding plant in temperate gardens. It is noted for its long flowering period, throughout the year in tropical conditions, and from spring to late autumn in warm temperate climates. Full sun and well-drained soil are preferred. Numerous cultivars have been selected, for variation in flower colour (white, mauve, peach, scarlet and reddish-orange), and also for tolerance of cooler growing conditions in temperate regions.

Notable cultivars include 'Albus' (white flowers), 'Grape Cooler' (rose-pink; cool-tolerant), the Ocellatus Group (various colours), and 'Peppermint Cooler' (white with a red centre; cool-tolerant). *Catharanthus roseus* is used in plant pathology as an experimental host for phytoplasmas. This is because it is easy to infect with a large majority of phytoplasmas, and also often has very distinctive symptoms such as phyllody and significantly reduced leaf size.

Inulin

Inulin are a group of naturally occurring polysaccharides produced by many types of plants. They belong to a class of fibers known as fructans. Inulin is used by some plants as a means of storing energy and is typically found in roots or rhizomes. Most plants that synthesize and store inulin do not store other materials such as starch.

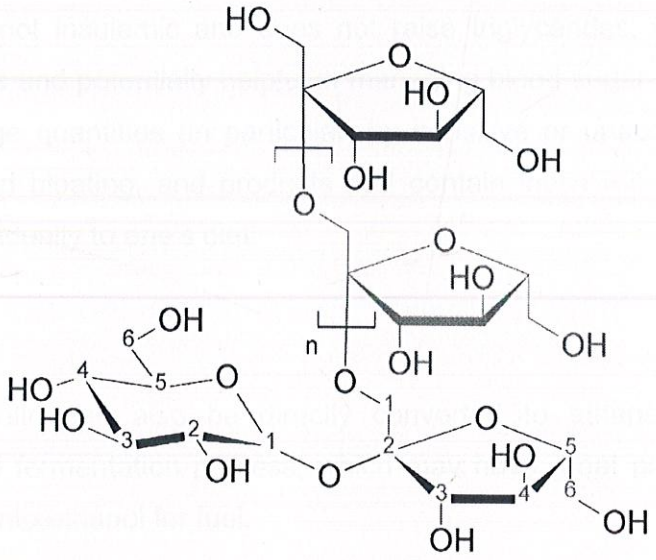

Fig. 2 Inulin staructure

Molecular formula- $C_{6n}H_{10n+2}O_{5n+1}$

Molar mass- Polymer; depends on n.

Uses

Processed foods

Inulin is used in processed foods because it has unusually adaptable characteristics. Its flavour ranges from bland to subtly sweet (approx. 10% sweetness of sugar/sucrose).It can be used to replace sugar, fat, and flour. This is particularly advantageous because inulin contains a quarter to a third of the food energy of sugar or other carbohydrates and a ninth to a sixth of the food energy of fat. While inulin is a versatile ingredient, it also has health benefits. Inulin increases calcium absorption and possibly magnesium absorption, while promoting the growth of intestinal bacteria. In terms of nutrition, it is considered a form of soluble fiber and is sometimes categorized as a prebiotic. Due to the body's limited ability to process polysaccharides, inulin has minimal increasing impact on blood sugar, and—unlike fructose—is not insulemic and does not raise triglycerides, making it considered suitable for diabetics and potentially helpful in managing blood sugar-related illnesses. The consumption of large quantities (in particular, by sensitive or unaccustomed individuals) can lead to gas and bloating, and products that contain inulin will sometimes include a warning to add it gradually to one's diet.

Industrial use

Non hydrolyzed inulin can also be directly converted to ethanol in a simultaneous saccharification and fermentation process, which may have great potential for converting crops high in inulin into ethanol for fuel.

Medical

Inulin is used to help measure kidney function by determining the glomerular filtration rate (GFR). GFR is the volume of fluid filtered from the renal (kidney) glomerular capillaries into the Bowman's capsule per unit time. Inulin is of particular use as it is not secreted or reabsorbed in any appreciable amount at the nephron allowing GFR to be calculated, rather than total renal filtration. However, due to clinical limitations, inulin is rarely used for this purpose and creatinine values are the standard for determining an approximate GFR.

Natural sources of Inulin

Onion (*Allium cepa*), Garlic (*Allium sativum*), Agave (*Agave spp.*)Chicory (*Cichoriumintybus*), Jicama (*Pachyrhizuserosus*), Burdock (*Arctiumlappa*), Coneflower, *Jerusalem artichoke*.

Difference between Inulin and Insulin

Inulin

Inulin is a storage food in the plants and a type of polysaccharide. Inulin tastes sweet, cannot be digested by humans, and is soluble (unlike cellulose). It gets transported to the large intestine where it feeds microbes and promotes fermentation. Inulin is recommended for diabetics. It is found naturally in asparagus, garlic, Jerusalem Artichokes, chicory root, and others.

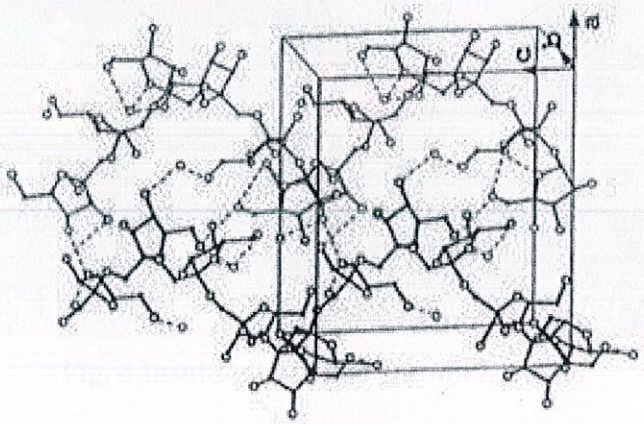

Fig. 3 Crystal structure of Inulin

Insulin

Insulin is a hormone central to regulating carbohydrate and fat metabolism in the body. When you digest food, your body changes most of the food you eat into glucose (a form of sugar). Insulin allows this glucose to enter all the cells of your body and be used as energy.

When you have diabetes, your body doesn't make enough insulin or can't use it properly, so the glucose builds up in your blood instead of moving into the cells. Too much glucose in the blood can lead to serious health problems.

If you take too much insulin it will lower your blood sugar level too much, and you may get hypoglycemia (also called an insulin reaction).

Insulin is composed of two peptide chains referred to as the A chain and B chain. A and B chains are linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain of 30 amino acids.

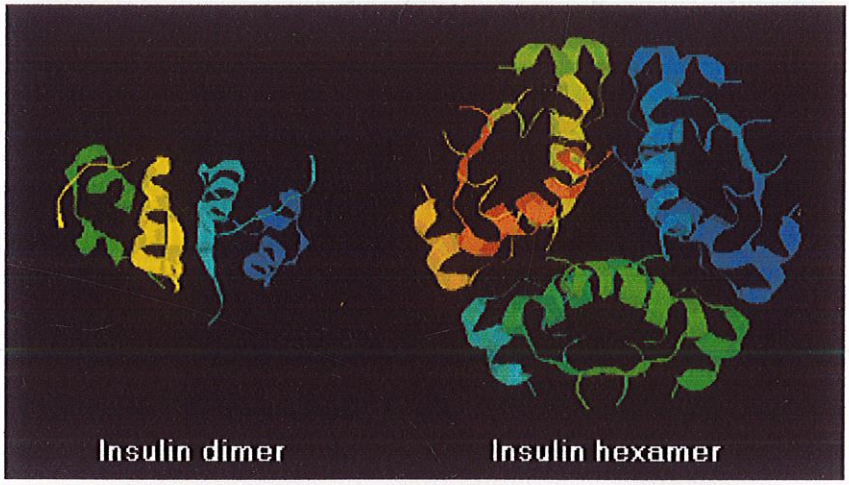

Fig. 4 Insulin dimer and Insulin hexamer

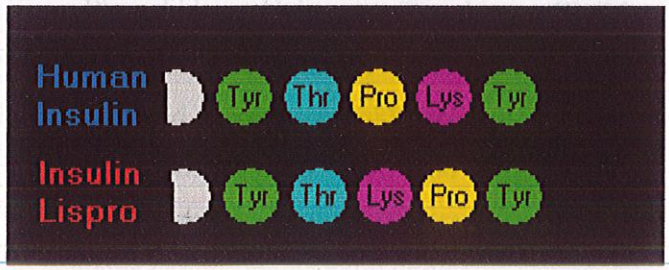

Fig. 5 Human Insulin and Insulin Lispro

Extraction of Inulin

A.M. Gaafar, M.F. Serag El-Din, E.A. Boudy and H.H. El-GazarFood Technology Research Institute, Agricultural Research Center, Giza, Nutrition and Food Science, Faculty of Home Economics, Minufiya University, National Nutrition Institute, Cairo, Egypt worked on extraction conditions of inulin from *Jerusalem Artichoke* Tubers and its effects on blood glucose and lipid profile in diabetic rats. This study aimed to analyze *Jerusalem artichoke* tubers to identify its contents and to optimize conventional extraction of inulin, various time extract, temperature, and solvent ratio were used. 30 male albino rats divided into 5 groups (6 rats) were used to evaluated the extricated inulin as Hypoglycemic agents. It could be concluded that, the highest yield of inulin was recovered from Jerusalem artichoke tuber by using the following condition, sample to solvent ratio was 1: 5 w/v at 80°C for 90 minutes. The crude inulin extracted from *Jerusalem artichoke* tubers were used for production of orange juice and chocolate and estimated by aid of 10 panelists. The reduction of glucose was observed after one week of feeding till the end of experimental period, also, high level of inulin 15% led to amore reduction of blood glucose level comparing with the low level especially in the end of experimental period. The crude inulin extracted from Jerusalem artichoke tubers were used in diet for diabetic rats on different levels of inulin (10 and 15%) had significantly lower in total cholesterol, triglyceride and total lipids in comparing to positive diabetic rats fed on control diet. Meanwhile, HDL level was increased significantly after fed on 10 and 15% inulin. On the other hand, LDL and VLDL levels were decreased significantly after fed on (10 and 15%) inulin in comparing to positive group rats fed on control diet.

Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India Department of Botany, Government Arts College, Karur, Tamil Nadu, India, Department of Biotechnology, J.J. Collage of Art and Sciences, Pudukkottai-622 404, Tamil Nadu, India performed a study on inulin and esculin Content of *In vitro* and *In vivo* plants of chicory. Chicory (*Cichoriumintybus*L.) is an important medicinal plant used traditionally to cure various diseases. The whole plant contains various medicinally important compounds and the concentrations of the compounds vary in different tissues.

In the present study, a comparison was made between the field grown and *in vitro* plantlets for the production of inulin and esculin. Highest amount of inulin was detected from the *in vitro* regenerated root followed by the callus, whereas esculin content was high in the *in vitro* root followed by *in vitro* leaves.

MaywanHariono, MuhamadFaizinAkba, Indah Sularsih, LailatunNajihah, Sarosa Purwadi, ArsaWahyuNugrahani done extraction, identification and acetylation of inulin from DAHLIA TUBER (*Dahlia Pinata*Cav.) The acetylation of the inulin was performed in polar aprotic organic solvents i.e. dimethyl formamide (DMF) and pyridine. According to the type of solvent, it were used and compared in amount of yield that was based on the assumption which the average of Degree Polimerization (DP) of inulin from dahlia was 20. The products were characterized by IR and H-NMR. The methods using pyridine presented 40% of yield, whereas the one using DMF showed a slight decrease in reaction efficiency to 35%. However, the method using pyridine proved to be the preferable way to graft the inulin backbone with acetylation on a bigger scale.The methods using DMF as a solvent were not attractive since the end products had a specific bad smell.

Marisa F. Mendes*, Luana F. Cataldo, Cristiano A. da Silva, Regina I. Nogueira, Suely P. Freitas extraction of the inulin from chicory roots (*Chicoriumintybus L.*) using supercritical carbon di oxide. The chicory, tuberous roots that store inulin, is a native plant from Europe, Asia, Africa and South America but it can be cultivate in all over the world. The inulin is a polysaccharide that can be found in a series of vegetable products like chicory roots (*Chicoriumintybus L.*). It is mostly used in the food industry for production of low caloric foods, acting as a substitute of sugar or fat. The inuline is also utilized as a functional ingredient in the food and pharmaceutical industries. The conventional technology used to produce inulin is the aqueous extraction, but the energetic costs of this process are very high due to the presence of separation steps including filtration, evaporation and drying using spray-dryers. Considering that Brazil is recognized as the most active country in Latin America involved in supercritical CO2 R&D and the other hand, does not have a commercial production of inulin, the motivation of this work is evaluate the operational parameters of supercritical extraction technology of inulin from chicory.

Extractions were done in an experimental apparatus containing a stainless steel extractor, a micrometer valve, a high-pressure pump and a heating bath. Approximately 16 grams of crushed chicory was fed to the extractor. The experimental conditions has been investigated the effect of temperature in the range of 40 to 80°C and pressure in the range of 62 to 170 bars in an operational time of 2 hours. The maximum solute recovery was reached at 40°C and 150 bars, for 2 hours of extraction. As occurred in many supercritical processes, the experimental results indicated that when the pressure is increased, the extracted mass increases at constant temperature and when the temperature increased at constant pressure, the extracted mass decreases due to the decrease of the solvent density.

Jean-Hugues Renault, Jean-Marc Nuzillard, Gae¨lle Le Croue´rour, Philippe The´penier, Monique Ze`ches-Hanrot, Louisette Le Men-Olivier isolated indole alkaloids from *Catharanthus roseus* by centrifugal partition chromatography in the pH-zone refining mode. Centrifugal partition chromatography (CPC) in the pH-zone refining mode allowed a preparative and efficient isolation of vindoline, vindolinine, catharanthine and vincaleukoblastine from a crude mixture of *Catharanthus roseus* alkaloids. The separation protocol was tested with a synthetic mixture of vindoline, catharanthine and vinca leukoblastine. The fraction content was analyzed and the results compared with theoretical chromatograms obtained by numerical simulation. The increase in injected sample mass results in an improvement of the purity of the isolated compounds. This observation, confirmed by theory, is of prime importance for the development of preparative pH-zone refining CPC as a preparative separation method.

For extracting and purification of inulin various instruments were used.

SONICATOR

Fig. 6 Sonicator

Sonication is the act of applying sound (usually ultrasound) energy to agitate particles in a sample, for various purposes. In the laboratory, it is usually applied using an ultrasonic bath or an ultrasonic probe, colloquially known as a sonicator. In a paper machine, an ultrasonic foil can distribute cellulose fibres more uniformly and strengthen the paper.

Sonication can be used to speed dissolution, by breaking intermolecular interactions. It is especially useful when it is not possible to stir the sample, as with NMR tubes. It may also be used to provide the energy for certain chemical reactions to proceed.

Sonication can be used to remove dissolved gases from liquids (degassing) by sonicating the liquid while it is under a vacuum. This is an alternative to the freeze-pump-thaw and sparging methods.

In biological applications, sonication may be sufficient to disrupt or deactivate a biological material. For example, sonication is often used to disrupt cell membranes and release cellular contents. This process is called sonoporation. Sonication is also use to fragment molecules of DNA.

Sonication is commonly used in nanotechnology for evenly dispersing nanoparticles in liquids.

Sonication can also be used to initiate crystallisation processes and even control polymorphic crystallisations[citation needed]. It is used to intervene in anti-solvent precipitations (crystallisation) to aid mixing and isolate small crystals.

Sonication is the mechanism used in ultrasonic cleaning; loosening particles adhering to surfaces. Outside the field of laboratory science, sonicating baths are used to clean objects such as spectacles and jewelry.

Sonication is also used to extract microfossils from rock.

Sonication can also refer to buzz pollination – the process that bees use to shake pollen from flowers by vibrating their wing muscles.

Sonication is the process of converting an electrical signal into a physical vibration that can be directed toward a substance. Sonicator are vital lab equipment and are used for a number of purposes. Sonication is usually performed to break apart compounds or cells for further examination. The vibration has a very powerful effect on solutions, causing their molecules to break apart and cells to rupture. A prime example is in DNA testing, where the cells that may contain DNA information are subjected to sonication to break them apart and release the DNA proteins so they can be tested.

The primary part of a sonication device is the ultrasonic electric generator. This device creates a signal (usually around 20 KHz) that powers a transducer. This transducer converts the electric signal by using piezoelectric crystals, or crystals that respond directly to the electricity by creating a mechanical vibration. This vibration, molecular in origin, is carefully preserved and amplified by the sonicator, until it is passed through to the probe.

The sonication probe transmits the vibration to the solution being sonicated. This probe is a carefully constructed tip that moves in time with the vibration, transmitting it into the solution. The probe moves up and down at a very high rate of speed, although the amplitude can be controlled by the operator and is chosen based on the qualities of the solution being sonicated.

The rapid movement of the probe creates an effect called cavitation. Cavitation occurs when the vibrations create a series of microscopic bubbles in the solution, pockets of space wedged between the molecules that form and then collapse again under the weight of the solution, sending out tiny shockwaves into the surrounding substance. Thousands of these bubbles forming and collapsing constantly create powerful waves of vibration that cycle into the solution and break apart the cells.

There are different-sized probe tips based on what sort of sonification process is desired. A very small tip will create excellent cavitation effects and easily disrupt surrounding cells, but it will have a limited area of effect based around the probe itself. Larger tips can reach a greater quantity of the solution but will not produce such an intense reaction.

Sonication rates as a very thorough cell-disrupter, sometimes too strong for the cells and proteins in question. If scientists need to use a more delicate procedure, they generally choose a traditional process such as enzyme digestion (disruption by chemical reaction) or grinding with a material such as sand.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase.After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via

capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

Thin layer chromatography can be used to:

- Monitor the progress of a reaction
- Identify compounds present in a given substance
- Determine the purity of a substance

Specific examples of these applications include:

- analyzing ceramides and fatty acids
- detection of pesticides or insecticides in food and water
- analyzing the dye composition of fibers in forensics, or
- assaying the radiochemical purity of radiopharmaceuticals
- identification of medicinal plants and their constituents [3]

A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantization. This method is referred to as HPTLC, or "high performance TLC".

PLATES PREPRATION

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

TECHNIQUE

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products.

To run a TLC, the following procedure is carried out:

- A small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off, otherwise a very poor or no separation will be achieved. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber.
- A small amount of an appropriate solvent (elutant) is poured in to a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper is put into the chamber, so that its bottom touches the solvent, and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results).
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the elutant in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample). When the solvent front reaches no higher than the top of the filter paper in the chamber, the plate should be removed (continuation of the elution will give a misleading result) and dried.

Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the R_f value) can be adjusted. Also, the separation achieved with a TLC plate can be used to estimate the separation of a flash chromatography column.[6]

Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. For instance, if normal phase silica gel is used as the stationary phase it can be considered polar. Given two compounds which differ in polarity, the more polar compound has a stronger interaction with the silica and is therefore more capable to dispel the mobile phase from the binding places. Consequently, the less polar compound moves higher up the plate (resulting in a higher Rf value). If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding places and all compounds on the TLC plate will move higher up the plate. It is commonly said that "strong" solvents (elutants) push the analyzed compounds up the plate, while "weak" elutants barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate. For silica gel coated TLC plates, the elutant strength increases in the following order: Perfluoroalkane (weakest), Hexane, Pentane, Carbon tetrachloride, Benzene/Toluene, Dichloromethane, Diethyl ether, Ethylacetate, Acetonitrile, Acetone, 2-Propanol/n-Butanol, Water, Methanol, Triethylamine, Acetic acid, Formic acid (strongest). For C18 coated plates the order is reverse. Practically this means that if you use a mixture of ethyl acetate and heptane as the mobile phase, adding more ethyl acetate results in higher Rf values for all compounds on the TLC plate. Changing the polarity of the mobile phase will normally not result in reversed order of running of the compounds on the TLC plate. An eluotropic series can be used as a guide in selecting a mobile phase. If a reversed order of running of the compounds is desired, an apolar stationary phase should be used, such as C18-functionalized silica.

PREPRATIVE TLC

TLC can also be used on a small semi-preparative scale to separate mixtures of up to a few hundred milligrams. The mixture is not "spotted" on the TLC plate as dots, but rather is applied to the plate as a thin even layer horizontally to and just above the solvent level. When developed with solvent the compounds separate in horizontal bands rather than horizontally separated spots. Each band (or a desired band) is scraped off the backing material. The backing material is then extracted with a suitable solvent (e.g. DCM) and filtered to give the isolated material upon removal of the solvent.

For small-scale reactions with easily separated products, preparative TLC can be a far more efficient in terms of time and cost than doing column chromatography. Obviously, the whole plate can not be chemically developed or the product will be chemically destroyed. Thus this technique is best used with compounds that are coloured, or visible under UV light. Alternatively, a small section of the plate can be chemically developed e.g. cutting a section out and chemically developing it, or masking most of the plate and exposing a small section to a chemical developer like iodine.

CHAPTER 3

MATERIALS & METHODS

MATERIALS

PLANT MATERIAL

Catharanthus **(Madagascar Periwinkle)** is a genus of eight species of herbaceous perennial plants, seven endemic to the island of Madagascar, the eighth native to the Indian subcontinent Asia *Catharanthus roseus* goes by its common name "sadaphuli" (perennially flowering) in parts of Western India.

Catharanthus roseus

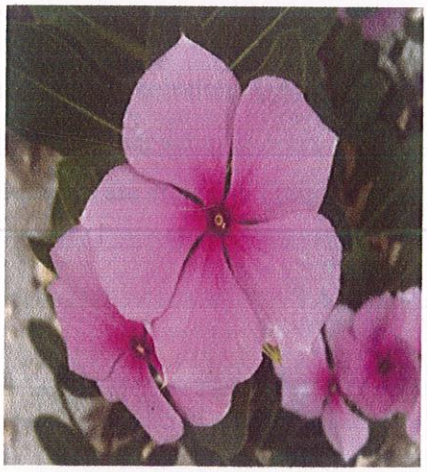

Fig. 7 Flower of C. Roseus

Fig.8 Catharanthus Roseus tubers

Family: Apocynaceae

Pronunciation: cath-ar-AN-thus ROW-zee-us

Common names

This species was formerly known as ***Vinca rosea*** .

METHODOLOGY

Preparation of *samples*:

The samples of *Catharanthus roseus* tubers were cleaned with tap water to remove dust and other undesirable materials. The cleaned tubers were cut into small pieces and used immediately.

Extraction of inulin from *Catharanthus roseus* by different methods

There are basically 2 methods for extracting inulin from *Catharanthus roseus.*

1. Hot water extraction method
2. Sonication

EXTRACTION OF INULIN

6 samples were prepared using sonication. Sample are having the ratio 5:1 i.e. 75ml of distilled water and 15 gms of chopped roots of *Catharanthus roseus*. Sonicator parameters were set. Pulse was kept at 20 sec on time and 10 sec off time. Sample is kept in the sonicator such that the probe is just dipped in the solution. Sonication for sample is done for 20 mins.

Progressively other samples were created having the ratio 6:1, 7:1, 8:1, 9:1 and 10:1.

These samples are stored in their flasks with aluminium foil around their opening.

TABLE -1 SONICATED SAMPLES

Sample no.	Ratio	Wt. of roots(in gm)	Distilled water(in ml)	Time (mins)
1	5:1	10	50	20
2	6:1	8.3	50	20
3	7:1	7.1	50	20
4	8:1	6.3	50	20
5	9:1	5.5	50	20
6	10:1	5	50	20

PARTIAL PURIFICATION

After extraction, Liquid portion was taken for further purification. Cantrifugation of all samples are done at 10,000 rpm for 10 mins at room temperature.

The main objective behind centrifugation is to purify the sample..

THIN LAYER CHROMATOGRAPHY

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate(gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. We're using glass sheet here. The resultant glass plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

Solvent (elutant) consists of 2-Butanol:isopropanol:Glacial acetic acid: methanol is the ratio of 60:30 :80 :2

To run a TLC, the following procedure is carried out

- A small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. Thesolvent is allowed to completely evaporate off, otherwise a very poor or no separation will be achieved. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vaccum chamber.
- A small amount of an appropriate solvent (elutant) is poured in to a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper is put into the chamber, so that its bottom touches the solvent, and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results).
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the elutant in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the

sample). When the solvent front reaches no higher than the top of the filter paper in the chamber, the plate should be removed (continuation of the elution will give a misleading result) and dried.

For visualizing the spots developing reagent is used.

It is made of 135ml Ethanol + 10ml 4-methoxy-benzaldehyde + 5ml Glacial acetic acid + 2ml conc. Sulphuric acid.

Yellow colored spots appeared on the strip of paper corresponding to the spot of inulin showing the yield of inulin.

CARBOHYDRATE ANALYSIS

There are two methods for doing carbohydrate quantitative test :-

1) Dinitrosalicylic acid test
2) Phenol sulphuric acid test

DINITROSALICYLIC ACID TEST

For sugar estimation dinitrosalicylic acid method is a simple, sensitive and adoptable during handling of a large number of samples at a time.

Fructose was taken as refrence.

MATERIALS :-

1) Dinitrosalicylic Acid Reagent (*DNS Reagent*)

Dissolve by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 mL 1% NaOH. Store at 4°C. Since the reagent deteriorates due to sodiumsulphite, if long storage is required, sodium sulphite may be added at the time of use.

2) 40% Rochelle salt solution (Potassium sodium tartrate).

PROCEDURE :-

- Analysis of standard
  1) Stock solution is prepared having fructose 10 mg/ml.
  2) Serial dilution is performed by taking .1, .2 .4, .6, .8. and 1 and water is added to make these dilutions upto 1 ml.
  3) Prepare DNS reagent by taking 1% NaOH solution (1 gm in 100 ml distilled water) and adding 1 gm DNS, 0.2 gm crystalline phenol, 0.05 gm sodium sulphide.
  4) Add 3 ml DNS reagent into each test tubes having dilution.
  5) Hot water bath is performed for 5 mins at 100°c.
  6) Add 1ml Rochelle salt and take OD at 510nm.
  7) Plot the standard curve for fructose.

- Analysis of sample
  1) Add 3ml DNS reagent into each test tubes having samples.
  2) Hot water bath is performed for 5 mins at 100°c.
  3) Add 1ml Rochelle salt and take OD at 510nm.
  4) Plot the graph having sample curve.

PHENOL SULPHURIC ACID TEST

In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green coloured product with phenol and has absorption maximum at 490 nm.

Inulin was taken as standard for making standard curve.

MATERIALS :-

1) Phenol 5%: Redistilled (reagent grade) phenol (50 g) dissolved in water and diluted to onelitre.
2) Sulphuric acid 96% reagent grade.
3) Standard inulin -10 mg in 10 mL of water.

PROCEDURE :-

- Analysis of standard

1) Stock solution is prepared having inulin 10mg/ml.
2) Serial dilution is performed by taking .1, .2 .4, .6, .8. and 1 and water is added to make these dilutions upto 1 ml.
3) Add 0.5ml phenol solution.
4) Add 0.25ml of 96% sulphuric acid (96ml in 100ml distilled water).
5) After adding sulphuric acid, vigourously shake it.
6) Keep it at 30°c for 20mins.
7) Now take OD at 490nm. Take the blank 1ml distilled water.

- Analysis of sample

1) Add 0.5ml phenol solution.
2) Add 0.25ml of 96% sulphuric acid (96ml in 100ml distilled water).
3) After adding sulphuric acid, vigourously shake it.
4) Keep it at 30°c for 20mins.
5) Now take OD at 490nm. Take the blank 1ml distilled water.

CHAPTER 4

RESULTS AND DISCUSSIONS

THIN LAYER CHROMATOGRAPHY

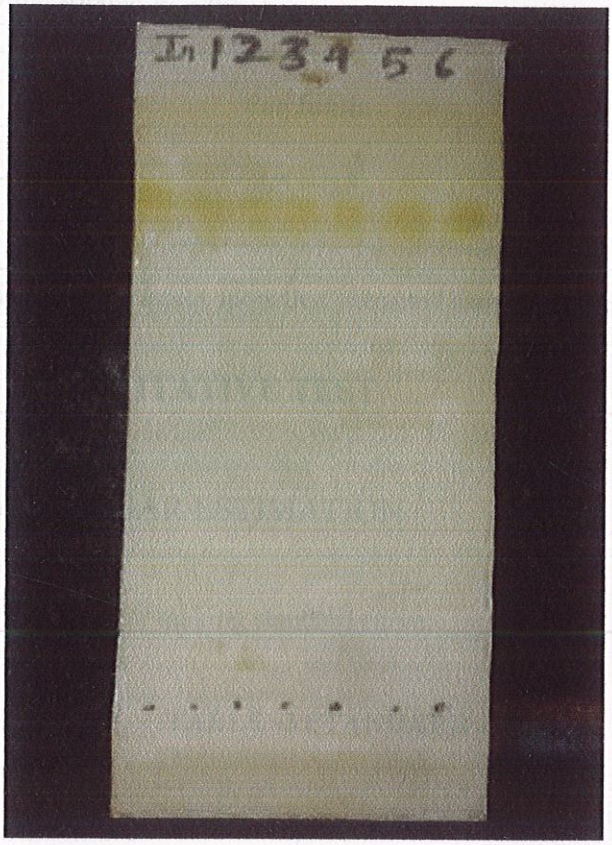

Fig. 9 Strip of paper showing yield

This strip of paper confirms the presence of inulin in the extracted samples. "In" mark on the paper is of pure inulin and the marks 1, 2, 3, 4, 5, 6 represent various extracted samples.

Retention factor of each spot can be determined by dividing the distance traveled by the product by the total distance traveled by the solvent (the solvent front).

The R_f value , or retention factor, of each spot can be determined by dividing the distance traveled by the product by the total distance traveled by the solvent (the solvent front). These values depend on the solvent used, and the type of TLC plate, and are not physical constants. Eluent on the thin layer is put on top of the plate.

The distance travelled by the product is 4cms.

The distance travelled by the solvent is 5cms.

	For Inulin	For Samples
R_f value	4/5 = 0.8	4/5 = 0.8

It is evident from the R_f factor of different spots that extracted samples contained inulin.

CARBOHYDRATE QUANTITATIVE TEST

1) REDUCING SUGAR ESTIMATION

In this experiment we use fructose for making standard curve.

TABLE -3 STANDARD OD

CONCENTRATION (µg/ml)	OD
10	0.078
20	0.158
40	0.293
60	0.425
80	0.489
100	0.591

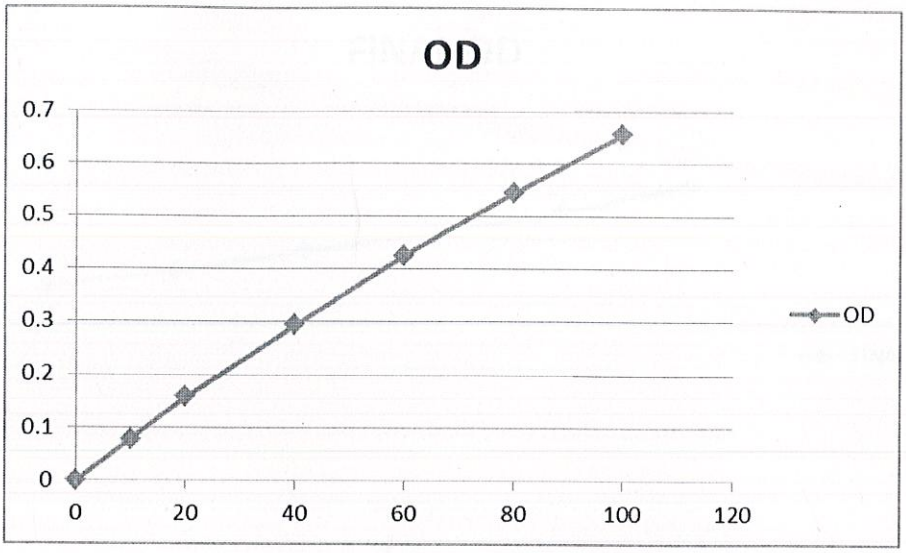

Fig. 10 FRUCTOSE STANDARD CURVE

TABLE – 4 SAMPLE OD

SAMPLE RATIO	OD
5:1	0.213
6:1	0.225
7:1	0.241
8:1	0.254
9:1	0.270
10:1	0.292

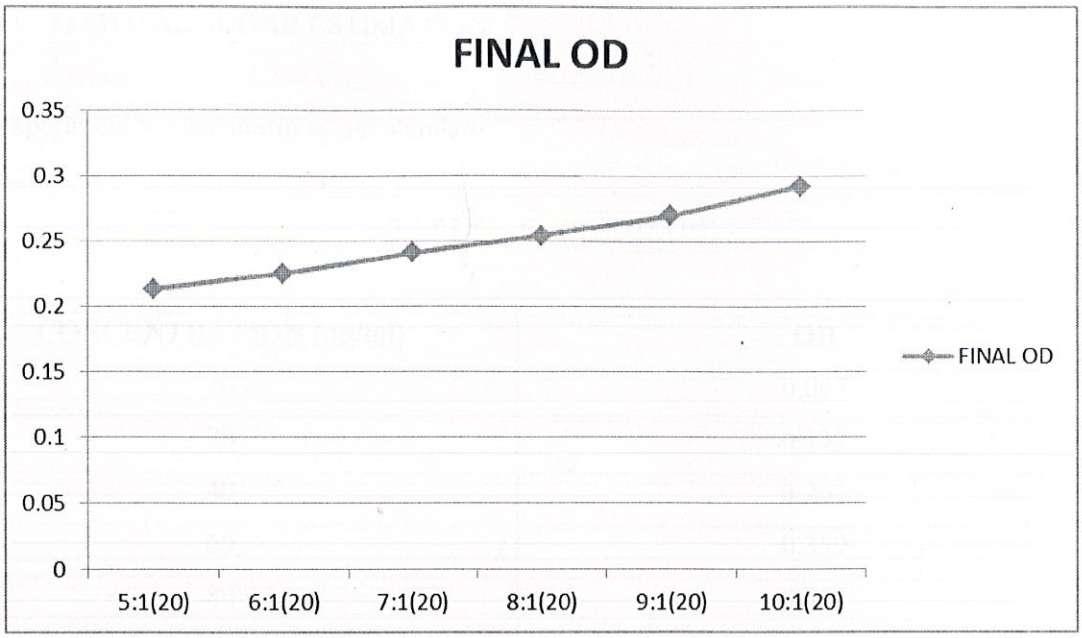

Fig. 11 SAMPLE CURVE

After compairing the standard curve and sample curve, the concentration of reducing sugar present in the samples are shown in the table below.

TABLE – 5 CONCENTRATION

SAMPLE RATIO	OD	CONCENTRATION (µg/ml)
5:1 (20)	0.213	28
6:1 (20)	0.225	30
7:1 (20)	0.241	32
8:1 (20)	0.254	33
9:1 (20)	0.270	36
10:1 (20)	0.292	38

2) TOTAL SUGAR ESTIMATION

In this experiment we use inulin as our standard.

TABLE – 6 STANDARDS

CONCENTRATION (µg/ml)	OD
10	0.067
20	0.132
40	0.261
60	0.359
80	0.450
100	0.567

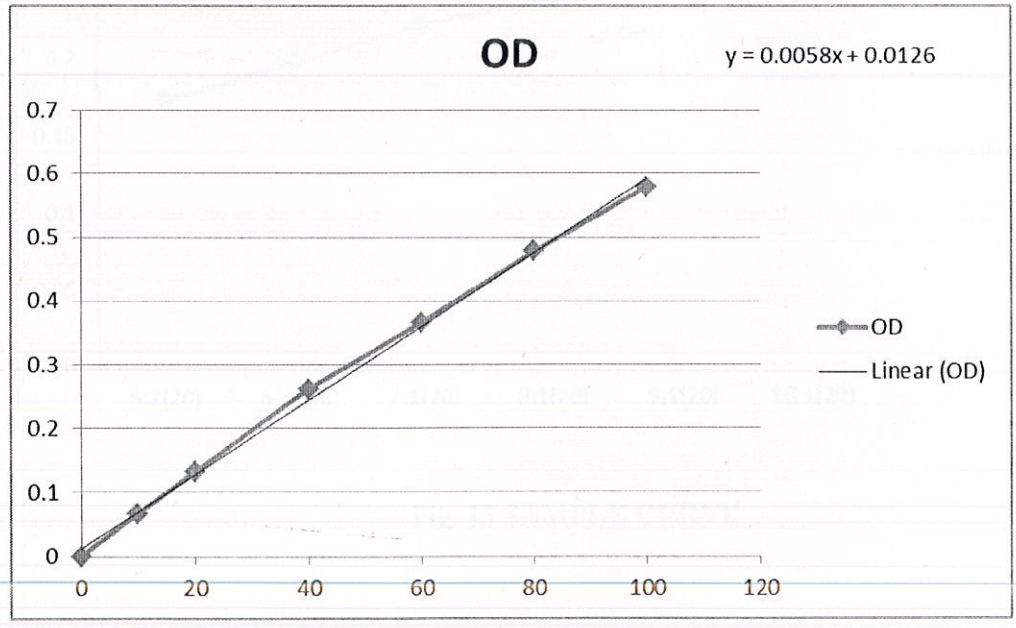

Fig. 12 INULIN STANDARD CURVE

TABLE – 7 SAMPLES

Sample ratio	OD
5:1(20)	0.173
6:1(20)	0.198
7:1(20)	0.222
8:1(20)	0.238
9:1(20)	0.249
10:1(20)	0.271

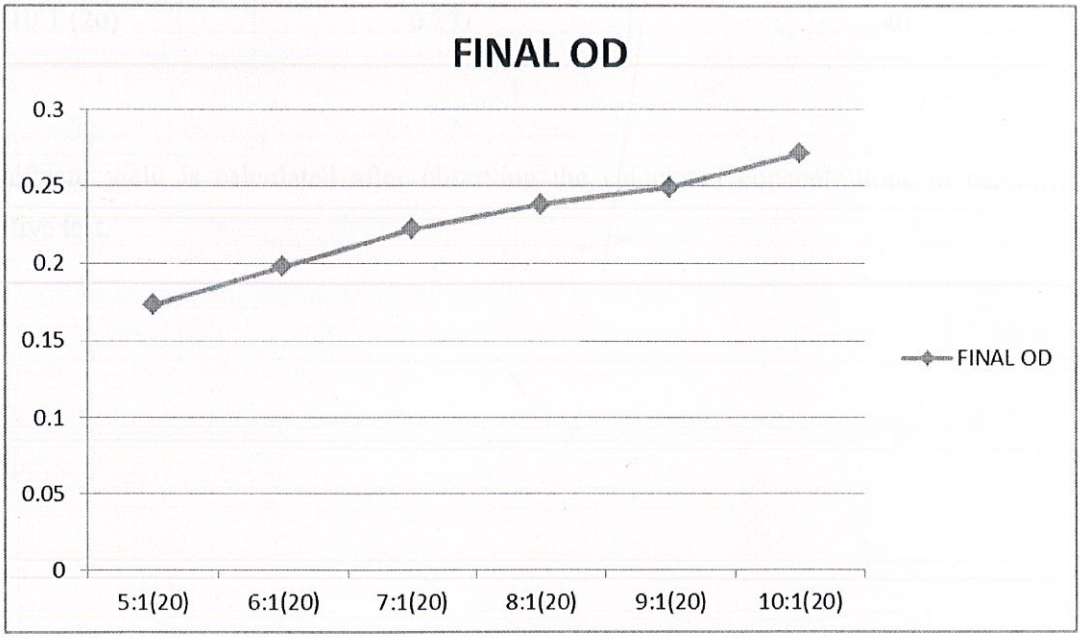

Fig. 13 SAMPLE CURVE

After compairing the standard curve and sample curve, the concentration of sugar present in the samples are shown in the table below.

TABLE – 8 CONCENTRATION

SAMPLE RATIO	OD	CONCENTRATION (µg/ml)
5:1 (20)	0.173	26
6:1 (20)	0.198	30
7:1 (20)	0.222	33
8:1 (20)	0.238	35
9:! (20)	0.249	37
10:1 (20)	0.271	40

No significant yield is calculated after observing the calculated concentrations in carbohydrate quantitative test.

CHAPTER – 5

CONCLUSIONS

- Inulin belongs to the family of fructans. It has immense medical importance. It helps in measuring kidney function. It is also used for diabetic patients.

  R_f factor shows the presence of inulin in *Catharanthus roseus* tubers but results shows no significant yield. It concludes that further purification and concentration steps are required.
  Different extraction and purification process is required.

CHAPTER – 6

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