MEDIA OPTIMIZATION BY CENTRAL COMPOSITE DESIGN BASED RSM AND CHARACTERIZATION OF INULINASE FROM SOIL FUNGAL ISOLATES

PROJECT REPORT

Submitted by

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Register No: 1020203008

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DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitled

MEDIA OPTIMIZATION BY CENTRAL COMPOSITE DESIGN BASED RSM AND CHARACTERIZATION OF INULINASE FROM SOIL FUNGAL ISOLATES

is the bonafide record of project work done by

S. MUTHU SELVI

Register No: 1020203008

of M.Tech (Biotechnology) during the year 2011-2012

…… ……..

Project Guide **Head of the Department**

Submitted for the project Viva-Voce examination held on .....

…….. ……

Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled "**MEDIA OPTIMIZATION BY CENTRAL COMPOSITE DESIGN BASED RSM AND CHARACTERIZATION OF INULINASE FROM SOIL FUNGAL ISOLATES**" being submitted in partial fulfilment for the award of M.Tech (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

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I certify that the declaration made above by the candidate is true.

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(MUTHU SELVI S)

ABSTRACT

Inulinase is a versatile enzyme used in many fields, especially in food industry, to produce high fructose syrups. Microbial inulinases (ß-D-fructopyranoside fructohydrolase, EC 3.2.1.80) are enzymes which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose). In this present study, Fungal species showing high high inulinase activity and isolated from rhizosphere region of soil samples collected from Non such estate, Coonoor and Yercaud. Two different species namely *Pencillium purpurogenum* (0.953 U/ml) and *Aspergillus tamarii* (0.432 U/ml) showed maximum inulinase activity. From the two species *Pencillium purpurogenum* possess maximum inulinase activity. The characterisation of the crude enzyme from *Pencillium purpurogenum* using coffee spent as a substrate was analysed. The optimum temperature and pH were found to be 60 ^{o}C and 5, respectively. The k_m and V_{max} of crude inulinase were 0.25 mg and 40 U/ml, respectively. The enzyme was thermostable for 4 hrs. Metals like Hg^{2+}(87.57%), Fe^{3+}(84.68%), Cu^{2+}(71.41%), Zn^{2+}(64.14%), Mg^{2+}(56.62%) inhibits the inulinase activity. The maximum yield of inulin extraction using coffee spent (Pretreatment) was achieved by the microwave assisted extraction. The yield was found to be 42.57 U/ml (20 fold increase) at optimum conditions of 540 watts with 1: 25 material ratio and 30 seconds. The optimisation of media using Response Surface Methodology revealed that the coffee spent (0.1 mg/ml), NH_4Cl (0.1 mg/ml) and Agitation rate (200 rpm) recorded the maximum inulinase activity (106.29 u/ml). The enzyme was partially isolated by ammonium sulphate precipitation and ultrafiltration and the fold purification was about 1.83 (88.5U/mg). Partially isolated inulinase activity was optimal at pH 5 and temperature 55^{o}C. TLC analysis revealed the presence of hydrolysed products like fructose. All these conditions make *Pencillium purpurogenum*, a potential candidate for food industries.

Keywords: Inulin, inulinase activity, coffee spent, *Pencillium purpurogenum* , metal salts

LIST OF TABLES

LIST OF FIGURES

ABBREVATIONS

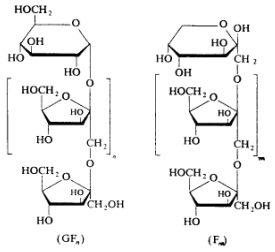
BSA	Bovine Serum Albumin
$CuSO_4$	Copper sulphate
DNS	Dinitro Salicylic Acid
$FeCl_3$	Ferric Chloride
$HgCl_2$	Mercuric Chloride
KCl	Potasssium Chloride
K_m	Michaelis Menton constant
$MgSO_4$	Magnesium Sulphate
NH_4Cl	Ammonium chloride
$ZnCl_2$	Zinc chloride
PBD	Plackett- Burman Design
RSM	Response Surface Methodology
PDA	Potato dextrose agar
PDB	Potato dextrose broth
TLC	Thin Layer Chromatography
V_{max}	Maximum Rate of Reaction
U	Enzyme Unit
CCD	Central composite design
MAE	Microwave assisted extraction
H	Hour
g	Gram
min	Minutes
ml	Millilitre
nm	Nanometer

CHAPTER 1

INTRODUCTION

1.1 Inulin

Inulin occurs as a reserve of carbohydrate in the roots or tubers of plants such as *Helianthus tuberosus* L, *Cichorium intybus* L *Jerusalem artichoke*, dahlia tubers, chicory root, yacon and *Dahlia variabilis*. Inulin is a group of widespread naturally occurring polysaccharide produced by many types of plants (Marchetti, 1993). They belong to a class of fibers known as fructans. Inulin is used by some plants as a storage polysaccharide and is typically found in roots or rhizomes. Most plants that synthesize and store inulin do not store other materials such as starch. Inulin is composed of linear chains of β-(2,1) linked fructose residues attached to a terminal sucrose molecule (Waterhouse and Chatterton, 1993). A starting glucose moiety can be present, but is not necessary. In contrast, levan which isformed by certain bacteria consists mainly or exclusively of (2,6) fructosyl-fructose links.



GF_n _ (α-D-glucopyranosyl1-[β-D-fructofuranosyl]$_{n-1}$-D-fructofuranoside)
F_n _ (β -D-glucopyranosyl1-[α -D-fructofuranosyl]$_{n-1}$-D-fructofuranoside)

n- degree of polymerization.

GFn and Fn compounds are considered to be included under this same nomenclature. In chicory inulin, n (the number of fructose units linked to a terminal glucose) can vary from two to 70 (De Leenheer and Hoebregs, 1994). This also means that inulin is a mixture of oligomers and polymers.The degree of polymerization (DP) of inulin, as well as the presence of branches are important properties since they influence the functionality of most inulin to a striking extent. Thus, a strict distinction must be made between inulin of plant origin and that of bacterial origin. The DP of plant inulin is rather low (maximally 200) and varies according to the plant species, weather conditions and the physiological age of the plant.

1.2 Biodegradation of inulin

Several fungal enzymes have been described to involve in the degradation or modification of inulin,that includes exoinulinases, endoinulinases, invertases, and fructosyl transferases. Exoinulinase (D-fructan fructohydrolase, EC 3.2.1.80), hydrolyzes the terminal β-(2,1) fructofurosidic bonds in inulin to produce mainly fructose and partially glucose; endoinulinase (D-fructan fructanohydrolase, EC 3.2.1.7), which is specific for inulin and hydrolyzes the internal linkages in inulin to release inulotriose, tetraose and pentaose as the main products. A third enzyme, invertase (D-fructofuranoside fructohydrolase, EC 3.2.1.26) hydrolyzes 2,1 fructosidic bond from sucrose to fructose and glucose. Because of the overlapping substrate specificity of invertases and exo inulinases, the enzymes are further classified by the substrates hydrolytic ratio. Fructosyl transferases (EC2.4.1.99, 2.4.1.9) transfers fructose residues from the non reducing terminal 2,1 fructofurosidic bonds in sucrose or inulin to another sucrose or inulin molecule to form ketose or higher fructooligosaccharides. The difference between these two enzymes EC 2.4.1.9 and EC 2.4.1.99 is clarified by the products; if the predominant product is kestose the enzyme is classified as EC 2.4.1.99, when the predominant products are higher fructooligosaccharides the enzyme is classified as EC2.4.1.9. All enzymes with above activities are considered as inulin modifying enzymes (IMEs).

1.3 Inulinase

Microbial inulinases are of considerable interest both in fundamental studies and in industrial applications. The production of ß-fructofuranosidases (inulinases) is widely distributed in some groups of microorganisms and its synthesis is dependent on growth conditions, mainly the carbon source. These enzymes are classified according to their mode of action on inulin. Endo-inulinases (ß-D-fructan:fructan hydrolase, EC 3.2.1.7) produce inulo-oligosaccharides from inulin, and exo-inulinases (ß-D-fructopyranoside fructohydrolase, EC 3.2.1.8) release fructose from the fructosyl terminal of inulin. Inulinase (2,1-β-D-fructanohydrolases EC 3.2.1.7) hydrolyses inulin into practically pure fructose, being an excellent alternative for the production of fructose syrup. Inulinases can be found in higher plants (Vandamme and Derycke, 1983; Claessens *et al.,*1990; Kaur et al., 1992) and microorganisms as filamentous fungi, yeasts and bacteria (Vandamme and Derycke, 1983; Belamri *et al.,* 1994; Wei *et al*., 1998; Kochhar *et al.,* 1998; Pessoa Júnior andVitolo, 1999; Kushi *et al*., 2000). Inulinases of many microorganisms, especially of filamentous fungi, are used to optimize process of hydrolysis of the inulin related to food industries for the production of alcohol, acetone and butanol (Vandamme and Derycke, 1983; Pandey *et al*., 1999); and are also used in the medical area as a tool for the diagnosis of renal problems (Kuehnle *et al*., 1992). Generally, the inulinase activity (I) is accompanied by invertase activity (S) and the enzymatic complex is characterized by I/S ratio. When I/S ratio is higher than 10-2, the enzyme complex has a preponderate inulinase activity, while for invertase activity the I/S ratio is lower than 10-4 (Sharma *et al*., 2006).

1.3 Sonication assisted extraction

Sound waves, which have frequencies higher than 20 kHz, are mechanical vibration in a solid, liquid and gas. Unlike electromagnetic waves, sound waves must travel in a matter and they involve expansion and compression cycles during travel in the medium. Expansion pulls molecules apart and compression pushes them together. The expansion can create bubbles in a liquid and produce negative pressure. The bubbles form, grow and finally collapse. Close to a solid boundary, cavity collapse is asymmetric and produces high-speed jets of liquid. The liquid jets have strong impact on the solid surface (Luque-Garcia and Luque de Castro, 2003).

1.4 Microwave-assisted extraction

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz. Domestic and industrial microwaves generally operate at 2.45 GHz, and occasion-ally at 0.915 GHz in the USA and at 0.896 GHz in Europe. Microwaves are transmitted as waves, which can penetrate biomaterials and interact with polar molecules such as water in the

biomaterials to create heat. Consequently, micro-waves can heat a whole material to penetration depth simultaneously.

Microwave-assisted extraction (MAE) offers a rapid delivery of energy to a total volume of solvent and solid plant matrix with subsequent heating of the solvent and solid matrix, efficiently and homogeneously. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix, improving the recovery of nutraceuticals (Kaufmann, Christen and Veuthey, 2001). Kratchanova, Pavlova and Panchev (2004) observed using scanning electron micrographs that microwave pretreatment of fresh orange peels led to destructive changes in the plant tissue. These changes in the plant tissue due to microwave heating gave a considerable increase in the yield of extractable pectin. Furthermore, the migration of dissolved ions increased solvent penetration into the matrix and thus facilitated the release of the chemicals. The effect of microwave energy is thus strongly dependent on the dielectric susceptibility of both the solvent and the solid plant matrix.

1.4 Origin of research problem

Conventional fructose production from starch require three enzymatic steps process which includes the action of α-amylase, amyloglucosidase and glucose isomerase that yields only 45% fructose (Mazutti *et al*, 2006). Therefore fructose can be obtained by acid hydrolysis of inulin, at low pH and the process gives rise to coloring of the inulin hydrolysate and by-product formation in the form of difructose anhydrides. Fructose formation from inulin offers advantage as it involves only a single enzymatic step yielding up to 95% fructose (Vandamme and Derycke, 1983; Pandey *et al* ., 1999; Luminita and Irina,2005).

1.5 OBJECTIVES

- To screen the inulinase producing soil fungal isolates.
- To characterize the kinetic parameters of crude inulinase (pH, Temperature k_m and metal inhibitors)
- To optimise (pretreatment) the inulin extraction from the coffee spent using microwave and ultrasonication assisted extraction using L_{16} orthogonal design of experiments.
- To optimize the inulinase producing media by Plackett-Burman and Central composite design of experiments
- To partially isolate the inulinase (Ammonium sulphate precipitation, Ultrafiltration) and characterize the partially isolated enzyme.
- To determine the hydrolysed products using thin layer chromatography.

CHAPTER 2
LITERATURE REVIEW

2.1 Inulin

Inulin belongs to the class of non-digestible carbohydrates known as fructans, which are linear and branched polymers of fructose (Luminita and Irina, 2005). It is a widespread naturally occurring polyfructan in plants (Carpita, Kanabus and Housley, 1989; Hendry, 1987; Marchetti, 1993; Arun *et al.,* 2006). About 15% of flowering plant species store fructans as a reserve in atleast one of their organs during their life cycle. It is produced naturally in over 36,000 plants worldwide, including 1200 native grasses belonging to 10 families (Marchetti, 1993; Arun *et al* , 2006). Among the plants that store fructans are many of significant economic importance, such as cereals (e.g. barley, wheat and oat), vegetables (e.g. dahlia and tulip) and forage grasses (e.g. Lolium and Festuca) (Hendry and Wallace, 1993; Luminita and Irina, 2005). Such inulin sources have received a great interest as they represent a relatively inexpensive and abundant substrates for the microbial production of high fructose syrup, which has gained importance in food, drink and pharmaceutical industries (Arun *et al* , 2006).

In higher plants five major classes of structurally different fructans can be distinguished: inulin, levan, mixed levan and inulin neoseries. Inulin has been defined as a polydisperse carbohydrate material consisting mainly, if not exclusively, consisting of linear chains of β-(2,1)-linked fructose residues attached to a terminal sucrose molecule (Luminita and Irina , 2005, Arun *et al* , 2006). A starting glucose moiety can be present, but is not necessary.

Fructans are widely spread within the family Liliaceae (3500 species) and most frequently among the Compositae (25000 species) (Hendry and Wallace, 1993; Luminita and Irina, 2005). The degree of polymerization of inulin should be 30 or greater, inulin has a low solubility in cold water but it is readily soluble in warm water. In addition, inulin has extensive documented in the historical human use through the consumption of edible plants and fruits (Marchetti, 1993). Small fructans have a sweet taste, whereas larger fructans chains form emulsion with a fat-like texture possess a neutral taste. Inulin has recently received a great interest as it represents a relatively inexpensive and abundant substrate for the production of high fructose syrup, which has gained importance in the pharmaceutical industries e.g. fructose syrup has beneficial effects in diabetic patients, increases the iron absorption in children, has high sweetening capacity and so it can be used in the diet of obese persons (Roberfroid and Delzenne, 1998, Prabhjot *et al* ., 2003), stimulates calcium absorption in postmenopausal women (Heuvel *et al* ., 2000; Prabhjot *et al* , 2003), stimulates growth of Bifidobacteria in large and small intestine (Durieux *et al.,* 2001; Prabhjot *et al*, 2003), prevents colon cancer (Rowland *et al* ., 1998; Prabhjot *et al* , 2003) and also used as dietary fibers because of its fat like texture (Roberfroid and Delzenne, 1998; Prabhjot *et al* , 2003). Plant that contain high concentrations of inulin include, Elacampane (*Inula helenium*), Dandelion (*Taraxacum officilale*), Jerusalem artichokes (*Helianthus tuderosus*), chicory (*Cichorium intybus*),Onion (*Allium cepa)* Garlic (*Allium sativum*) Agave (*Agave species*) Wildyarm (*Dioscorea spp.*) Yacon (*Smallamthu sonchifolius*).

2.2 Microbial sources of Inulinase

Microbial inulinases (2,1-b-D fructan fructanohydrolase,EC 2.2.1.7) are usually inducible and exo-acting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) (Vandamme and Derycke, 1983; Prabhjot *et al* , 2003). Inulinase (β-2,1-D-fructan fructanohydrolase (EC 2.2. 1.7) targets the β-2,1 linkage of inulin and hydrolyzes it into fructose. Several research reports have documented the inulinase activity that used pure inulin as a carbon source; were *Fusarium oxysporum* (8 IU/L) (Kaur and Singh, 1990); *Panaeolus papillonaceous* (230 IU/L) (Mukherjee and Sengupta, 1987) ; *Aspergillus fumigatus* (400 IU/L) (Sharma *et al* ., 1998) and comparable to *Penicillium sp*. TN-85 (560 IU/L) (Nakamura *et al* ., 1997), *Candida pseudotropicalis* (2.8U/ml), *Candida kefyr* (14.6 U/ml), *K. marxianus* YS-1 (55.4U/ml), (18.7 U/ml) *C. pseudotropicalis*, (18.4 U/ml) *Kluyveromyces marxianus* var. *bulgaricus*, (14.3 U/ml) *K. fragilis, K. marxianus* var. *bulgaricus* (40.5U/ml).

Table: 2.1 Comparision of properties of fungal endo inulinases (Vandamme and Derycke, 1983; Prabhjot *et al* , 2003)

Fungal strain	M_r* (kDa)	pH optimum (stability)	Temperature optimum (stability)	K_m (mM)	Specific activity (U/mg) toward: inulin	sucrose	Inulin hydrolysis (%)	Hydrolysis products
Aspergillus niger strain 12	66	5.3 (4.0–7.5)	45°C (<40°C)	1.25	101	0.00	45	F_3, F_4, F_5
Aspergillus niger strain 817								
P-IA	70	5.3 (5.0–7.0)	50°C (<50°C)	0.48	352	0.00	53	F_3, F_4
P-IB	68	5.3 (3.5–9.0)	50–55°C (<50°C)	0.50	338	0.00	51	F_3, F_4
Aspergillus ficuum	64	4.8–5.2 (NA**)	NA** (<60°C)	8.1	348	0.00	NA**	F_3
Cryosporium pannorum	58	6.0–7.0 (4.5–8.5)	50°C (<45°C)	NA**	106	0.00	NA**	F_3, F_4, F_5
Penicillium purpurogenum	64	5.1 (5.0–7.5)	NA** (<55°C)	0.21	82.8	0.02	32	F_3, F_4, F_5
Penicillium sp. strain TN-88	68	5.2 (5.0–7.0)	50°C (<40°C)	0.20	105	0.00	70	F_3

*M_r, determined by SDS-PAGE. **NA, not available.

Table: 2.2 Comparision of properties of fungal exo inulinases (Vandamme and Derycke, 1983; Prabhjot *et al* , 2003)

Fungal strain	M_r (kDa)	pH optimum (stability)	Temperature optimum (stability)	K_m (mM)	Specific activity (U/mg) toward: inulin	sucrose	Activity toward levan	I/S
Aspergillus awamori	72	4.5 (3.0–5.0)	60°C (<45°C)	0.003	75	NA*	+	NA*
Aspergillus niger strain 12								
P-I	70	4.0 (4.0–7.0)	55°C (<50°C)	0.4	1.68	7.30	–	0.23
P-II	59	5.0 (4.0–7.0)	55°C (<50°C)	1.87	7.60	9.50	–	0.80
Aspergillus ficuum								
Exo I	76	4.7	60°C	15	NA*	NA*	NA*	0.16
Exo II	74	4.7	60°C	11	NA*	NA*	NA*	0.36
Crysosporium pannorum								
F2	84	5.0 (5.0–7.5)	55°C (<50°C)	NA*	41.5	62.3	–	0.67
F3	70	6.0 (5.0–8.5)	55°C (<45°C)	NA*	10.50	36.2	+	0.29
Penicillium sp. strain 1								
P-I	86	4.5 (4.0–6.0)	45°C (<40°C)	0.17	44.3	22.7	+	2.0
P-II	64	5.0 (4.0–6.0)	50°C (<40°C)	0.23	16.3	25.0	+	0.65
P-III	66	4.0 (4.0–6.0)	45°C (<40°C)	0.16	1.39	1.22	+	1.1
Penicillium trzebinskii	87	5.2 (4.0–9.0)	NA* (<50°C)	0.04	103	61.7	–	1.7
Penicillium sp. strain TN-88	81	4.0 (5.0–7.0)	55°C (<50°C)	0.09	743	93.8	–	7.9

*NA, not available.

2.3 Inulin/sucrose (I/S) ratio

The fast enzyme production for the new selected microorganism is suitable property in industrial processes and represents an advantage of this on the other microorganisms already proposed for such purpose. It is generally accepted that the ratio of the activity on inulin/sucrose (I/S ratio) characterizes the enzymes: for inulinase I/S ratio is higher than 10^{-2} while for invertase it is lower than 10^{-4}. A close relationship was observed between inulinase activity and I/S ratio in a time dependent manner. As compared with pure inulin, I/S ratio was significantly higher with garlic, which further confirmed the advantage of using garlic over inulin. The ratios in the range of 0.02–7.9 have been reported in literature (Moriyama *et al.*, 2002).

2.4 Substrates for inulinase production

Industrial application of inulinase, however, would only be feasible if the carbon sources were available in large quantities at competitive price. A reduction in the production cost can be achieved by the usage of inexpensive inulin-containing substrates such as chicory, dahlia, onion, garlic, banana, wheat, rye and barley, which are often abundant. Microbial inulinase having interesting alternative, since, these carbon sources are cheap, easily available, which are an attractive feedstock for large-scale fermentation, whereas pure inulin is only available in limited quantities and at very high cost. Among different substrates, maximum inulinase activity was observed with garlic (524 IU/L). However, the inulinase activities observed with other substrates, viz: rye (313 IU/L); barley (244 IU/L); banana (283 IU/L); wheat (320 IU/L); chicory (328 IU/L) and onion (300 IU/L) were comparable to the activity obtained by pure inulin (321 IU/L) (Arun *et al* , 2006).

2.5 Microorganisms producing inulinase

Inulinases are encountered in plants and many microorganisms. Inulinase producing yeasts, moulds and bacteria have been reported (Vandamme and Derycke, 1983; Prabhjot *et al* , 2003). Among fungi, some well-known sources of these enzymes include *Aspergillus sp (*Vandamme and derycke*, 1983), A.niger* (Nakamura *et al .*, 1994), *A .niveus* (Nandagopal *et al .*, 2006), *A.ficuum (*Ettalibi and Baratti*, 1987), A.awamori, Penicillium sp (* Nakamura *et al ., 1997), Penicillium purpurogenum* (Nandagopal *et al .*, 2006), *Penicillium sp strain 1, Penicillium strain TN 88, Penicillium trezbenszki, Fusarium oxysporum (*Kaur *et al ., 1992), Kluveromyces marxianus (*Ongen-Baysal and Sukan *1996), Candida kefr (*Negoro and Kito*, 1973), Candida pseudoropicalis* (Manzoni *et al.*, 1996), *Pichia pastoris, Pichia guilliermondii, Clostridium acetobutylicum ABKn8 (*Estathion *et al ., 1986), Pseudomonas Sp (*Kim *et al ., 1997), Staphylococcu sp RRL1 (* Selva kumar *et al ., 1999), Streptomyces rochei, Bacillus polymyxa Geobacillus stearothermophilus, Chryosporium pannorum, Cryptococcus aureus, Candida pseudotropicalis* var. *lactosa*, (Manzoni *et al.*, 1996), *Kluyveromyces cicerisporus* (Manzoni *et al*., 1996) and *Kluyveromyces fragilis* (Manzoni *et al.*, 1996). Among yeasts, the best-known producers are *Kluyveromyces marxianus*, *Candida kefyr, Debaryomyces cantarellii and Pichia polymorpha* (Sarote *et al.*,2007). These yeasts appear to produce only exo-inulinases whereas most inulin-hydrolysing molds produce both endo- and exo-inulinases (Guiraud and Galzy (1990); Barta (1993); Hensing *et al.*, (1993); Frank and Leenheer (2002); Sarote *et al.,* 2007. Exoinulinase producing microbes are *Streptomyces rochei, Geobacillus stearothermophilus.* The endo-inulinase producing micro organisms are *A.ficcum, P.purprogenum* and *Chryosporium pannorum.*

2.6 Characterisation studies for inulinase

Inulinase from different microbial cultivations have been characterized up to now mainly in terms of pH, temperature,substrate concentration.

2.6.1 Effect of pH on inulinase activity

The optimal pH is in agreement with the general range of many microbial sources reported so far: *Streptomyces* sp (pH 6.0) (Arun *et al* ., 2006), *Staphylococcus* sp.(pH6.5) (Selvakumar, Ashok pandey ,1999) *Kluyveromyces marxianus* (pH 6.0) (Selvakumar, Ashok pandey ,1999) *A. niger* (pH 4.4) (Derycke and Vandamme, 1984), *A. versicolor* (5.5) (Kochhar *et al.,* 1997) , *Penicillium janczewskii* (pH 4.8–5.0) (Pessoni, Figueiredo and Braga, 1999), *Pichia pastoris* (pH 4.5) (Linghua *et al* ., 2004) *C. Pannorum* (pH 5.6) (Prabhjeet Singh and Prabhjot Kaur, 2006).

2.6.2 Effect of Temperature on inulinase activity

Rhizopus sp. strain TN-96 (Kazuyoshi *et al.*, 2002) and *Acetobacter diazotropicus* SRT4 (5.5) (Tambara *et al.*, 1999). The optimal temperature is in agreement with the general range of many microbial sources reported so far: *Streptomyces* sp (60^0C) (Arun *et al* ., 2006), *F. oxysporum, P. janczewskii, P. aculeatum* and *P.digitatum* which have temperature optima of 35–45^0C, 55^0C,respectively (Kaur *et al* ., 1992; Pessoni *et al* ., 1999) *C. Pannorum* (50^0C) (Prabhjeet Singh and Prabhjot Kaur, 2006) *Pichia pastoris* (55^0C) (Linghua *et al* ., 2004*) Arthrobacter sp*. (50^0*C*) (Su-Il Kang *et al* ., 1998). Thermostable inulinase activity was identified in the extracellular extract of *Aspergillus fumigatus.* At its optimum temperature of 60 ^{0}C, the ammonium sulphate fraction retained approximately 70% of its maximum activity after 72 h incubation in the absence of inulin. The two isoforms of *A. fumigatus* inulinase were purified and their thermostability was studied. In the presence of inulin, isoform II was more thermostable when compared to other two fractions and retained approximately 54% activity after 3 hr at 60 ^{0}*C.(* Prabhjot *et al* ., 2006). Higher temperature optimum of inulinases is an extremely important factor for the application of these enzymes for commercial production of fructose or fructooligosaccharides from inulin, since high temperatures (60 °C or higher) ensure proper solubility of inulin and also prevent microbial contamination. (Vandamme and Derycke, 1983;Prabhjeet Singh and Prabhjot Kaur, *2006*). Higher temperature optimum of inulinases is an extremely important factor for the application of these enzymes for commercial production of fructose or fructooligosaccharides from inulin, since high temperatures (60 °C or higher) ensure proper solubility of inulin and also prevent microbial contamination. Higher thermostability of the industrially important enzymes also brings down the cost of production because lower amount of enzyme is required to produce the desired product. Inulinases from yeasts, fungi and bacteria have been studied but only a few of these enzymes have an optimum temperature of 60°C or higher, as required for industrial applications Between yeasts and *Aspergillus* spp., which are the most versatile sources of inulinases, the inulin hydrolytic activity from the latter is more thermostable. Among the different fungal strains reported, the exoinulinase isoform of *A. fumigates*,due to its higher thermostability, appears to be more suitable for commercial hydrolysis of inulin than inulinases from *A. niger* , *A. ficuum* and *Scytalidium acidophilum (*Prabhjeet Singh and Prabhjot Kaur Gill,2004). Between yeasts and *Aspergillus* spp., which are the most versatile sources of inulinases, the inulin hydrolytic activity from the latter is more thermostable. Although the inulinase from the thermophilic bacterium *T. Maritime* is the most thermostable (Liebl *et al* ., 1998; Prabhjeet Singh and Prabhjot Kaur, 2006), its low production may be a limiting factor for inulin hydrolysis at industrial level. Inulinases from *Aspergillus ficuum* were stable below 50 ^{0}C with optimum activity at 45 ^{0}C, and were stable at a pH range of 4–8 with an optimum pH at 4.5 for exoinulinase and at 5.0 for endoinulinase. (Han *et al* ,2009). Three different extracellular ß-fructofuranosidases (two inulinases and one invertase) obtained from *Penicillium janczewskii* were purified from fungal cultures grown on sucrose or inulin, through precipitation with ammonium sulfate, and anion-exchange, hydrophobic interaction and gel filtration chromatographies. (Marcia *et al* ., 2007) The endo-inulinase obtained from *Arthrobacter* sp. S37 hydrolyzed inulin mainly into inulo-triose (F3), inulo-tetraose (F4) and inulo-pentaose (F5) optimally at pH 7.5 and 50°C. (Su-II Kang *et al* ,1998).

2.6.3 Kinetic constants for the inulinase activity

Using inulin as the substrate, Km values for inulinase from *Aspergillus ficuum* JNSP5-06 were 42.1 mg/ml, 31.5 mg/ml, 25.3 mg/ml, 14.8 mg/ml, and 25.6 mg/ml, respectively. (Han *et al* ,2009).The values of km for the two inulinases from *Penicillium janczewskii* were 8.11 10–4 and 2.62 10–3 M, being lower for inulin (Marcia *et al* ., 2007).The purified inulinase obtained from *Streptomyces* sp had a lower Km (1.63 mM) and higher Vmax (450 mM) for inulin. (Arun and Prabhjot ,2007) For the extracellular inulinase fron *Rhizopus* sp. the k_m value was found to be 9.0 mM for inulin. (Kazuyoshi *et al* ,2002).

2.7 Optimisation of inulin extraction from coffee spent through L_{16} Orthogonal design of expriments

2.7.1 Microwave assisted extraction

Microwave energy has tremendous heating potential, use of microwave extraction technology have recently appeared in analytical laboratories (Zuloaga *et al*., 1999). The microwave heating leads to the expansion and rupture of cell walls and is followed by the liberation of chemicals into the solvent (Spar Eskilsson and Bjorklund *et al*., 2000). Plant particle size and size distribution usually have a significant influence on the efficiency of MAE. The particle sizes of the extracted materials are usually in the range of 100 mm–2 mm (Spar Eskilsson and Bjorklund *et al*., 2000). The extracting selectivity and the ability of the solvent to interact with microwaves can be modulated by using mixtures of solvents (Brachet *et al*., 2002). One of the most commonly used mixtures is hexane-acetone (Spar Eskilsson and Bjorklund *et al*., 2000). A small amount of water (e.g. 10%) can also be incorporated in non-polar solvents such as hexane, xylene, or toluene to improve the heating rate (Spar Eskilsson and Bjorklund *et al*., 2000).Williams, Raghavan, Orsat, and Dai (2004) found MAE was efficient in recovering approxi-mately 95% of the total capsaicinoid fraction from capsicum fruit in 15 min compared with 2 h for the reflux and 24 h for the shaken flask methods.Solvents such as ethanol, methanol and water are sufficiently polar to be heated by microwave energy (Brachet *et al*., 2002).

Potential applications of microwave-assisted extraction

MAE can extract nutraceutical products from plant sources in a faster manner than conventional solid–liquid extractions. MAE of the puerarin from the herb Radix puerariae could be completed within 1 min (Guo *et al*., 2001). MAE (80% methanol) could dramatically reduce the extraction time of ginseng saponins from 12 h using conventional extraction methods to a few seconds (Kwon, 2003). It took only 30 s to extract cocaine from leaves with the assistance of microwave energy quantitatively similar to those obtained by conventional solid–liquid extraction for several hours (Brachet *et al*., 2002). Microwave-assisted extraction can also reduce solvent consumption. Focused MAE was applied to the extraction of withanolides from air-dried leaves of Iochroma gesner-ioides (Kaufmann *et al*., 2001). The main advantages of MAE over Soxhlet extraction are associated with the drastic reduction in organic solvent consumption (5 vs 100 ml) and extraction time (40s vs 6 h). It was also found that the presence of water in the solvent of methanol had a beneficial effect and allowed faster extractions than with organic solvent alone.

2.7.2 Ultrasonication assisted extraction

Ultrasound assisted technology using high intensity ultrasound waves has caught the attention of food scientist due to its promising effects on biological systems which can be taken as an advantage for product modification and process improvement. High intensity ultrasound waves produce intense pressure, shear and temperature gradients within the material leading to a physical disruption of its structure (McClements, 1995). Both biological cell walls and cell membranes are disrupted due to cavitation (implosion of gas bubbles within the material) caused by considerable changes in temperature and pressure during a very short period of time (Knorr, 2004). Additionally, cavitation causes particle size reduction which increases the surface area in contact between the solid and the liquid phase. Therefore,ultrasound induces a better penetration of solvent into cellular materials which improves mass transport rates within the tissue and facilitates the transfer of components from the cell into the solvent leadind to the enchancement of the extraction operation (Wang and Weller *et al*., 2006). The higher temperature attainedby the cell.

Potential applications of sonication-assisted extraction

Ultrasound-assisted extraction has been used to extract nutraceuticals from plants such as essential oils and lipids (Chemat *et al*., 2004; Li *et al.*, 2004; Luque-Garcia and Luque de Castro, 2004; Sharma and Gupta *et al*.,2004), dietary supplements (Hui, Etsuzo and Masao, 1994; Wu *et al*., 2001). An overview of the uses of ultrasound in food technology was prepared by Mason *et al*., (1996). Ultrasound can increase extraction yield. Sharma and Gupta (2004) found that ultrasonication was a critical pretreatment to obtain high yields of oils from almond, apricot and rice bran. The yield of oil extracted from soybeans also increased significantly using ultrasound (Li *et al.*, 2004). For ultrasound-assisted extraction of saponin from ginseng, the observed total yield and saponin yield increased by 15 and 30%, respectively (Hui *et al*., 1994). Ceng. (2010) was also applied to ultra-high purity fructose syrup with different parameters to get the influence law of ultrasound on high inulinase production by *Aspergillus niger* and the yield of inulin extracted by ultrasound reached 94.23% at an optimum conditions.

2.8 Applications

2.8.1 High fructose syrup

Industries uses a large amount of natural polysaccharides and in the last few decades attention has been directed toward producing these polysaccharides using microbial fermentation. Fructose is the sweetest of all naturally occurring carbohydrates and is often produced by enzymatic process from starch. Conversion of starch to fructose involves the use of three different enzymes and the maximum yield is 45% (Pandey *et al.,* 1999). A simple and high productivity method to obtain high fructose syrup is the enzymatic hydrolysis of inulin, a single step process that utilized in the production of chocolates, ice cream and sauces (Chi *et al.,* 2011). It was found that the major IOS obtained after inulin hydrolysis with endo-inulinases have a degree of polymerization of 3 and 4. Inulooligosaccharides can act as prebiotics and their positive effect on human health has been widely acknowledged (Rocha *et al.,* 2006, Chi *et al.,* 2009).

2.8.2 Bioethanol production

Biochemical and thermo-chemical conversion technologies can convert biomass into carbon containing biofuels such as biodiesel and other liquids. The primary feedstock for ethanol production worldwide remains sugar or starch from agricultural crops, and its primary use is as a blend with gasoline (at 5-90% blend). Nowadays, studies concerning bioethanol production from various unconventional feedstock, such are lignocelluloses materials or kitchen refuse, are increasing. Alcohol production from inulin rich feedstock has been studied since the end of 19th century. Although more widely recognized now, the dramatic environmental, economic, strategic and infrastructure advantages offered by the production of ethanol were not appreciated in the past. Inulin rich raw materials gained researchers attention for bioethanol production. The microbial exo-inulinases can remove the terminal fructose residues from the non-reducing end of inulin molecule, producing fructose and glucose, which can easily be fermented to ethanol by *Saccharomyces* spp. yeast strains (Chi *et al.,* 2011). Some yeast strains can perform simultaneous hydrolysis and fermentation of the inulin: *Kluyveromyces marxianus* and some *Saccharomyces* spp. yeasts can produce both active inulinase and ethanol (Chi *et al.,* 2011, Rosa *et al.,* 1986, Kim *et al.,* 1998, Lim *et al.,* 2011).

2.8.3 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 (Fleck, 1999).

2.8.4 Other applications of inulinases

Inulinases have also found their application for inulin substrates hydrolysis for single-cell oil and single-cell protein production (Chi *et al.,* 2011). The marine yeast *Cryptococcus aureus* can be used for single cell protein production by cultivation on inulin hydrolysates from Jerusalem artichoke tubers. The same applications are important to produce citric acid, 2,3 butanediol, lactic acid and sugar alcohols, like mannitol (Chi *et al.,* 2011; Saha, 2006; Liu *et al.,* 2010).

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals

Pure inulin was obtained from Sri Ayyappa and Scientific Instrumentation, Sivakasi. All other reagents and chemicals used were of analytical grade.

3.2 Isolation of Microorganisms

The coffee soil samples were collected from Non such estate,Coonoor and Yercaud. According to Warcup (1950), the procedure was followed. The rhizosphere soil samples (1g) were suspended in 100ml of sterilized distilled water (1:100 dilution) and subsequently 1ml of this suspension was added into 9ml of sterilized distilled water. Petridishes containing potato dextrose medium plus streptomycin were inoculated with 1ml of 1:100 diluted soil suspensions. The plates were kept at room temperature (approx 28^0C) and colonies were accompanied upto 72h. Fragments of the individual colonies were transferred separately to the same medium containing 10 mg of streptomycin and the growth was accompanied for 72 h. The strains were maintained at 4^0C.

3.2.1 Screening Procedure

3.2.1.1 Primary screening

All fungal colonies were point inoculated on agar plates. The plates were incubated at 30°C for 5 days. Colonies that displayed rapid growth, i.e. attained large colony diameter per unit growth time, were selected for further experiments and transferred to fresh plates**.**

3.2.1.2 Secondary screening

Selected fungi and other isolates were inoculated into separate 250-ml shake flasks that
contained 50 ml of liquid medium.Flasks were incubated at 30°C, 200 rpm, for 5 days.
Samples were withdrawn periodically and analysed for activity of inulinases.

3.2.2 Staining of screened cultures by lactophenol cotton blue

The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which kill any live organisms; lactic acid which preserves fungal structures, and cotton blue stains the chitin in the fungal cell walls.

Materials

Sterile Petri dish, glass rod, Microscope slides and coverslips (Sterile), Potato dextrose agar plate with mixed culture of fungi, Lactophenol cotton blue stain, Glass capillary tube, Scalpel, Inoculating needle, Sterile distilled water, 70% ethanol, Forceps.

Procedure

A sheet of sterile filter paper was kept in a Petri dish with a pair of forceps above which a sterilised U-shaped rod was placed. About 4 ml of sterile distilled water was poured on the filter paper to completely moisten it. A sterile slide was placed on the U-shaped rod. Using a sterilised scalpel, a 5 mm square block of medium was cut from PDA agar plate. The block was aseptically transferred to the centre of the slide and the four sides of the agar square were inoculated with spores or mycelial fragments of the fungus to be examined. A sterile cover glass was placed aseptically on the upper surface of the agar cube. The Petri dish was covered and incubated at room temperature for 48 hours after which the slide was examined under low power.

3.3 Sample extraction

To know the amount of inulin present in the substrate, it was extracted as follows. 5g of coffee spent wash was taken and was extracted in 50ml of 80% of absolute alcohol for 12 hours to remove free sugars. From the dried sample, 500mg was taken in a 100ml conical flask. It was made upto 20ml and heated in a water bath at 90ºC for 10min.The extract was collected and made upto 70ml with dis.H_2O. The flask was replaced for another 30min with occasional shaking to dissolve the fructosan and then it was removed and cooled at room temperature. The extract was filtered and made upto 100ml in a standard flask. Inulin content in the extract was estimated following the procedure for fructose estimation.The amount of inulin was expressed in terms of fructose concentration. (Sadasivam and Manickam,1991).

3.4 Quantification of inulin

Standard fructose solution was prepared. 0.2, 0.4, 0.6, 0.8, 1.0ml of working standard was pipette out and the volume was made up to 2ml.0.4, 0.8 ml of extracts were taken and the volume was made up to 2ml. To that 1ml of resorcinol reagent was added followed by 7ml of dilute HCl. All tubes were heated in a water bath at 80 ^{0}C for exactly 10min.It was removed and cooled by immersing in tap water for exactly 5min. The orange colour product was formed was recorded spectrophotometrically at 520nm within 30min. The amount of inulin is expressed in terms of fructose concentration. (Ashwell, 1957).

3.5 Screening for enzyme extract for inulinase

Quantitative screening was done.The substrate was added to the medium (Potato Dextrose Broth).Erlenmeyer flasks (250 ml) containing 100 ml of the medium were inoculated from the 7 days old stock culture grown PDA and incubated at 37°C for 24 .hr on a rotary shaker at 200 rpm.The culture broth was centrifuged at 8000 rpm for 20 min at 4°C and stored at 20°C till further analysis. The enzyme activity was checked till 14^{th} day.

3.6 Enzyme extraction

The strain showed higher activity was cultivated in submerged culture at 28^0C on rotary shaker (150 rpm), in Erlenmayer flasks containing potato dextrose salts. After incubation, the mycelium was separated by filtration and the filtrate was stored at 4 ºC till further analysis.

3.7 Assay for inulinase and invertase

Inulinase activity on inulin (I) is commonly compared with the invertase activity on sucrose (S) displayed by the same enzyme preparation and the I/S ratio is used to characterize inulinases (Ohta *et al*., 2002).

3.7.1 Inulinase assay

Inulinase assay was carried out as follows; 2 ml of 0.2 % inulin (pH 3.5) and 0.050 ml enzyme were added and incubated at 37ºC for 20 min. (Modified method of Marcin and Jan, 2004). The same mixture, with inactivated crude enzyme extract was used as the control. The amount of the reducing sugars in the reaction mixture was assayed using the DNS method.
The reaction mixture was assayed for reducing sugars as fructose by the DNS method (Miller, 1959) by reading the absorbance at 510 nm. The orange red colour was formed and the calibration curve was drawn with fructose (20 – 100 mg). One unit of inulinase activity (U) was defined as the amount of enzyme, which forms 1 µmol fructose per min under special conditions.(Dilipkumar *et al*., 2011).

Determination of enzyme activity

The enzyme activity can be determined as

Enzyme Activity$=\frac{(OD(Sample)-OD(Control))\ x\ Reactionvolume\ \ x\ 2\ x\ Dilution\ factor}{Sample\ volume\ x\ mM\ extinction\ coefficient\ x\ Time\ of\ incubation}$

U/ml

3.7.2 Invertase assay

Crude enzyme extract (0.5 ml) was diluted to the required level with 0.2 M sodium acetate buffer at pH 3.5. Diluted extract (0.5 ml) was mixed with a 2 ml solution of 0.5% w/v sucrose in the mentioned buffer. The mixture was incubated and analysed for reducing sugars, as described earlier for the inulinase assay. One unit of invertase activity was defined as that liberating 1 µmol of sucrose per min under assay conditions.The amount of reducing sugars was estimated by comparison with a calibration curves made with fructose for inulinase and equimolar solution of glucose and fructose for invertase (Saber *et al*., 2009).

3.8 Protein quantification

Protein concentration was measured by the method using Bovine serum albumin (BSA) as standard. Protein assay mixture consisted of 0.05ml of ample and standard of 0.05mg of BSA prepared were taken as working standards of different volumes, 0.15 ml of distilled water and 0.1 ml of 1N Folin`s reagent was added. Then the contents were incubated at room temperature for 30 minutes. A reaction mixture containing distilled water was used as blank. Blue colour developed in the standard and tests were measured at 660nm. (Lowry *et al*., 1951)

3.9 Characterization of crude inulinase enzyme

3.9.1 Effect of time on enzyme activity

Inulinase activity was determined upto 14 days with the interval of 24h. After finding the optimum day, the activity was checked to find the optimum hour.

3.9.2 Effect of pH on enzyme activity and its stability

The effect of pH on Inulinase activity was determined in the pH range of 3-10.5, using 100 mM citrate(pH 3-4) sodium acetate (pH 3.5-5.5) and sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.5-9.0) Glycine-NaOH(9.5-10.5) buffers.The enzyme activity was measured at 37 ^{o}C for 20 min.

3.9.3Effect of temperature on enzyme activity and its stability

The effect of temperature on enzyme activity was measured at pH 3.5 in 100 mM sodium acetate buffer, over a temperature range of 35-80^{o}C. After incubation at appropriate temperature for 20 min, the reaction was stopped and reducing sugars were estimated by DNS method. Thermostability was determined by measuring the enzyme activity at different intervals at optimum pH and temperature (Bharathi *et al*., 2011).

3.9.4 Determination of kinetic parameters

The k_m value and maximum reaction velocity (V_{max}) for inulin was determined by the method of Lineweaver-Burk plot.

3.9.5 Effect of metal ions and reagents in enzyme activity

The effect of different metal ions and reagents in inulinase activity was examined by incubating it with enzyme extract in 100mM sodium acetate buffer (pH3.5) at 30^{0}C for 1hr. The residual activity was determined under assay conditions (Arun *et al*. 2007).

3.10 Determination of hydrolysis products

In order to analyse the products of inulin hydrolysis, the crude enzyme was incubated at 60^{0}C for 1h with 0.2% inulin in 0.1M sodium acetate buffer pH 3.5. The hydrolysis products were analysed by thin layer chromatography (TLC) (Arun *et al*., 2007).

3.10.1Thin layer chromatography

The hydrolysis products and also the presence of sugars in agave were analysed by thin layer chromatography (TLC).Thin layer chromatography was used for qualitative analysis of the reaction products. Pre-coated TLC plates (Merck, Germany) spotted with samples, were developed 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^{0}C after spraying with orcinol sulphuric acid reagent prepared by adding 0.2% (w/v) orcinol prepared in ethanol–water–sulphuric acid (40:50:10) . The colour developed after heating for 15 min at 120^{0} C.(Arun *et al.,* 2007).

3.11 Optimization of inulin extraction from coffee spent (pretreatment) by L_{16} orthogonal design of experiments

3.11.1 Microwave Assisted Extraction

Extraction of inulin from pretreated coffee spent was obtained by L_{16} $(4)^3$ orthogonal designs i.e., four levels and three different variables. Variables including the microwave power,solid:liquid ratio and irradiation time was tuned to deliver desired product attributes and optimize process economics. Power varies from 180 to 720 Watts. Extraction time varies from 10 to 40 seconds and the solid:liquid ratio variying fom 1:25 to 1:100 g:ml (Khizar Hayat *et al.,* 2009).

Table 3.1 Orthogonal design parameters in microwave assisted extraction

LEVELS	POWER (A) (watts)	SOLID:LIQUID RATIO (B) (g:ml)	TIME (C) (secs)
1	180	1:25	10
2	360	1:50	20
3	540	1:75	30
4	720	1:100	40

3.11.2 Ultrasonication Assited Extraction

Extraction of inulin from pretreated coffee spent was obtained by L_{16} $(4)^3$ orthogonal designs i.e., four levels and three different variables (Solvent percentage, Extraction time,Solid Liquid ratio). Solvent percentage varied from 65 to 95. The solvent used for the extraction purpose was ethanol. Extraction time varied from 3 to 12 min and the solid liquid ratio varied from 1:25 to 1:100 (Lan Zhang *et al*., 2009).

Table 3.2 Orthogonal design parameters in Ultrasonication assisted extraction

LEVELS	CONCENTRATION OF ETHANOL (A) (%)	SOLID:LIQUID RATIO (B) (g:ml)	TIME (C) (mins)
1	65	1:25	3
2	75	1:50	6
3	85	1:75	9
4	95	1:100	12

3.12 Optimisation of inulinase production

3.12.1 Plackett Burman design

Randomized block design was utilised for the optimisation of 7 variables (coffee spent, Agitation rate, Orange peel, NH_4Cl , KCl, MgSO4, pH) to maximise the inulinase production by *Pencillium purpurogenum.* The software Minitab16 (trial version) and Design expert 8 (trial version) was used for this purpose. Total runs is 8. Each of the independent variables is tested at two levels, a high level (H) and a low level (L), as shown in the table 3.3.

Table: 3.3 Level of variables for Plackett Burman design

Variables	Low level (-)	High level (+)
Coffee spent	0.5 g/ml	1 g/ml
NH4Cl	0.01 g/l	0.03 g/l
MgSO4	0.002 g/ml	0.012 g/ml
pH	3.5	5.5
Agitation rate	100 rpm	200 rpm
KCl	0.5 g/ml	1.5 g/ml
Orange peel	0.5 g/ml	1 g/ml

Table 3.4 Randomized block design

Run	Blk	Coffee spent	NH4Cl	MgSO4	pH	Agitation rate	KCl	Orange peel
1	1	-	+	+	-	-	+	-
2	1	+	+	-	+	-	-	-
3	1	-	-	-	+	+	+	-
4	1	+	-	-	-	-	+	+
5	1	+	+	+	+	+	+	+
6	1	-	+	-	-	+	-	+
7	1	+	-	+	-	+	-	-
8	1	-	-	+	+	-	-	+
13	13	-1	1	0.075	0.075	65.91036	85.14	
12	14	-1	1	0.075	0.117045	150	91.98	
11	15	-1	1	0.075	0.032955	150	80.5	
19	16	0	1	0.075	0.075	150	96.07	
14	17	-1	1	0.075	0.075	233.0896	93.41	
15	18	0	1	0.075	0.075	150	92.52	
8	**19**	**1**	**1**	**0.1**	**0.1**	**200**	**106.29**	
17	20	0	1	0.075	0.075	150	91.57	

3.13 Partial isolation of enzyme

Under aseptic conditions, *Pencillium purpurogenum* was cultivated in submerged culture at 28^{0}C on rotary shaker (150 rpm), in Erlenmeyer flasks containing optimal media found from RSM studies. After incubation, the mycelium was separated by filtration and the filtrate was stored at 4 ^{0}C and was used for partial isolation studies. Enzymes are unstable molecules with a definite physico chemical organization. Even a slight change in this organization reduces the activity of enzyme and sometimes the enzyme is totally inactivated. Therefore, the enzymes have to be isolated under controlled conditions of pH, ionic strength and temperature.

3.13.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as out. Ammonium is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed.

Procedure:

Crude enzyme solution was precipitated at an Ammonium sulphate concentration of 20-80%.Ammonium sulphate was added slowly to the 5ml of enzyme extract with stirring and the mixture was kept aside at 4^{0}C for 2h. At the end of 2 h, the mixture was centrifuged at 10000 rpm for 10min and the supernatant was collected. The pellet was dissolved in a minimum amount of sodium acetate buffer (0.1M, pH 3.5). The enzyme activity and the protein content were determined. The fraction showing higher specific activity was taken to the next step of purification **(**Carlier *et al*., 1980).

3.12.2 Response surface designs

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analysing the significance or the influence where the independent variables have on the dependent variable or response. There are several choices for second-order designs in the RSM. One of the most popular methods is the central composite design (CCD), which finds the optimal levels of the design variables by adding a few more experiments to a full factorial design. All the design variables must vary continuously.

Table: 3.5 RSM design table

The software Minitab (trial version) and Design expert 8 was used for this purpose. Three factors (Coffee spent, NH_4Cl, Agitation rate) were taken. Total runs is 20.

StdOrder	RunOrder	PtType	Blocks	Coffee spent	NH_4Cl	Agitation rate	Output (U/ml)
3	1	1	1	0.05	0.1	100	91.84
20	2	0	1	0.075	0.075	150	91.71
4	3	1	1	0.1	0.1	100	85.23
7	4	1	1	0.05	0.1	200	88.56
16	5	0	1	0.075	0.075	150	90.67
10	6	-1	1	0.117045	0.075	150	92.38
9	7	-1	1	0.032955	0.075	150	80.64
2	8	1	1	0.1	0.05	100	95.58
18	9	0	1	0.075	0.075	150	90.54
5	10	1	1	0.05	0.05	200	70.42
6	11	1	1	0.1	0.05	200	100.44
1	12	1	1	0.05	0.05	100	92.11

3.13.2 Ultrafiltration

Ultrafiltration is a technique for separating dissolved molecules in solution on the basis of size which means that molecules larger than the membrane pore size rating will be retained at the surface of the membrane.

Procedure:

The fermented broth wascentrifuged at 10000 rpm for 15 minutes. The purified pellet was subjected to ultra-filtration(membrane cut-off is 50kDa, PALL Life science, India). The permeate and retendate was obtained by ultrafiltration. The inulinase activity was performed with inulin as substrate. The absorbance were measured spectrophotometrically using ELICO Spectrophotometer.

3.14 Characterization of inulinase by partial isolation

3.13.1 Effect of pH on partially isolated enzyme

The effect of pH on Inulinase activity was determined in the pH range of 3.5-5.5 using 100 mM sodium acetate buffer. The enzyme activity was measured at 37^{o}C for 20 min.

3.13.2 Effect of temperature on partially isolated enzyme

The effect of temperature on enzyme activity was measured at pH 3.5 in 100 mM sodium acetate buffer, over a temperature range of 45-65^{o}C. After incubation at appropriate temperature for 20 min, the reaction was stopped and reducing sugars were estimated by DNS method.

3.13.3 Determination of kinetic parameters on partially isolated enzyme

The k_m value and maximum reaction velocity (V_{max}) for inulin was determined by the method of Lineweaver-Burk plot.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 ISOLATION OF MICROORGANISMS

Coffee soil samples are collected from Non such estate,Coonoor and Yercaud and the soil samples was serially diluted in the petri dishes containing the PDA agar. Many different wildtype species were obtained. From different colonies (Coonoor soil sample), isolate 1 has proved to possess maximum inulinase activity (0.732 U/ml) than isolate 2 (0.432U/ml).Similarly, colonies screened in Yercaud samples, isolate 1 possess maximum inulinase activity (0.953 U/ml) than isolate 2 (0.238 U/ml).

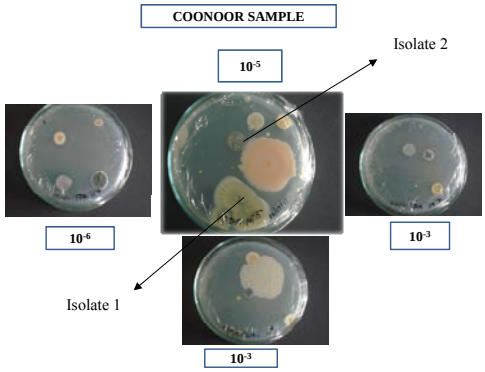


Fig 4.1A ISOLATION OF FUNGAL MICROORGANISMS FROM SOIL SAMPLE COLLECTED FROM NON SUCH ESTATE,COONOOR

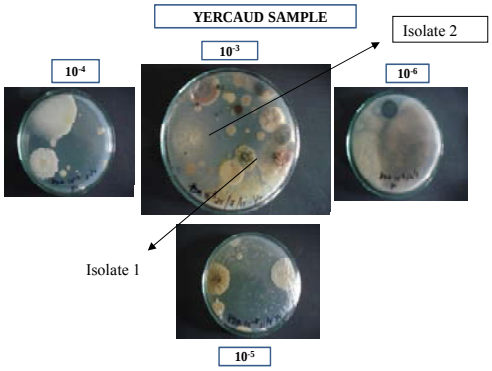


Fig 4.1 B ISOLATION OF FUNGAL MICROORGANISMS FROM SOIL SAMPLE COLLECTED FROM YERCAUD

4.2 IDENTIFICATION OF FUNGAL ISOLATES

The mold isolate 1 and isolate 2 were identified as *Pencillium purpurogenum* and *Aspergillus tamarii*, based on the macroscopic (colour, texture, appearance) and microscopic characteristics (microstructures) according to the standard protocols, Udayaprakash 2004.

Pencillium purpurogenum

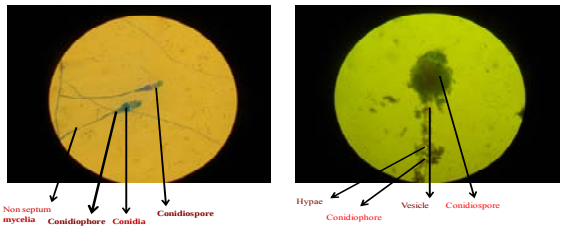
Colour: Blue or green mold

Texture: Conidio-phores that branch out into racemules at the apex, where chains of colored, unicellular spores, or conidia, were observed.

Aspergillus tamarii

Colour: Black mold

Texture: Black mold showing the hypae structure with conidiospores were observed.



Pencillium purpurogenum *Aspergillus tamarii*

Fig : 4.2 MICROSCOPIC EXAMINATION OF FUNGAL ISOLATES

4.3 I/S ratio

Inulinase activity on inulin (I) is commonly compared with the invertase activity on sucrose (S) displayed by the same enzyme preparation and I/S ratio was used for characterisation studies (Ohta *et al*.,2002).

Table: 4.1 I/S ratio

Fungal isolate	Inulinase activity (U/ml)	Invertase activity (U/ml)	I/S ratio
Pencillium purpurogenum	23.54	14.38	1.51

In this present study, the I/S ratio was found to be 1.51. Ertan *et al*., 2003 have reported that the maximum I/S ratios for *A.parasiticus,P.spinulosum* were 1.61,1.79 under optimal conditions respectively.

4.4 CHARACTERIZATION OF CRUDE INULINASE FROM *Pencillium purpurogenum*

4.4.1 Effect of time on enzyme activity

The maximum inulinase activity (2.882 U/mL) was observed at the 7th day old inoculum. Previous reports were available for the growth time for obtaining the maximum inulinase activity from different microorganisms and were well agreed with the present results. *Fusarium oxysporum* (8 IU/L after 9 days) (Gupta *et al*., 1990); *Panaeolus papillonaceous* (6 days) (Mukherjee and Sengupta, 1987); *Aspergillus fumigatus* (5 days) (Sharma *et al* ., 1998); *Penicillium sp*. TN-85 (5 days) (Nakamura *et al* ., 1997); *Streptomuces sp*. (2 days) (Arun *et al* .,2006); *Penicillium purpurogenum* (5 days) and *Aspergillus niveus* (5 days) (Nandagopal and Ranjitha Kumari,2006); *Aspergillus ficuum* (5 days) (Han *et al* , 2009); *Penicillium Janczewskii* (8 days) (Pessoni,2007); *Rhizopus* sp strain TN -96 (5 days) (Kazuyoshi *et al* .,2002).

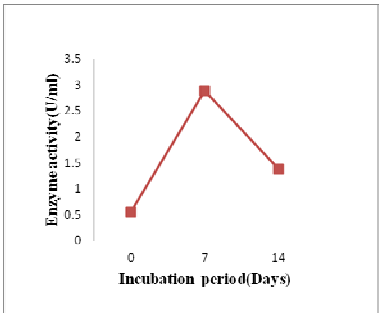


Fig: 4.3. Effect of growth time on inulinase activity from *Pencillium purpurogenum*

4.4.2 Effect of pH and temperature on enzyme activity

Inulinase from *Pencillium purpurogenum* was found to be active in a pH range between 3.5 to 9.0 , the optimum being **pH 5** with inulin as substrate in acetate buffer(Fig 4.3). The optimal pH of the purified inulinases from fungi and yeasts are in the range of 4.5–6.0 (Sheng *et al* . 2008; Singh *et al.,* 2007).Optimum pH for the production of Inulinase from *Kluyveromyces has* been reported between 6.0 and 7.0. For *Streptomyces* sp. it was reported as 6.0 (Arun *et al.,* 2006). *Pichia guilliermondii* have optimum pH in the range of 4.0 to 6.0. (Fang Gong *et al* . 2007).

Inulinase from *Pencillium purpurogenum* was found to be active in a temperature at 30 to 80°C , the optimum temperature was found to be **60 °C** (Fig: 4.4 **).** Simillar observation was obtained from *K. fragilis* and *K. Marxianus* were 55 °C (Kushi *et al* . 2000). The maximum inulinase activities for *K. marxianus* var. *Bulgaricus* were observed at 50 °C and 60 °C (Cazetta *et al* .., 2005). *P. janczewskii, P. aculeatum and P. digitatum*, which have temperature optimal of 35 °C, 45 °C, 55 °C respectively (Kaur *et al* . 1992; Pessoni *et al* . 1999).Nandhagopal *et al*., 2006 reported the optimum temperature of about 60°C for *pencillium purpurogenum*.

In *pencillium purpurogenum* at 60 °C, inulinase was stable over 4hrs at optimum pH range (5) (Fig: 4.6**).** In *Aspergillus ficuum*, inulinase showed stability over wide pH range (4.0-8.0) at 60 °C and there was decrease in activity beyond that range (Chen *et al* ., 2009). Nandhagopal *et al*., 2006 have reported the higher thermostability for *pencillium purpurogenum* by maintaining the residual activity of 95% after 1hr incubation at optimal conditions.

The affinity of the inulinase for inulin and sucrose were determined at 60°C and pH 5 by a Line weaver Burk plot. The k_m and V_{max} values for inulin were 0.25 mg/ml 40 U/ml respectively. Marcia *et al* ., 2007 have reported the values of km for the two inulinases from *Penicillium janczewskii* were 8.11 10–4 and 2.62 10–3 M.

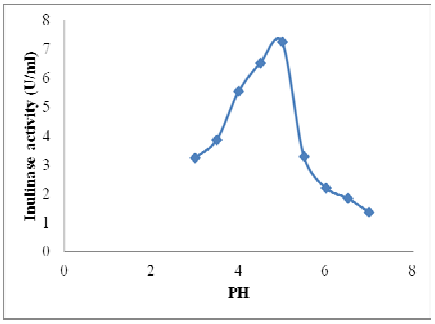


Fig 4.4 Effect of pH on the activity of inulinase

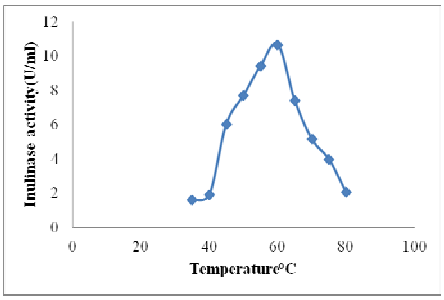


Fig: 4.5 Effect of temperature on the activity of inulinase

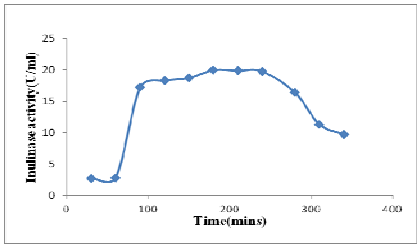


Fig 4.6 Effect of thermostability on the activity of inulinase

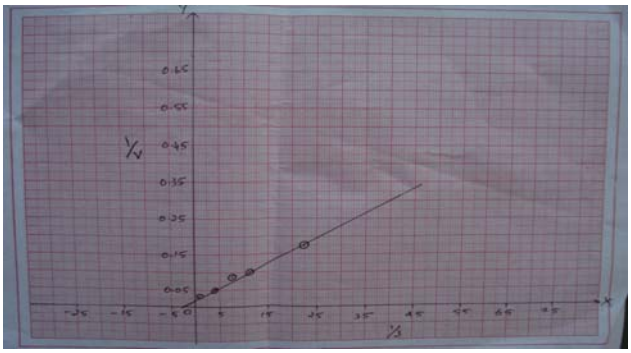


Fig 4.7 Kinetic Characterisation on Inulinase Activity –Lineweaver Burk Plot

4.4.3 Effect of metal ions and on enzyme activity

The effect of metal ions on inulinase was shown in Fig 4.8. Among the ions tested, $HgCl_2$ has highly inulinase activity. Hg^{2+} to about 88% indicating that trytophan residue appears to play in the catalytic processes of the enzyme and some –SH in protein might be responsible for activity.. The effect of various ions and agents on Inulinase activity from *Streptomyces* sp.was investigated at a concentration of 1 mM. Only CoCl2 positively modulated Inulinase activity, whereas the inhibitory effect of $AgNO_3$ and $NaHCO_3$ was marginal. The Hg2+, which is known to affect thiol groups, and $Fe2^+$ completely abolished the inulinase activity. (Arun *et al.*, 2006) The strong inhibitory effect observed with $Hg2^+$. This has been earlier observed for other microbial inulinases also (Ettalibi & Baratti, 1987; Kochhar *et al* ., 1997;Arun *et al* ., 2006). The purified inulinases produced by the marine yeast *C.aureus* G7a and *P. guilliermondii* strain 1 are activated by Ca^{2+}, K^+, Na^+, $Fe^{2+,}$ and Cu^{2+} while Mg^{2+}, Hg^{2+}, and Ag^+(at concentrations of 1.0 mM) act as inhibitors in Gong *et al* . (2008). This means that the purified inulinases are metalloenzyme and Ser and Cys residues of the enzymes are essential for the enzymes' active sites.

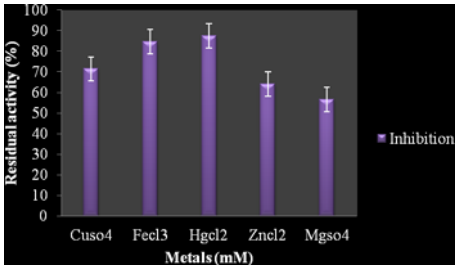


Figure 4.8 Effect of metal ions on inulinase activity

4.5 Optimization of inulin extraction from coffee spent (pretreatment) using microwave and assisted L_{16} orthogonal design of experiments

The effect of microwave energy is strongly dependent on the dielectric susceptibility of both solvent and solid plant matrix. Most of the time,the sample is immersed in a single solvent or mixture of solvents that absorb microwave energy strongly. Temperature increases

concentration of solvent into the matrix and constituents are released into the surrounding hot solvent. However in some cases only selective heating of sample matrix is brought about by immersing the sample in a microwave transparent solvent (hexane,chloroform). This approach is particularly useful for thermoliable components to prevent their degradation. Although microwave energy has tremendous heating potential, use of microwave extraction technology have only recently appeared in analytical laboratories (Zuloaga *et al.*,1999).

Even though dried plant material is used for extraction in most cases, but stills plant cells contain minute microscopic traces of moisture that serves as the target for microwave heating. The moisture when heated up inside the plant cell due to microwave effect, evaporates and generates tremendous pressure on the cell wall due to swelling of the plant cell (Wang and Weller 2006). The pressure pushes the cell wall from inside,stretching and ultimately rupturing it, which facilitates leaching out of the active constituents from the ruptures cells to the surrounding solvent thus improving the yield of constituents. This phenomenon can even be more intensified if the plant matrix is impregnated with sovents with higher heating efficiency under microwave. Higher temperature attained by microwave radiation can hydrolyze ether linkages of cellulose, which is the main constituent of plant cell wall, and can convert into soluble fractions within 1 to 2 min. The higher temperature attained by the cell wall, during extraction, enchances the dehydration of cellulose and redices its mechanical strength and this in turn helps solvent to access easily to compounds inside the cell (Latha 2006).

However, the surface of the sample was found greatly destroyed after extraction. This observation suggests that microwave treatment affects the structure of the cell due to sudden temperature rise and internal pressure increase in the yield of extractable pectin. Furthermore, the migration of dissolved ions increases solvent penetration into the matrix and thus facilitates the release of chemicals.

The optimum extraction conditions for inulin extraction was found to be 540 W, 1:25 material ratio and 30 seconds with the maximum activity of 42.57 U/ml . This is about 20 times fold increase in inulinase activity conferred with inulinase activity. A significant difference at 5% level (P≤ 0.05) was observed for solid:liquid ratio proving its privotal role .

Table 4.2 One way ANOVA of microwave assisted extraction

Levels	Sum of squares	Degrees of freedom	Mean square	F-value
A	100.19	3	33.39	0.20
B	598.95	3	199.65	1.24
C	250.35	3	83.45	0.56

Table 4.3 Experimental results and range analysis in microwave assisted extraction

Experiments	A	B	C	Enzyme activity (U/ml)
1	1	1	1	12.42
2	1	2	2	16.33
3	1	3	3	20.07
4	1	4	4	19.12
5	2	1	2	31.86
6	2	2	1	18.40
7	2	3	4	10.35
8	2	4	3	4.99
9	**3**	**1**	**3**	**42.57**
10	3	2	4	24.21
11	3	3	1	11.79
12	3	4	2	11.47
13	4	1	4	31.00
14	4	2	3	20.08
15	4	3	2	11.43
16	4	4	1	4.99
K1	67.95	117.85	47.61	
K2	64.61	79.03	71.10	
K3	90.04	53.64	87.71	
K4	67.51	40.59	84.69	
k1	16.98	29.46	11.90	
k2	16.40	19.75	17.77	
k3	22.51	13.41	21.92	
k4	16.87	10.41	21.17	
R	6.11	19.32	10.02	

4.4.1 Effect of microwave power on extraction of inulin

The power must be chosen correctly to avoid the excessive temperature, which could lead to solute degradation and over preesure inside the vessel. Rapid rupture of cell wall takes place at higher temperature when kept at higher power, as a result together with the desired analytes impurities are also leached out in to the solvent whereas at low power levels the cell wall rupture might take place gradually. The effect of power on the extraction of pretreated coffee spent. The extraction efficiency was improved by raising the microwave power from 180 to 720 watts. The yield of pretreated coffee spent were enchanced with a short irradiation time ranges from 10 to 40 seconds. When the extraction solutions were raised to 40 seconds, the yields were low. The difference in the yield of coffee spent between 180 to 540 watts shows more significant with short irradiation times. Significant increase in extraction yield was observed at 540watt microwave power for all extraction time and found to be optimum. More electromagnetic energy was transferred to the extraction system quickly improved the extraction efficiency when the microwave power increased from 180 to 720 watts (Weihua 2008).

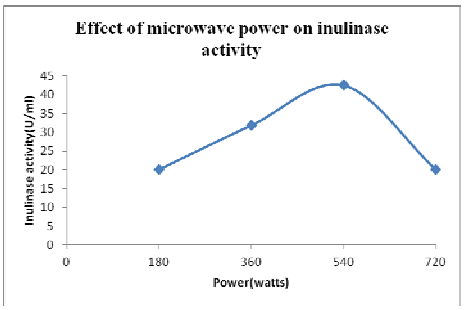


Fig 4.9 Effect of microwave power on the extraction of inulin

4.4.2 Effect of material ratio on the extraction of inulin

The volume of solvent must be sufficient to ensure that the entire sample is immersed, especially when having a matrix that will swell during the extraction process. In conventional extraction techniques, a higher solvent volume may give lower yield (Vivekanandha, 2008). Fig 4.10 showed the contents of inulin extracted were maxima at 1:25 materials ratio. Further increase in the material ratio leads to a gradual decrease in the inulin content revealing a saturated condition. A significant rise in the inulin content was observed with the material

ratio of 1:24. However, a gradual decrease in the inulin content was noticed when there is an increase in the material ratio. This decrease might be due to the fact that when the material ratio reached a certain level, the extract has well dissolved in the solution that may lead the contents of the extract become saturated and prevent further increase (Yaqin Xu *et al.*, 2005).

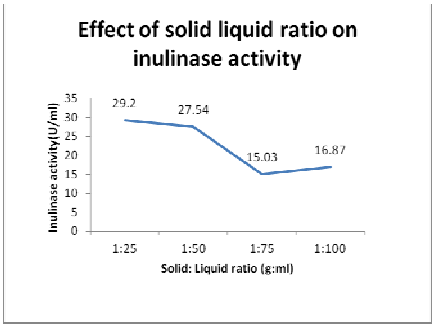


Fig 4.10 Effect of material ratio on the extraction of inulin

A higher extraction yield can be achieved in a shorter extraction time using Microwave assited extraction. A 12-min MAE could recover 92.1% of artemisinin from Artemisia annua L, while several-hour Soxhlet extraction could only achieve about 60% recovery (Hao, Han, Huang, Xue and Deng, 2002). A 4 to 5 min MAE (ethanol–water) of glycyrrhizic acid from licorice root achieved a higher extraction yield than extraction (ethanol–water) at room temperature for 20 to 24 hrs (Pan, Liu, Jia and Shu, 2000). For the extraction of tea polyphenols and caffeine from green tea leaves, a 4 min MAE achieved a high extraction yield than an extraction at room temperature for 20 h, ultrasonic extraction for 90 min and heat reflux extraction for 45 min, respectively, (Pan, Niu and Liu, 2003).

4.4.3 Effect of irradiation time on the extraction of inulin using coffee spent

Fig: 4.11 Effect of microwave irradiation time on inulinase activity

4.6 Optimization of inulin extraction from the coffee spent (pretreatment) using ultrasonication assisted extraction using L_{16} orthogonal design of experiments (Zhang *et al.*, 2009)

Extraction of inulin could be obtained by $L_{16}(4)^3$ orthogonal design i.e., four levels and three different variables (Solvent percentage (A), Solid Liquid ratio (B), Extraction time (C)). Solvent percentage varied from 65 to 94. Extraction time varied from 4 to 16 min and the solid liquid ratio varied from 1:25 to 1:100 g : ml.

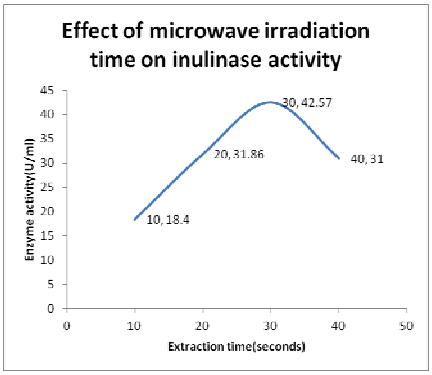
Table 4.4 One way ANOVA of Ultrasonication assisted Extraction

Levels	Sum of squares	Degrees of freedom	Mean square	F-value
A	3090.8	3	1030.29	0.81
B	424.74	3	141.58	0.11
C	232.01	3	77.338	0.06

Table 4.5 Expermimental Results And Analysis In Ultrasonication Assisted Extraction

Experiments	**A**	**B**	**C**	**Enzyme activity (U/ml)**
1	1	1	1	14.53

As in other technique, time is another parameter whose influence needs to be taken into account. Generally, by increasing the extraction time, the quantity of analytes extracted is increased, although there is the risk that degradation may occur. A proper study on optimization of extraction time is vital because extraction time may vary with different plant part used. Irradiation time is also influenced by the dielectric properties of the solvent. Solvents like water, ethanol, and methanol may heat up tremendously on longer exposure thus risking the future of thermoliable constituents. The result of Fig.511 showed that the contents of inulin extracted for 30 seconds reached maxima and prolonged extraction may not yield an increased content. The contents of inulin extracted for reached its maxima. Furthermore a decrease in the flavonoids content was noticed for 40 seconds extraction and a sudden increase in their content was observed for 30 seconds extraction time. This increase in the inulin content may be due to the synergistic effect of other parameters involved. A similar report by Chen *et al.,* 2007 revealed that 2hrs was the optimal extraction time for the extraction of a hypotensive drug geneposide from the bark of *Eucommia ulmoides* tree. Shu, Ko, and Chang (2003) reported that the extraction yield of ginsenosides from ginseng root obtained by a 15-min MAE (ethanol–water) was higher than that obtained by conventional solvent extraction (ethanol–water).



2	1	2	2	24.79
3	1	3	3	19.26
4	1	4	4	24.25
5	**2**	**1**	**2**	**37.57**
6	2	2	1	20.88
7	2	3	4	16.74
8	2	4	3	26.82
9	3	1	3	34.24
10	3	2	4	22.90
11	3	3	1	13.36
12	3	4	2	8.86
13	4	1	4	30.51
14	4	2	3	19.75
15	4	3	2	13.90
16	4	4	1	10.89
K1	82.82	116.85	59.66	
K2	102.01	89.32	84.12	
K3	79.36	63.26	100.07	
K4	74.05	70.82	94.4	
K1	20.70	29.21	14.91	
K2	24.50	22.08	21.28	
K3	19.84	14.81	24.01	
K4	18.76	17.70	23.6	

R	6.74	13.4	10.1

4.6.1 Effect of ethanol concentration in the extraction of inulin

Figure 4.12 showed the extraction of inulin with the ethanol concentration varying from 65 - 95%. The extraction of inulin gradually increased from 65 to 85 %. Ethanol was used for extracting the inulin among various solvents, since it is environmentally benign and relatively safe to human health (He *et al*., 2005). The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In this study, the results indicated that the optimal ethanol concentration for extraction of inulin from coffee spent was found to be 75%.

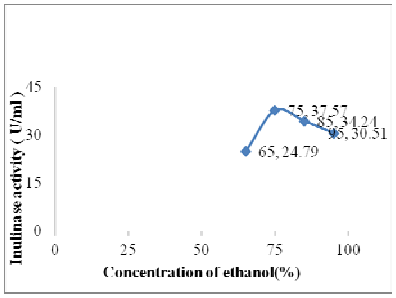


Fig 4.12 Effect of ethanol concentration in the extraction of inulin

4.6.2 Effect of material ratio (w/v) in the extraction of inulin

Extraction of inulin increased significantly with the ratio of solvent to raw material was between in the range of 1: 25 to 1: 100 g : ml. Figure 4.13 showed the contents of inulin extracted were maxima at 1: 25 materials ratio. Further increase in the material ratio leads to a gradual decrease in the inulin content. A significant rise in the inulin content was observed with the material ratio of 1:24. However, a gradual decrease in the inulin content was noticed when there is an increase in the material ratio. This decrease might be due to the fact that when the material ratio reached a certain level, the extract has well dissolved in the solution that may lead the contents of the extract become saturated and prevent further increase (Yaqin Xu *et al.,* 2005). Sathishkumar *et al*., 2009 reported that when the ratio of solvent to raw material was between 1 : 5 to 1:20 g : ml phenolic acids were extracted fully with the rise of volume the extracing agent so that the extracting agent so that extraction yields increased.

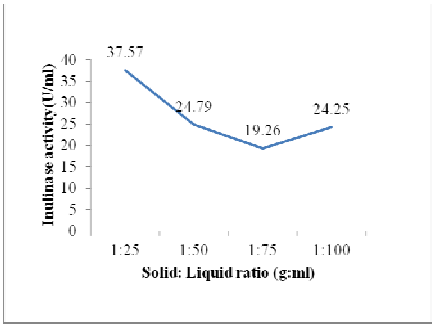


Fig 4.13 Effect of material ratio on the extraction of inulin

4.6.3 Effect of extraction time in the extraction of inulin

Maximum extraction of inulinase activity was found at 8 min and then decreased with prolonging ultrasound time. Cravotto *et al*., 2004 found that rice bran oil extraction can be efficiently performed in 30 min under high-intensity ultrasound either using hexane or a basic aqueous solution. Ceng, 2010 has applied to ultra-high purity fructose syrup with different parameters to get the influence of ultrasound on high inulinase production by *Aspergillus niger* and the yield of inulin extracted by ultrasound has reported as 94.23% under optimum conditions. Ultrasound can also reduce the operating temperature allowing the extraction of thermolabile compounds.

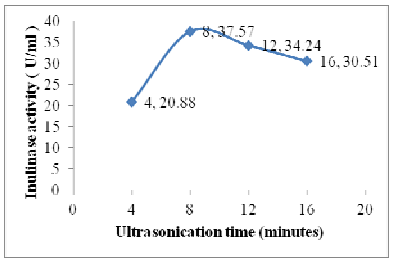


Fig 4.14 Effect of Extraction time in the extraction of inulin

Microwave irradiation showed advantages, such as shorter time, less solvent, high extraction rate, savings of energy and better products with lower cost compared to ultrasonication method. The disadvantage of ultrasonication method is that the ultrasonic extraction mainly depemds on the ultrasonic effects of acoustic cavitations and the uneven distribution of which leads to low activity of inulinase.

4.7 Effect of coffee spent on inulinase activity

Coffee spent was treated by different methods and the inulinase assay was carried out under assay conditions. Coffee spent (without pretreatment) under optimal conditions the inulinase activity was found to be 23.94 U/ml. Inulin extraction of coffee spent (pretreatment) with 70% ethanol and dilute HCl, the inulinase activity was found to be 28.08 U/ml.

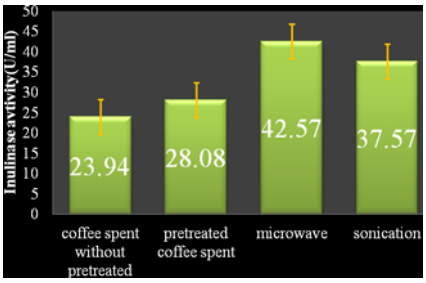


Fig : 4.15 Effect of coffee spent on inulinase activity

4.8 Media optimisation of inulinase production

4.8.1 Optimisation of inulinase production (Plackett-Burman Design)

The significant variables affecting the production of inulinase were identified using a 2-factorial system i.e., Plackett-Burman design (PBD) . The effect of individual parameters on enzyme production was calculated by the following equations:

$E_o = (\Sigma M_+ - \Sigma M_-) / N$ ---------- (Eqn.1)

$E = \beta_1 + \Sigma \beta_2 + \Sigma \beta_3 + \beta_{123}$ ---------- (Eqn.2)

In Eqn. 1, E_o is the effect of first parameter under study while M+ and M− are responses of enzyme production by the fungal strain. N is the total number of optimizations. In Eqn. 2, E is the significant parameter, β_1 is the linear coefficient, β_2 the quadratic coefficient while β_3 is the interaction coefficient among significant process parameters. The PBD was used to investigate the effects of seven variables and submerged fermentation (SuF) technology was adopted for inulinase production. The design matrix selected for screening the significant variables and their corresponding responses are given in Table 4.6.

TABLE 4.6 Plackett Burman design and the results (Enzyme activity) of experiments

Among the variables screened, agitation rate (200 rpm), NH_4Cl (0.03 g/ml) and Coffee spent (0.5 g/ml) were identified as the most significant variables influencing inulinase production and the maximum activity was observed as 42.07 U/ml. In previous literature, the variables corn steep liquour, FeSo4. 7H2O, Urea were selected on their positive influence on

Run	Coffee spent	NH4Cl	MgSO4	Ph	Agitation rate	KCl	Orange peel	Enzyme activity (U/ml)
1	-1	+1	+1	-1	-1	+1	-1	21.71
2	+1	+1	-1	+1	-1	-1	-1	21.37
3	-1	-1	-1	+1	+1	+1	-1	**42.07**
4	+1	-1	-1	-1	-1	+1	+1	20.36
5	+1	+1	+1	+1	+1	+1	+1	27.22
6	-1	+1	-1	-1	+1	-1	+1	40.72
7	+1	-1	+1	-1	+1	-1	-1	12.60
8	-1	-1	+1	+1	-1	-1	+1	20.92

inulinase production (Dilipkumar *et al*., 2011). Camelia *et al*., 2011 has reported that the agitation rate, Yeast extract, buckwheat flour, K_2HPO_4 concentration from fermentative medium composition, were determined as the most infleutial variables that have positive effects on fructofuranosidases biosynthesis and the maximum yield was found to be 6.2198 UI.

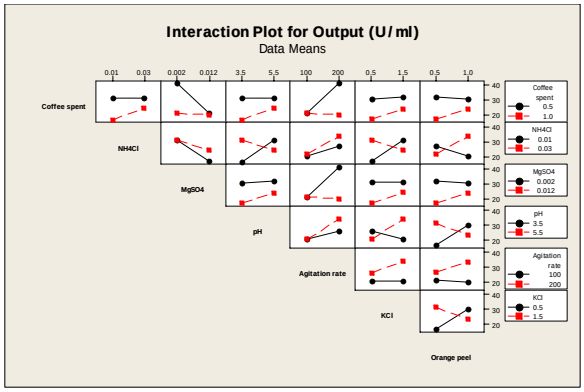


Figure :4.16 Intraction plot for Plackett Burman Design

Among the variables coffee spent and NH_4Cl were found to exert a positive effect i.e., when the concentration of coffee spent decreased and NH_4Cl was increased then the inulinase production was increased. $MgSo_4$ also found to be significant, but due to the nitrogen source we have chosen NH_4Cl for further design. In contrast, the agitation rate was also observed to have a positive effect on inulinase production, when the rpm was increased the inulinase production was increased and these variables were found to be significant than other variables.Therfore these three variables (Coffee spent, NH_4Cl, Agitation rate) were screened for futher CCD design of experiments.

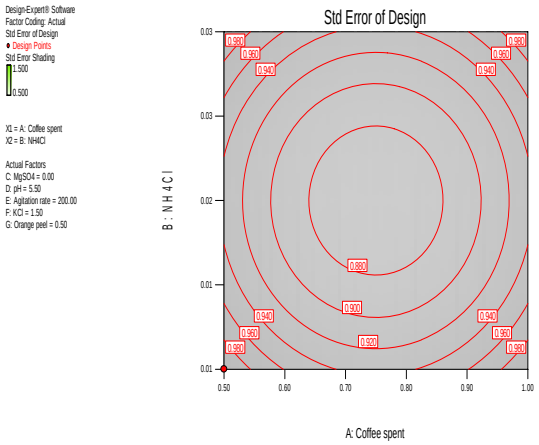


Figure :4.17 Contour plot for Plackett Burman Design

4.8.2 Optimizing the medium by Response Surface Methodology (RSM)

CCD is a very useful tool for determining the optimal level of medium constituents and their interaction. The levels of CCD were designed based upon the results obtained from PBD and SuF technology was adopted for inulinase production. By keeping 10 day old inoculam as fixed, the optimum levels of the selected variables according to CCD were found to be 0.1 g/ml (coffee spent , 200 rpm (agitation rate) and 0.1 g/ml (NH_4Cl) and the maximum activity was recorded as 106.29 U/ml at run No. 19.

Table 4.7 Central composite design consisting of various experiments for the study of three experimental factors (media constituents) in coded units and results.

Run order	**Coffee spent (g/ml)**	**NH_4Cl (g/ml)**	**Agitation rate Rpm**	**Output (U/ml)**
1	0.05	0.1	100	91.84
2	0.075	0.075	150	91.71
3	0.1	0.1	100	84.23
4	0.05	0.1	200	88.56
5	0.075	0.075	150	90.67
6	0.117045	0.075	150	92.38
7	0.032955	0.075	150	80.64
8	0.1	0.05	100	94.58
9	0.075	0.075	150	90.54
10	0.05	0.05	200	70.42
11	0.1	0.05	200	100.44
12	0.05	0.05	100	92.11
13	0.075	0.075	64.91036	84.14
14	0.075	0.117045	150	91.98
15	0.075	0.032955	150	80.5
16	0.075	0.075	150	96.07
17	0.075	0.075	234.0896	94.41
18	0.075	0.075	150	92.52
19	**0.1**	**0.1**	**200**	**106.29**
20	0.075	0.075	150	91.57

Table 4.8 ANOVA for Response Surface Model

Source	**Sum of squares**	**DF**	**Mean square**	**F value**	**p-value**
Model	937.48	6	156.25	12.68	< 0.0001
A-Coffee spent	303.25	1	303.25	24.61	0.0003
B-NH_4Cl	78.19	1	78.19	6.34	0.0257

C-Agitation rate	20.03	1	20.03	1.63	0.2246
AB	1	62.55	4.08	0.0422	
AC	1	323.72	26.27	0.0002	
BC	1	149.73	12.15	0.0040	

The Model F-value of 12.68 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.In this case **A, B, AB, AC, BC** are significant model terms.Values of"Prob>F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant.

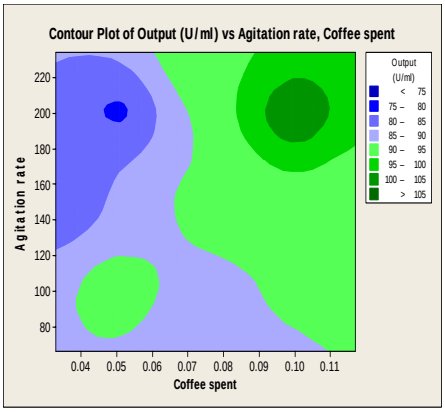


Fig 4.18 Contour plot of the inulinase production by *P.purpurogenum* (Minitab 16 trial version)

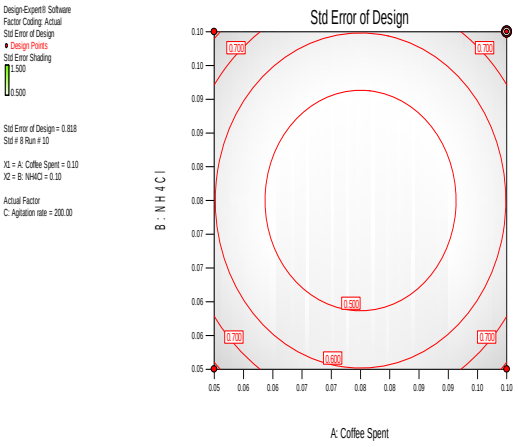


Fig 4.19 Contour plot of the inulinase production by *P.purpurogenum* (Design expert 8 trial version)

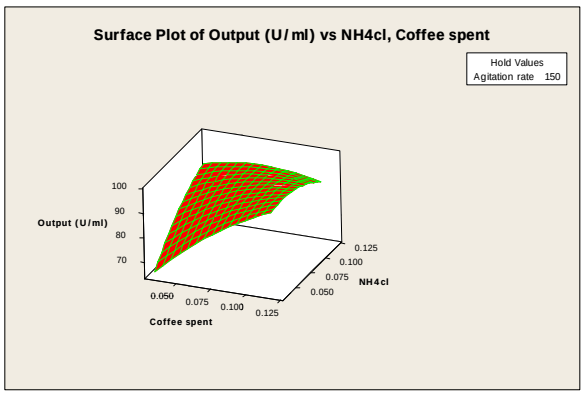


Fig 4.20 Response surface of the inulinase production by *P.purpurogenum*

The goodness of fit manifested by determination coefficient (R^2 = 0.88) showed that the sample variation of 88% for inulinase production is characteristic to the independent variables and only 12% of the total variation cannot be explained by the model.Three-dimensional graphs were generated for the pair-wise combination of the three variables, while keeping the other one at their optimum levels for the inulinase production by *pencillium* purpurogenum. The response surface and contour plots were employed to determine the interaction between the variables and the optimum levels that have the most significant effect on inulinase production . The surface and contour diagrams indicated that maximum inulinase activity (> 100U/ml) can be obtained by a combination of 0.05% (coffee spent), 200 rpm (agitation rate). To our knowledge, it is the first report on inulinase production from *Penicillium* species using coffee spent as a sole carbon source and it was found that 0.05 g/ml of coffee spent was the optimum concentration for inulinase production.In previous literature studies, Dilipkumar et al., 2011 reported that the optimum conditions are corn steep liquor (0.05813 g/gds), FeSO4.7H2O (0.00011 g/gds) and Urea (0.0211 g/gds) and the yield of inulinase was found to be 268 U/gds.

4.9 Partial isolation of inulinase from *Pencillium purpurogenum*

The partial isolation steps for inulinase were given in the table 4.9. The fraction 40% presented higher inulinase activity, with recovery of the 80.46% and purification of 1.67 fold.The fractions with inulinase activity was pooled and subjected to dialysis and utrafiltration and the specific activity of inulinase was determined to be 88.5 U/mg protein and the purification fold to be 1.83. Balayan *et al* ., (1996) reached yields of 16% and 10% of inulinase produced by *Penicillium palitans* T-1 and *P. cyclopium* T-18, respectively, using ultrafiltration, ammonium sulphate precipitation (0.8% of saturation), DE-32 Cellulose and Sephadex G-150. Kochhar *et al*., (1997) using precipitation with ammonium sulphate, DEAE Cellulose, CM Cellulose and Sephadex G 150 obtained a purification of 50 fold for inulinase of *Aspergillus versicolor*. Values higher than our results were mentioned by Kochhar *et al*., (1999) for the purification of the extracellular inulinase of *Aspergillus candidus*, using ammonium sulphate precipitation and DEAE Cellulose and Sephadex G-150, resulting a purification of 56 fold. Chen *et al*., (1997) purified the inulinase of A. niger using precipitation with ammonium sulphate, DEAE Cellulose and Sephadex G-100 with 67 fold purification. Derycke and Vandamme (1983), Cruz *et al*., 1998 and Pandey *et al*., (1999) descried that for industrial application, inulinase showed have maximum activity at pH lower than 4.0 and at higher temperatures, as produced by *A. niveus* 4128URM.

Table 4.9 Partial isolation of inulinase from *Pencillium purpurogenum*

Fraction	Volume(ml)	Inulinase activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Fold Purification
Crude	100	54.81	1.159	48.15	1
Ammonium sulphate precipitation	15	38.27	0.513	80.46	1.67
Ultrafiltration	50	28.32	0.32	88.5	**1.83**

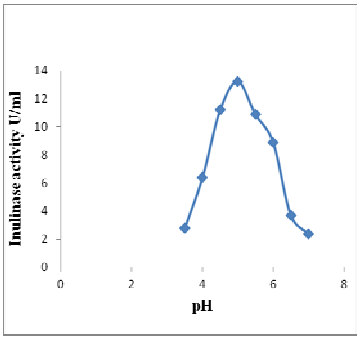


Fig: 4.21 Effect of pH on the activity of partial isolation Inulinase from *P.purpurogenum*

4.10.2 Effect of temperature

The optimum temperature was found as 55°C (Figure 4.22). As it is near to room temperature this enzyme can be easily used. Similar temperature was reported for purified inulinase from *A.niger* 20 OSM (Marcin *et al*., 2006) and *Penicillium Janczewskii* (Pessoni, 2007). Lower temperature was reported with *Rhizopus sp*. strain TN -96 (40°C) .(Kazhuyoshi *et al*., 2002) *A.ficuum* JNSP5-06(45°C) (Han *et al*., 2009) and *Arthrobacter* sp.(50°C) .(Su-II Kang *et al*., 1998). However, higher temperature was reported for purified inulinase from *Streptomyces* sp (70°C) (Arun *et al*., 2007). The k_m and V_{max} were found to be same as in case of crude enzyme shown in fig: 4.7

4.10 Characterization of partially Purified inulinase

4.10.1 Effect of pH

The optimum pH of purified inulinase was found to be 5 (Figure 4.21). The pH obtained for crude was also the same. The pH optimum of many microbial sources reported so far: *A. niger* (4.4) (Derycke and Vandamme, 1984), *A. versicolor* (4.5) (Kochhar *et al*., 1997), *Penicillium janczewskii (*4.8–4.0) (Pessoni and Braga, 1999) and *Acetobacter diazotropicus* SRT4 (4.5) (Tambara *et al*., 1999). *Penicillium Janczewskii (5-4.5)(Pessoni,2007)Rhizopus sp*. strain TN -96 (6.0) .(Kazhuyoshi *et al*., 2002) *A.niger* 20 OSM (4.5)(Marcin et al., 2006) *A.ficuum* JNSP5-06 (4.5-4.0) (Han et al.,2009).

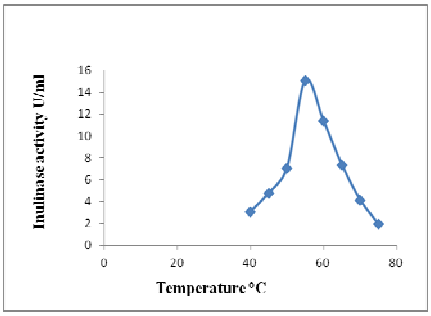
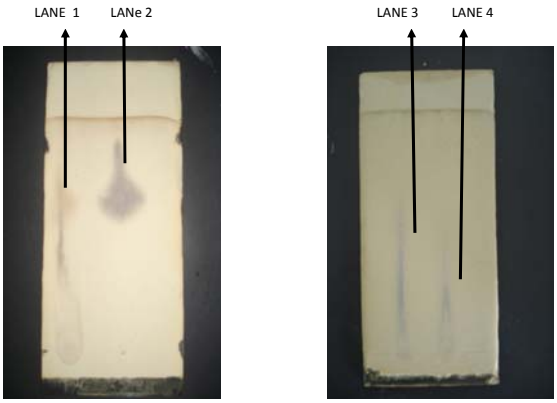


Figure: 4.22 Effect of temperature on the activity of partially isolated inulinase

4.11 Determination of hydrolysis products (Thin layer chromatography)

TLC analysis was done with reaction products with the inulin treated with inulinase (Figure 4.6). Fructose was the major sugar produced during hydrolysis, supported the view that inulinase was an end group cleaving enzyme. According to the TLC results, it was determined that *P.purpurogenum* inulinases is exoinulinase because their hydrolyse product is only free fructose. Exoinulinase split off terminal fructose units successively from non-reducing end of the inulin molecule. Thus, exoinulinases are used for the production og high fructose syrup. The R_f value is similar for pure inulin treated with dialysed enzyme(0.614) and that of pure inulin treated with crude enzyme(0.688). Ertan *et al*., 2003 have reported that the *A.parasiticus* and *P.spinulosum* inulinases are exoinulinases because their hydrolysed product is free fructose using thin layer chromatography.



LANE 1:Pure inulin treated with dialysed enzyme

LANE 2: Pure inulin

LANE 3: Pure inulin treated with crude enzyme

LANE 4: Pure fructose

Figure 4.23 Determination of hydrolysed products (TLC)

CONCLUSION

- Out of several soil isolates screened, *Pencillium purpurogenum,* proved to produce high inulinase activity(0.934 U/ml). The crude inulinase recorded the optimum pH (5) and temperature (60^{o}C). The enzyme was thermostable for 4hrs (19.97 U/ml) under assay conditions. The k_m and V_{max} values were observed as 0.25 mg and 40 U/ml respectively.
- Microwave irradiation showed maximum activity of 42.57 U/ml than the ultrasonic mediated extraction (37.57U/ml). The optimum conditions of 540 W with 1:25 material ratio and 30 seconds showed high inulinase activity.
- The maximum inulinase activity (42.57 U/ml) can be obtained by a combination of coffee spent (0.5 g/ml), Aagitation rate (200 rpm), NH_4Cl (0.003g/ml), $MgSo_4$ (0.002g/ml), pH (5.5), KCl (1.5 g/ml), orange peel (0.5 g/ml) by Placket- burman design of experiment.
- The maximum inulinase activity (106.29 U/ml) was obtained by the combination of coffee spent (0.1 g/ml), NH_4Cl (0.1 g/ml) and agitation rate (200 rpm) was carried out using Response Surface Methodology.
- The partial isolation was carried out by ammonium sulfate precipitation and ultrafiltration and the fold purification 1.83 (88.50 U/mg) was obtained.
- The partially isolated inulinase was characterized, its optimum pH was found as 5 and its optimum temperature was 55^{o}C.
- The hydrolysed products present in the enzyme extract were analysed by TLC.

APPENDICES

1. Resorcinol reagent (100ml)

Resorcinol - 1g

Thiourea - 0.25g

Glacial acetic acid - 100ml

2. DNS reagent (100ml)

3,5- dinitro salicylic acid - 1g

Crystalline phenol - 200mg

1% Sodium hydroxide - 100ml

Sodium sulphite - 50mg

3. Potato dextrose agar

Components	**Amount (g/l)**
Potato infusion	200
Dextrose	20
Agar	15

4. **Potato dextrose Broth**

Components	**Amount (g/l)**
Potato infusion	200
Dextrose	20

5. Lactophenol cotton blue stain

Ingredients	**In 100ml aqueous solution**
Methylene blue(cotton blue,aniline blue)	50mg
Phenol	25g
L(+) Lactic acid	25g
Glycerol	50g

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