MICROBIAL ENZYMES

# Studies on β-fructofuranosidase from a thermotolerant strain of *Kluyveromyces marxianus* NCYC 2675

**A THESIS**
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By

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*-------- Dedicated to my beloved parents*

*Aai and Dada*

# DECLARATION BY RESEARCH GUIDE

Certified that the work incorporated in the thesis entitled: **Microbial enzymes: Studies on β- fructofuranosidase from thermotolerant strain of *Kluyveromyces marxianus* NCYC 2675,** submitted by Mrs. Sulabha K Karandikar for the Degree of Doctor of Philosophy, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune 411018, India. Material that has been obtained from other sources is duly acknowledged in the thesis.

**Dr. (Mrs.) Asmita Prabhune**
(Research Supervisor)

# DECLARATION BY RESEARCH STUDENT

I hearby declare that the thesis entitled: **Microbial enzymes: Studies on β-fructofuranosidase from thermotolerant strain of *Kluyveromyces marxianus* NCYC 2675,** submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune 411018, India under the supervision of Dr. Asmita Prabhune. This work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

**Mrs. Sulabha K Karandikar**
(Research Student)

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SULABHA

## ABSTRACT

The major objective of the research was to get hands on training of classical enzymolgy with respect to methods of purification of enzyme, its characterization, study of active site and the applied aspects namely immobilization. The THESIS embodies work on a thermotolerant yeast strain *Kluyveromyces marxianus* NCYC 2675 and attempts were made to explore the enzyme as described below. The investigations include optimization of fermentation parameters for production of the enzyme, its purification and characterization, inhibitor studies using sucrose analogues and immobilization using different matrices for enzyme and whole cells.


## Chapter 1

### Introduction

Invertase (β-D-fructofuranosidase fructohydrolase, (EC 3.2.1.26) hydrolyses the O-glycosyl bond forming an equimolar mixture of glucose and fructose (invert syrup) from sucrose. Invert syrup is non crystallizable and is approximately 1.5 times sweeter than sucrose. It has wide applications in the food and pharmaceutical industry because of its functionally more desirable properties like high solubility and hygroscopic nature.

Though considerable attention has been paid to the production of high fructose syrup (HFS), very little importance has been given to invertase for the production of isomerase's syrup. Production of high fructose syrup requires starch hydrolysate as its starting material. In this respect, in countries like India where sucrose is readily available, utilization of sucrose will be advantageous. Additionally, molasses a by-product of the sugar industry can serve as a source of sucrose due to its high sucrose content (55%).

Several bacteria, yeast and fungi have been shown to produce invertase. Though there is ubiquitous distribution, the enzyme of commercial interest originates from yeast species. However, the difficulty of obtaining the source of the enzyme in quantities sufficient for commercial exploitation eliminates many of these from consideration. A preliminary survey was made in the present studies to identify a thermotolerant strain producing high levels of enzyme activity. Isolates and standard type cultures were screened preliminarily for invertase production before attempting the isolation and characterization of its kinetic and molecular properties.

Most of the invertases reported so far are from mesophilic strains and very few reports are on thermophilic/thermotolerant organisms. Thermotolerant/thermophilic organisms not only prevent microbial contamination but also permit optimum enzyme production in shorter time compared to mesophilic organisms.

**Chapter 2**

**Isolation Characterization and Optimization of Fermentation parameters**

The yeast *Kluyveromyces marxianus* was isolated from over ripe grapes and identified and deposited at NCYC as NCYC 2675.The effect of growth and cultural conditions on over production of invertase was studied in detail. The effect of pH, temperature, inoculum size, dispensing volume, and rate of invertase production were investigated under shake flask conditions. The effect of sucrose concentration, metal ions, organic/ inorganic nitrogen on invertase production and cationic surfactant CTAB (cetyl trimethyl ammonium bromide) were investigated. For cost effective and accelerated production of this enzyme/invert syrup, the effect of cheap, renewable carbon sources and synthetic zeolites as additives were studied.

**Chapter 3**

**Purification, Chemical Modification and Characterization**

To study the enzyme and its structure function relationship, a homogenous preparation of the enzyme was obtained using octyl Sepharose, an affinity chromatography column. The molecular weight was determined by gel filtration using Sephadex G-200 column. This was confirmed by MALDI-TOF and SDS-PAGE. The homogenized enzyme preparation was subjected to pH profile, temperature profile, pH stability, temperature stability and substrate stability studies. Similarly, various metal ions, EDTA, and urea were used to test their effect on enzyme activity. Km, Vmax and pI (iso electric point) of the enzyme were determined.

Effect of amino acid specific modifying agents was studied to determine the amino acid residue at the catalytic site. NBS (N Bromosuccinimide) a chemical modifier of tryptophan resulted in enzyme inactivation. The protection provided by substrate and a competitive inhibitor during modification reaction of NBS was determined. Similar studies were performed using HNBBr (2-Hydroxy-5-Nitro Benzyl Bromide) a chemical modifier of tryptophan to confirm the results. The inactivation kinetics is indicative of the involvement of tryptophan residue in the hydrolysis of sucrose by invertase from *K marxianus*.

**Chapter 4**

**Sucrose mimetics and their role as Invertase inhibitors**

The synthesis of sucrose mimetics is particularly attractive, due to the serious and widespread sucrose metabolism disorders, such as diabetes mellitus. The effect of four synthetic sucrose mimetics on invertase activity in presence of substrate sucrose was studied. Two of the sucrose mimetics showed greater than 50% inhibition of invertase activity.

## Chapter 5

## Immobilization studies

*Using SBA 15*

Silica supports have number of advantages, including chemical and mechanical stability, non-hydrophobicity, biological inertness and resistance to microbial attack, and are stable at elevated temperatures  Attempts were made to immobilize invertase from *K marxianus* on functionalized silicalite and cross linked with glutaraldehyde to avoid leaching and to obtain a highly active and stable immobilized enzyme preparation suitable for commercial exploitation.

*Using Gellan Gum*

Gellan gum is a linear extracellular, anionic polysaccharide having a wide range of applications in the food and pharmaceutical industry. Whole cells were entrapped into Gellan beads linked by bifunctional reagent glutaraldehyde. Rheological behaviour of Gellan gum with/without cells was determined. Gel entrapment of cells and optimization of invertase production was performed using a $3^2$ factorial design to study the joint effect of two variables, the polymer concentration and TMACl concentration on various dependant variables like enzyme activity with/without glutaraldehyde, particle size, and gelling temperature.

*Using aerogels*

Silica aerogel is unique among solid matrices, having a plethora of applications High surface area, filigrane solid network and biocompatibility, makes it a promising material for immobilization of biologically active molecules notably enzymes. However it's brittle nature limits the performance as support material. We have reinforced silica aerogel with glasswool to increase its tensile and mechanical strength. This makes handling of aerogel easier while loading the cells. *K marxianus* was successfully immobilized on glass wool reinforced silica aerogel.

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# ABBREVIATIONS

| | |
|---|---|
| APTES | 3 amino propyl triethoxysilane |
| FTIR | Fourier transform infrared spectroscopy |
| CD | Circular Dichroism |
| XRD | X-ray diffraction |
| M-E | mesoporous silica and enzyme |
| M-E-G | mesoporous silicas enzyme and glutaraldrhyde |
| CTAB | N-cetyl-N,N,N-trimethyl ammonium bromide |
| NBS | N-bromosuccinimide |
| HNBBr | 2-Hydroxy-5-Nitrobenzyl bromide |
| TRIS | Tris(hydroxymethyl)amino-methane |
| TPTZ | Triphenyltetrazolium chloride |
| TMACl | Tetramethyl ammonium chloride |
| BPB | Bromophenol blue |
| ACN | Acetonitrile |
| TFA | Trifluoroacetic acid |
| DTNB | Dithiobis(2-nitrobenzoic acid) |
| PMSF | Phenyl methyl sulfonyl fluoride |
| TNBS | Trinitrobenzene sulfonic acid |
| DEP | Diethyl pyrocarbonate |
| NAI | N acetyl imidazole |
| AFM | Atomic Force Microscopy |
| SEM | Scanning Electron Microscopy |

# CHAPTER 1

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# GENERAL INTRODUCTION

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### 1.1 Carbohydrolases:

Glycoside hydrolases EC 3.2.1 are a widespread group of enzymes which hydrolyze α or β glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non carbohydrate moiety. Glycoside hydrolases are also known as carbohydrolases which transfer the glycosyl bond to a water molecule. Some of the enzyme representatives of this group are β galactosidase, invertase, α galactosidase, β glucosidase and amylase.

### 1.1a INVERTASE:

Invertase or β-fructofuranosidase (E C 3.2.1.26) catalyzes the irreversible hydrolysis of sucrose (α-D glucopyranosyl-[1-2] β D fructofuranoside) and is one of the earliest discovered enzymes in the second half of the 19$^{th}$century. Hydrolysis of sucrose by invertase results in an equimolar mixture of glucose and fructose (invert syrup) which causes the inversion of the anomeric carbon (**Scheme 1**). The anomeric carbon atoms of glucose and a fructose residue are in α-glycosidic linkage in sucrose. Consequently sucrose lacks a free reducing group (an aldehyde group), in contrast with other sugars. Invert syrup is non crystallizable and is approximately 1.5 times sweeter than sucrose and has numerous applications in the food and pharmaceutical industry. Sucrose is obtained commercially from cane or beet. Additionally, molasses a by-product of the sugar industry can serve as a source of sucrose. The replacement of continuous acid hydrolysis by a continuous immobilized enzyme process for invert sugar production seems to be the most attractive industrial application of invertase. Enzymatic hydrolysis of sucrose to invert syrup is preferential to acid hydrolysis, as it does not result in the production of furfural, highly neurotoxic hydroxyl methyl furfural and other undesirable flavors and oligosaccharides.

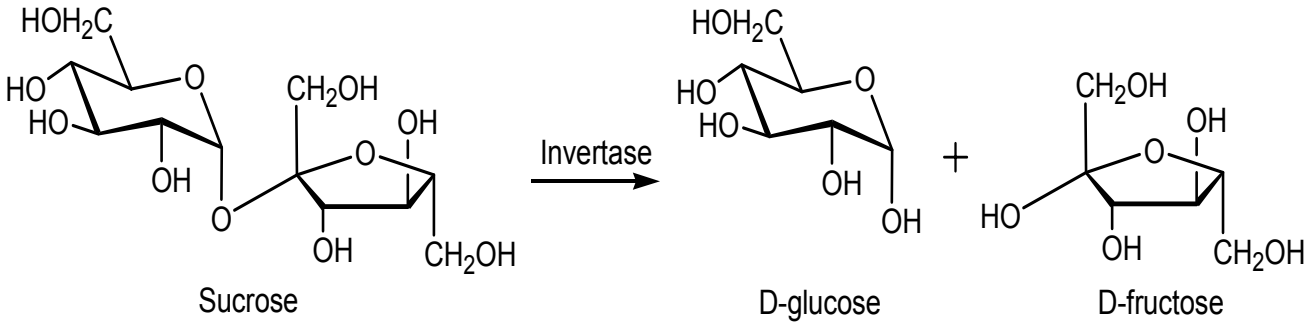
### 1.1b INULINASE:

Inulinase EC 3.2.1.7 catalyzes endohydrolysis of β-(2-1) D-fructosidic linkages from oligosaccharides or from linear or branched fructans. The enzyme can hydrolyze inulin, the fructose portion of raffinose, stachyose, sucrose and branched bacterial levans of high molecular weight. Inulin is a plant polymer which acts as a reserve food in the tubers of the Compositae and Gramineae families. Inulin is a linear fructan oligosaccharide consisting of multiple fructose units with a terminal D-glucose unit. It can generally contain 2 to 140 fructose units.

**Action of Invertase**

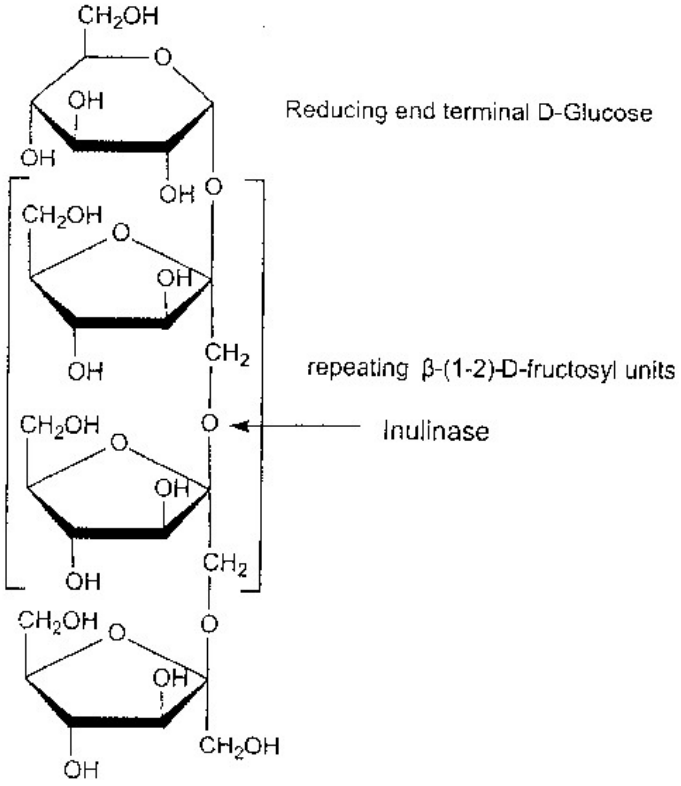
R-β fructofuranoside +$H_2O$ ⟶ ROH +Fructose

R=any sugar




**Scheme 1.1**

**Action of Inulinase**




**Scheme 1.2**

**Table 1.1**
**Other Sucrases**

| Enzyme | EC no | Reaction catalyzed |
|---|---|---|
| Sucrose alpha-glucosidase | EC 3.2.1.48 | Hydrolysis of sucrose and maltose by an alpha-D-glucosidase type reaction |
| Alternansucrase | EC 2.4.1.140 | Transfers alternatively an alpha D glucose residue from sucrose to the $6^{th}$ position and $3^{rd}$ position of alpha d glucose |
| Levansucrase | EC 2.4.1.10 | Transfer of fructose from sucrose to levan |
| Inulosucrase | EC 2.4.1.9 | Transfer of fructose from sucrose to inulin |
| Amylosucrase | EC 2.4.1.4 | Transfer of glucose from sucrose to glucan |
| Dextran sucrase | EC 2.4.1.5 | Transfer of glucose from sucrose to dextran |

**1.2 I/S ratio**:

Inulinases are classified as 2, 1-β-D fructan fructanohydrolase. It is generally accepted that the ratio of the hydrolytic activity at pH 5.0 on inulin versus sucrose (I/S ratio) characterizes the enzymes as inulinase or invertase: for inulinase the I/S ratio is higher than $10^{-2}$ while for invertase it is $10^{-2}$ or lower [Ettalibi and Baratti 1987].Invertase preparations containing α glucosidase could contribute to the hydrolysis rate of sucrose. This can be avoided if raffinose is used as substrate, in which α glucosidic bond is not located terminally [Synder and Phaff 1960].

**1.3 Occurrence and Physiological role**:

Invertase catalyzes the cleavage between fructose carbon and the bridge oxygen rather than between the glucose carbon and the bridge oxygen [Koshland and Stein 1954]. The enzyme is known to catalyze the hydrolysis of the β-fructofuranosides sucrose, stachyose, raffinose, verbascose, methyl fructofuranoside, benzyl fructofuranoside and to a certain extent inulin but does not act on α-fructofuranosides, fructopyranosides or 3 substituted β-fructofuranosides.

The physiological function of β fructofuranosidase could be the hydrolysis of fructose containing disaccharides and oligosaccharides present in the growth medium or in the natural environment liberating assimiable end products glucose and fructose which serve as a carbon source for growth of the organism. There are number of bacteria, yeast, fungi, and actinonomycetes which are known to produce intracellular/extra-cellular invertases. A few of them are listed in **Table 1.2**-**Table1.5**.

There have been very many reports in the literature of the detection of invertase activity in micro-organisms. The difficulty of obtaining the source of the enzyme in quantities sufficient for commercial exploitation eliminates many of them from consideration here. In general, microbial invertases have broad pH optima slightly below neutrality, and are reasonably thermostable.

**1.4 Physiology**:

To clarify the genes required for tolerance to high-sucrose stress, genome-wide screening was undertaken using the complete deletion strain collection of diploid *Saccharomyces cerevisiae*. The screening identified 273 deletions that yielded high sucrose sensitivity Cross-sensitivity of the high-sucrose-sensitive mutants to high concentrations of NaCl and sorbitol was studied. The genes identified in this study might be important for tolerance to high-sucrose stress, and therefore should be target genes in future research into molecular modification for breeding of yeast tolerant to high-sucrose stress [Ando *et al* 2006].

A low level of osmotic stress (NaCl) induces invertase synthesis at very high levels in *P anomala* and *K lactis*. Glucose consumption was not influenced at significant levels by the hyper osmotic stress in *P anomala* [Turkel et *al* 2006].Low amounts of NaCl in the growth medium (0.2M) increased the invertase activities of yeast strains up to 50%. Industrial strains are more sensitive to hyper osmotic stress than the laboratory strains of *Saccharomyces cerevisiae* [Turkel and Turgut 2002].

Commercially available invertase preparations from *Saccharomyces cerevisiae* are reversibly deactivated by high ethanol concentrations and by NaCl concentrations that occur in industrial molasses media [Zech and Gorisch 1995].

Glucose repression was not the only factor affecting the invertase expression. Invertase expression was also strongly dependant on oxygen availability. Moving from anaerobic to aerobic conditions led to five-fold increase in specific invertase activity [Pyun *et al*

1999]. Invertase secretion was mediated by inositol and phosphatidylinositol type signaling pathway [Zhen Ming *et al* 2004].

*Kluyveromyces lactis* can grow on a wider variety of substrates and shows less sensitivity to glucose repression than does *Saccharomyces cerevisiae*. Many genes that are subject to glucose repression in *S cerevisiae* are repressed only weakly or not at all in *K lactis*.

The SUC2 gene, which encodes invertase in *S cerevisiae*, is strongly regulated by glucose and serves as a model system for studies on glucose repression. The invertase gene of *K lactis*, KI INV1 is also strongly repressed in the presence of glucose [Georis *et al* 1999].

**Table 1.2**

**Invertase from Fungi/Actinomycetes**

| **Organism** | **Enzyme** | **Reference** |
|---|---|---|
| *Aspergillus niger* | intracellular/extracellular | Aranda *et al* 2006 |
| *Aspergillus japonicus* | extracellular | Chen and Liu 1995,Hayashi *et al* 1992 |
| *Thermomyces lanuginosus* | intracellular | Maheshwari *et al* 1983 |
| *Fusarium oxysporum* | extracellular | Kaur *et al* 1992 |
| *Neurospora crassa* | extracellular | Casanova *et al* 1987 |
| *Monographella nivalis* | intracellular | Cairns *et al* 1995 |
| *Fusarium solani* | extracellular | Bhatti *et al* 2006 |
| *Aspergillus nidulans* | extracellular | Chen Jee-song *et al*1996 |
| *Pycnporus sanguineus* | extracellular | Quiroga *et al* 1995 |
| *Aspergillus ficuum* | extracellular | Ettalibi and Baratti 1987 |
| *Aspergillus fumigatus* | extracellular/intracellular | Fiedurek *et al* 2000 |
| *Sclerotium rolfsii* | extracellular | Kotwal and Shankar 1995 |
| *Phytophthora parasitica* | intracellular | Hankin and McIntyre 1980 |
| *Puccinia graminis* | intracellular | Williams *et al* 1984 |
| *Streptomyces* | extracellular | Kaur and Sharma 2005 |
| *Aspergillus niger* | intracellular | Nguyen *et al* 2005 |
| *Aspergillus ochraceus* | extracellular | Ghosh *et al* 2001 |
| *Claviceps purpurea* | intracellular | Dickerson 1972 |

**Table 1.3**

**Invertase from Bacteria**

| **Organism** | **Enzyme** | **Reference** |
|---|---|---|
| *Zymomonas mobilis* | extracellular | Yanase *et al* 1995 |
| *Lactobacillus reuteri* | intracellular | DeGines *et al* 2000 |
| *Escherichia coli* | intracellular | Olusanya and Olutiola1994 |
| *Glutamate producing bacterium* | intracellular | Keisou *et al* 1986 |
| *Bacillus macerans* | extracellular | Kim *et al* 2000 |
| *Arthrobacter species* | extracellular | Fujita *et al* 1990 |
| *Thermotoga maritima* | intracellular | Liebl *et a*l 1998 |
| *Streptococcus mutans* | intracellular | Kuramitsu 1973 |
| *Clostridium perfringens* | intracellular | Ishimoto and Nakamura 1997 |
| *Bifidibacterium infantis* | extracellular | Warchol *et al* 2002 |
| *Azotabacter chroococcum* | extracellular | De la Vega *et al* 1991 |

**Table 1.4**

**Invertase from yeast**

| Organism | Enzyme | Reference |
|---|---|---|
| *Candida utilis* | extracellular | Chavez *et al* 1997 |
| *Saccharomyces fibuligera* | extracellular | Gogoi *et al* 1998 |
| *Torulaspora pretoriensis* | extracellular | Oda and Tonomura1994 |
| *Pichia anomala* | intracellular | Rodriguez *et al* 1995 |
| *Candida kefyr* | intracellular/extracellular | Negoro and Kito1973 |
| *Rhodotorula glutinis* | intracellular | Rubio *et al* 2002 |
| *Schwanniomyces occidentalis* | extracellular | Costaglioli *et al* 1997 |
| *Leucosporidium antarcticum*(marine ) | intracellular | Turkiewicz *et al 2005* |
| *Saccharomyces carlsbergensis* | intracellular | Toda 1976 |
| *Schizosaccharomyces pombe* | intracellular | Tanaka *et al* 1998 |

**Table 1.5**

**Heterologous/Recombinant Invertase**

| Organism | Enzyme | Reference |
|---|---|---|
| *Hansenula polymorpha* | intracellular | Narciandi *et al* 1995 |
| *Saccharomyces cerevisiae* | intracellular | Nam *et al* 1993 |
| *Schizosaccharomyces pombe* | intracellular | Zarate and Belda 1997 |
| *Yarrowa lipolytica* | extracellular | Nicaud *et al* 1989 |

## 1.5 Molecular Aspects:

### 1.5.1 Glycoprotein:

The role of carbohydrate chains for the structure, function, stability, and folding of glycoproteins have been investigated using invertase as a model [Kern *et al* 1992]. Several different genes encode the proteinand its carbohydrate moiety is heterogeneous. Both properties complicate physicochemical comparisons. Glycosylation is found to stabilize the protein with respect to thermal denaturation and chaotropic solvent components; the stabilizing effect does not differ for the external and the core-glycosylated forms. Unlike the internal enzyme, the glycosylated forms are protected from aggregation. Core-glycosylation is necessary for oligomerization to tetramers and octamers. Dimerization is required and sufficient to generate enzymatic activity; further association does not alter the specific activity of core-glycosylated invertase, suggesting that the active sites of invertase are not affected by the association of the dimeric units.

### 1.5.2 Glycosylation:

Glycosylation is one of the major naturally occurring modifications of the covalent structure of proteins. Most of the proteins which are transported along the secretory pathway become glycosylated in the course of synthesis and/or transport [Sharon and Lia 1982].Two different kinds of carbohydrate transfer are observed. O-glycosylation at the hydroxyl groups of serine and threonine residues and N-glycosylation at the asparagine residues. The role of glycosylation for the structure and function of glycoproteins is not entirely clear. Specific residues appear to be important for correct targeting of some proteins [Neufeld and Ashwell 1980], for the biological function and for receptor mediated clearance. A comparison of carbohydrate-free and variously glycosylated pancreatic ribonucleases indicated that in the case of small, single domain proteins, the presence of the carbohydrate chains is not important for the stability and mechanism of protein folding.

*Saccharomyces cerevisiae* invertase exists in two different forms. The cytoplasmic enzyme is nonglycosylated; whereas the external invertase contains about 50% carbohydrate of the mannan type. The protein moieties of both enzymes are identical. The ranges of stability of the two invertases were found to be essentially identical, indicating that the presence of a high amount of carbohydrate does not significantly contribute to the stability of external invertase. Earlier findings that invertase is stabilized by glycosylation could not be confirmed. Unlike the glycosylated form, the carbohydrate–

free invertase is prone to aggregation in the denatured state at high temperature and in a partially unfolded form in the presence of intermediate concentrations of guanidinium chloride [Schulke and Schmid 1988].

### 1.5.3 Hyperglycosylation:

N-oligosaccharides of *Saccharomyces cerevisiae* glycoproteins are classified as core and mannan types. The former contain 13-14 mannoses whereas mannan-type structures consist of an inner core extended with an outer chain of up to 200-300 mannoses, a process known as hyperglycosylation. On the basis of the three dimensional structure of exoglucanase 1, it was proposed by Conde *et el* in 2004 that amino acid charge influences the orientation of the inner core making it accessible to mannan polymerase 1 in the appropriate position for the addition of $\alpha$ 1-6 –mannoses.

### 1.5.4 Deglycosylation:

Chemical deglycosylation of the S and F forms of invertase (named due to their different migration ratio in polyacrylamide gel electrophoresis) was carried out using trifluoromethane sulfonic acid (TFMS). Enzymatic deglycosylation can be carried out using Endoglycosidase H exposure for 24 to 60 hours and subjecting the sample to SDS-PAGE (Belcarz *et al* 2002). It is known that Endo H catalyzes the hydrolysis of asparagine-linked oligomannose, but not complex oligosaccharides from glycoproteins. Gallili and Lampen in 1977 have shown the effect of Tunicamycin on glycosylation. Tunicamycin is an N-acetylglucosamine containing antibiotic which specifically inhibits the first step in the lipid-linked oligosaccharides pathway which produces Glc N Ac-pyrophosphoryl dolichol (Mahoney and Duksin 1979).

### 1.5.5 Octameric invertase:

Yeast external invertase is a glycoprotein that exists as a dimer that can associate to form tetramers, hexamers, and a process that is facilitated by the attached oligosaccharide chains. Tammi et *al* in 1987 have studied this association by high performance liquid chromatography on a gel filtration matrix. Investigation of aggregate formation by electron microscopy revealed that all invertases, including the internal nonglycosylated enzyme, form octamers under appropriate conditions.

Yeast invertase forms a homo-octamer of core glycosylated subunits during assembly in the lumen of the endoplasmic reticulum. This form has been purified from mutant cells in

which transport of secreted proteins from the endoplasmic reticulum is blocked [Esmon et *al* 1987]. Purified octameric invertase is dissociated into dimer units which reassociate in the presence of polyethylene glycol.

Assembly of the octamer in vitro and in vivo is facilitated by the presence of N-linked carbohydrate. Selective release of the dimeric glycosylated invertase from intact cells suggests that oligomerization helps retain the enzyme in the periplasmic space.

Cytoplasmic invertase is produced from the same gene as secreted invertase. The unglycosylated form of invertase is expressed constitutively, whereas synthesis of secreted invertase is subject to catabolite repression.

**1.5.6 Isozymes:**

The properties of purified preparations of the internal and external invertases of yeast, designated by their location relative to the cell membrane, show similarities and marked disparities [Gascon et *al* 1968]. Both invertases have the same $k_m$ for sucrose and raffinose, and neither showed an appreciable transferase activity. The pH optima for enzymatic activity are the same, but their pH stability curves differ, the internal invertase showing reversible inactivation at acidic pH.

The internal invertase has a higher mobility than the external in polyacrylamide gel electrophoresis at pH 6.8 and 8.6.The enzymes are immunologically related, and sucrose-negative mutants lack both external and internal invertase activity.

The most striking difference was in their renaturation following guanidine treatment where it was shown that inactivated external invertase could be renatured completely. Endo-β-N-acetylglucosaminidase H treated external invertase was restored to 40% of its original activity while internal invertase remained completely inactive [Chu *et al* 1985].

Three types of invertase (invertase 1, invertase 2 and invertase 3) were separated by Hoshino et *al* in1964 from bakers' yeast using DEAE column chromatography.

The intracellular invertase *of Saccharomyces cerevisiae* is mainly found in a soluble form, while only minor amounts are found bound to the plasma membranes. In the processes of derepression or repression, inhibition of RNA or protein synthesis, or in the presence of 2 deoxy glucose, the levels of the membrane bound and external activities are modified in a way in which their relation is clear, while the soluble enzyme does not change at all. These results, together with the fact that the membrane bound and the external enzymes are glycoproteins, suggest a precursor-product relationship between the enzymic forms [Rodriguez *et al* 1978].

Intracellularly, the manna protein form of invertase is largely localized in vacuoles whereas the small isoenzyme is largely present in the soluble cell fraction. These findings indicate that vesicles are not involved in the secretion of invertase [Meyer and Matile 1975].

**1.6 Glycosyl /Glycoside hydrolase**:

Invertases are found in family GH32 of the sequence-based classification of glycoside hydrolases. This family which includes over 370 members from plant, fungal, and bacterial origin, contains not only invertases but also other fructofuranosidases such as inulinase (EC 3.2.1.7), levanase (EC 3.2.1.65), exo-inulinase (EC 3.2.1.80), and transfructosidases such as sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99) and fructan: fructan 1-fructosyltransferase (EC 2.4.1.100).

Enzymes with β-fructosidase activity are classified in two families, based on their amino acid sequences: one (glycosyl hydrolase family 32) including pro-and eukaryotic sucrases and fructanases, and the other include bacterial levansucrases. Naumov and Doroshenko in 1998 have compared the sequences of several representatives of the two families. Nine regions similar to previously known highly conserved regions of sucrases and fructanases have been found in levansucrases, indicating that the two families have evolved from a common precursor and should be grouped into a super family.

A structural model is presented for family 32 of the glycosyl-hydrolase enzymes based on the beta-propeller fold (Pons *et al* 1998). The analysis of the position of the active site residues in this model is compatible with the catalytic mechanism proposed by Reddy and Maley 1996, which includes three conserved residues Asp, Glu, and Cys. Based on this analysis, the authors propose the participation of one more conserved residue (Asp 163) in the catalytic mechanism.

**Table1.6**

**Early landmarks in Invertase research**

| Year | Contribution | Reference |
|---|---|---|
| 1860 | Invertase Purification | Berthelot |
| 1890 | Invertase a contribution to the history of an enzyme or unorganized ferment | O'Sullivan |
| 1910 | The inactivation of invertase by alkali | Hudson |
| 1913 | Kinetic studies of an enzyme and it's substrate conversion | Michaelis and Menten |
| 1916 | Invertase adsorbed to charcoal | Nelson and Griffin |
| 1924 | Computation of the acidity-pH curve of sucrase | Von Euler |
| 1952 | Polymeric genes for maltose, raffinose and sucrose fermentation in yeast | Winge and Roberts |
| 1954 | Correlation of bond breaking with enzyme specificity cleavage point of invertase | Koshland and Stein |
| 1961 | Production of Extracellular and total invertase by *C utilis, S cerevisiae*, and other yeasts | Dworschack and Wickerham |
| 1967 | Invertase isozymes | Gascon and Ottolenghi |
| 1968 | Purification of the Internal Invertase of Yeast | Gascon and Lampen |
| 1981 | Inversion of sugar | Geison |

**1.7 Release of Invertase from Whole Cells**:

Cell disruption is one of the most important steps in the downstream processing of intracellular enzymes from microorganisms. Physical methods are often favored due to operational and economical limitations of the chemical and enzymatic methods. The classic isolation procedure for bakers' yeast invertase involving plasmolysis of the yeast with hot toluene would destroy many enzymes. The invertase, at least in crude form, is extremely stable and can be stored for long periods, especially in 55% glycerol or 70 % sorbitol. The intracellular enzyme was easily released by use of cationic detergents such as alkyl dimethyl-benzyl ammonium chloride [Wiseman1978].

Invertase can be released by disruption in an industrial homogenizer [Follows *et al* 1971], cell disruption chamber of a bead mill [Melendres *et al* 1993] and electric pulses [Ganeva *et al* 2002]. Lin *et al* in 1991 has reported disintegration of yeast cells by pressurized CO2.

Mercaptoethanol (thio1) induced release of invertase has been demonstrated in *S fragilis* [Kidby and Davis 1970]. Sonication, high-pressure homogenization and hydrodynamic cavitation has been studied and compared. Hydrodynamic cavitation has been shown to be energy efficient than sonication and has been reported for large-scale disruption of cells [Balasundaram and Harrison 2006]. High osmotic shock using concentrated solutions of mannitol, EDTA, 2 mercaptoethanol and resuspension in a solution of $MgCl_2$ of low osmotic strength causes rapid and specific release of extracellular enzymes [Schwencke *et al* 1971]. Invertase release selectivity has been calculated by taking the ratio between the maximum concentrations of enzyme released to that of maximum concentration of total soluble proteins released.

**1.8 Screening Methods**:

The methods used for invertase activity determination are based on the measurement of glucose or reducing sugar produced by the enzymatic hydrolysis of sucrose into glucose and fructose. When whole yeast cells are used in these assays, the monosaccharides formed by the action of the periplasmic enzyme can be taken up and metabolized, leading to errors on the enzyme activity determination. Silveira *et al* in 1996 have reported a method for a more accurate invertase activity measurement by blocking the enolase enzyme (glycolytic pathway) using the inhibitor sodium fluoride. This *in vivo* method is particularly useful for cells with low invertase activity.

The determination of invertase activity of intact cells presents a critical point, that is, the blockage of the enzyme action at a given moment [Vitolo and Borzani 1983]. Sucrose hydrolysis can be stopped using 0.01M NaOH solution or 0.01M $Na_2CO_3$ or immersion of the mixture in boiling water bath or addition of Somogyi reagent followed by boiling. Most of the proposed methods correlate the invertase activity with the amount of reducing sugars produced under standardized conditions.

Invertase activity *in situ* can be located using nitro-blue tetrazolium NBT staining [Jeng *et al* 2006] and TPTZ [Yamanaka 1975].Goldstein and Lampen in1975 have developed a calorimetric method using glucose oxidase, peroxidase and O-dianisidine.

Spectrophotometric method for measuring fructose using tryptamine in hydrochloric acid was reported. The assay shows low interference from other sugars [Taylor and Kimberley 1995].

Invertase activity has also been determined using enzyme units termed 'invertons', which are based upon the initial rate of inversion of the sucrose substrate [Johnston *et al* 1935].

A rapid enzyme linked calorimetric assay for the determination of nanomole quantities of glucose and fructose was developed by Cairns in 1987 which utilizes the intermediary electron carrier, phenazine methosulfate to couple NADP reduction to the production of a formazan dye from the tetrazolium salt thiazolyl blue, in a form suitable for measurement using a microtiter plate reader.

Other methods of estimation of reducing sugar are described by Nelson in 1944, Somogyi in1952, Kidby in1973, and Miller in1959.

**1.9 Regulation**:

The synthesis of invertase in *A niger* was induced by substrates such as raffinose, sucrose, turanose and inulin. Glucose and fructose are repressors of the synthesis (Rubio 2006).The β link and the fructose located at the end of the molecule are involved in the induction mechanism.

Invertase production in *A niger* during solid state fermentation increased when glucose was present in the medium ($100gl^{-1}$) however, higher concentrations reduced enzyme production [Aranda *et al* 2006].

Invertase synthesis in *Schwanniomyces occidentalis* is regulated by catabolite repression and is derepressed by raffinose (0.5%) and low concentration of glucose (0.05%).The invertase activity in the culture medium of *S occidentalis* is 10 to 80 times

greater than that of *S cerevisiae*. No activity was detected in either strain when cells were grown in a medium containing 2% glucose [Costaglioli *et al* 1997]. Similarly invertase biosynthesis appeared regulated by catabolite repression, and induced by sucrose in Antarctica marine yeast *Leucosporidium antarcticum* [Turkiewicz *et al* 2005].

Mormeneo and Sentandreu in 1982 have studied the synthesis and secretion of external invertase under conditions of repression after incubation at higher temperatures than that of growth. They reported that invertase is continuously synthesized both in the presence and absence of glucose, but under repressible conditions is degraded before secretion takes place.

Elorza *et al* in 1977 suggested that the catabolite repression of invertase synthesis produced by glucose operates at the level of transcription and translation and produces an increase in the rate of mRNA degradation. The catabolite repression has no effect on secretion and does not interfere with the catalytic activity of invertase.

### 1.9.1 Enzyme Purification and Kinetic properties:

Invertases have been purified and characterized in several fungi/yeast/bacteria including *Neurospora crassa*, *Candida utilis*, *Fusarium oxysporum*, *Phytophthora meganosperma*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Schwanniomyces occidentalis*. The purified enzymes exhibit a great range of differences in characterization including molecular size, degree of glycosylation and subunit structure (**Table 1.7**)

The invertase from *Arthrobacter species* had a molecular weight of 52 kDa, pH optima of 6.7 and IEP of 4.3 [Fujita *et al* 1950].The enzymes from *Azotobacter chroococcum* had a molecular weight of 57 kDa and amino acid analysis revealed a high proportion of acidic residues [De la Vega *et al* 1991].

The enzyme from *Lactobacillus reuteri* is a glycoprotein composed of a single subunit of 58 kDa. The invertase activity was significantly inhibited by bivalent metal ions, β mercaptoethanol and dihiothreitol [De Gines *et al* 2000]. Intracellular invertase from *Streptococcus mutans* was purified to homogeneity by gel filtration and ion-exchange chromatography having a molecular weight of 48 kDa. Invertase was purified by ammonium sulphate fractionation and column chromatography on DEAE cellulose and Sephadex G 200.The molecular mass of the enzyme was 65 kDa. The optimum pH and temperature for activity were 2.6 and 50˚C respectively and $k_m$ was 3.5 [Kuramitsu 1973].

*Streptomyces* sp ALKC8 produced 350 units$l^{-1}$of extracellular invertase. The optimum temperature and pH for enzyme activity were 60˚C and 5.0 respectively [Kaur and Sharma 2005]. The enzyme from hyperthermophilic bacterium *Thermotoga maritima* was expressed in *E coli* and the recombinant enzyme displayed similar catalytic efficiencies for the hydrolysis of sucrose and inulin [Liebl *et al* 1998].

Bakers' yeast invertase is a glycoprotein where the sugar moiety is phosphorylated mannan. The mannan content is 50%.The high thermal stability of the enzyme would seem to be associated with the conformation achieved during biosynthesis, perhaps with the assistance from the mannan addition stage. Highly purified invertase from bakers yeast has pH optima of 4.5 to 5.5, isoelectric point pH 4, and $k_m$ for sucrose of 26mM [Wiseman 1978]. The acidic residues are in excess of basic residues. The mannan is attached to an asparagine in invertase by a dimer of N acetylglucoseamine, probably through a glycosylaminyl-aspargine bond. 18-19 mannan chains (of varying length) are attached to the protein at different points. Salt linkages may be important in maintaining the conformational stability of invertase. Modification of either amino or carboxyl groups chemically or by immobilization onto charged supports, resulted in the loss of conformational stability, unless these stabilizing salt linkages were preserved [Woodward and Wiseman1978].

Bakers' yeast invertase was found to catalyze transfructosylation reactions in aqueous and anhydrous organic media with sucrose as a substrate, leading to the formation of five intermediate products in addition to the release of D glucose and D fructose. The products were identified as 1-kestose, fructofuranosyl glucose, inulobiose, 6 kestose and neokestose.

In the presence of anhydrous toluene and 1.46 M sucrose and 1000U of invertase as suspended powder, invertase was found to exhibit cyclic behavior, where sucrose was degraded and subsequently synthesized [Farine 2001].

**Table 1.7: Characterization of Invertase from different yeast**

| Organism | Molecular Weight | $K_m$ for sucrose | Glycosylation | Method of purification | Specific activity units/mg | Reference |
|---|---|---|---|---|---|---|
| Candida utilis | 60 kDa<br><br>300 kDa | 1.54 mM<br><br>2 mM | Non gly – F form<br><br>gly – S form | Ion exchange, affinity chromatography, Preparative column electrophoresis | 1496.8<br><br>2560 | Belcarz *et al* 2002 |
| Pichia anomala | 254 kDa Multimeric Subunit 86.5 kDa | 16.0 mM | gly 30 % | DEAE sephacel chromatogaphy | 1482 | Rodriguiz *et al* 1995 |
| *Schizosaccharomyces pombe* | 205 kDa 5-6 subunits 60kDa | ----- | gly- 67% | DEAE Sephadex Bio-Gel-A | 1590 | Moreno *et al* 1990 |
| *Rhodotorula glutinis* | 100 kDa Homodimer 47kDa | 227.0 mM | gly 19 % | Sephadex G-150,DEAE-Sephacell | 0.012 | Rubio *et al* 2002 |
| *Saccharomyces cerevisiae* | 135 kDa<br><br>270 kDa | 25mM<br><br>26mM | nongly<br><br>gly 50% | Diethyl aminoethyl sephadex | 2900 | Rodriguez *et al* 1978 |
| *Schwanniomyces occidentalis* | 125 kDa dimer | 20.0 mM | gly 17 % | Superose gel filtration Mono Q anion exchange chromatography | 698 | Klein *et al* 1989 |

### 1.9.2 Strain Improvement:

Several hyperproducing strains with desirable characteristics have been claimed in patents/papers although details of the performances of industrial strains are not available. The main focus and objectives of strain improvement programmes have been directed towards induced mutagenesis and recombinant DNA techniques.

Conidia of the selected strain of *Aspergillus fumigatus* were subjected to mutagenesis with both UV and NTG (N- methyl N'nitro-N-nitrosoguanidine) and the products were analyzed for invertase activity. A mutant M-7 showed intra and extracellular invertase activities to have increased about 2-fold compared with the parental strain [Fiedurek *et al* 2000]

### 1.9.3 Recombinant Invertase:

The SUC2 gene from *Saccharomyces cerevisiae* coding for the enzyme invertase was cloned in *Hansenula polymorpha* under the control of the alcohol-oxidase (AOX) promoter of *Pichia pastoris*. More than $1.5X10^4$U/ml of invertase were found to be secreted to cellular periplasmic membrane [Narciandi *et al* 1995].Recombinant *S cerevisiae* harboring plasmid pRB58 with the SUC2 gene were encapsulated in liquid-core alginate capsules.95% conversion at a productivity of $48gl^{-1}$ per hour was obtained using 0.5M sucrose solution [Chang *et al* 2000].

Invertase expressed in the methylotrophic yeast *Hansenula polymorpha* and *Pichia pastoris* has been studied by Acosta *et al* in 2000 in terms of enzyme conformational stability and structural behavior. The enzyme produced in both host was very stable over a broad range of pH values. Nicaud *et al* in 1989 have shown the expression of invertase activity in *Yarrowia lipolytica* and has been used as a selective marker.

Modeling and optimization of cloned invertase expression has been reported in *Saccharomyces cerevisiae* by Patkar *et al* in 1972.

Fed batch cultures of recombinant microorganisms have attracted attention as they can separate cell growth stage from cloned-gene expression phase during fermentations. The SUC2 gene (cloned on plasmid pRB58) expression was derepressed at glucose concentrations below $2gl^{-1}$ [Patkar and Seo 1992].

### 1.10 Applied Aspects

#### 1.10.1 Industrial Applications:

Analytical Probes/ Biosensors

- Determination of sucrose in serum and urine.
  The method entailed the coupling of invertase catalyzed sucrose hydrolysis with a fructose dehydrogenase catalyzed oxidation of the liberated fructose. Fructose was estimated calorimetrically using a tetrazolium salt [Holmes 1997].
- Screening of fish tissue for methyl mercury.
  Methyl mercury reacts with the thiol groups of invertase and the resulting inhibition of enzymatic activity serves as a measure of methyl mercury concentration. Concentrations as low as 10 ppb of methyl mercury were detected [Mohammadi *et al* 2005].
- Phenyl mercury in pharmaceutical samples
  Copper modified gold electrodes have been used for determination of phenyl mercury in pharmaceutical samples [Mohammadi et *al* 2004].
- Determination of glucose and sucrose in fruit juice using flow injection analysis [Guemas *et al* 2000].
- Hybrid Biosensors for determination of sucrose.
  Biocatalytic layer was prepared by coimmobilizing yeast cell walls and glucose oxidase on a nylon network via glutaraldehyde and was fixed to the Clark oxygen electrode [Barlikova *et al* 1991]. Co-immobilization of glucose oxidase and invertase led to the development of amperometric sucrose sensor.
- Glucose in molasses
  A technique for the determination of glucose in molasses based alcoholic fermentation broths using an enzyme electrode was assessed. Electrochemical interference produced during the alcoholic fermentation was measured as glucose [Garcia *et al* 1993].

Production of alcohol**:**

A thermotolerant strain of yeast was developed by UV treatment to produce 19.0 g ethanol per g of cells. The improved ethanol productivity was directly correlated with titers of intracellular and extracellular invertase activities [Rajoka *et al* 2005].

Continuous alcoholic fermentation of sucrose using flocculating yeast has been studied by Fontana et al in 1992.The hydrolysis rate is imposed by diffusion limitations in the biomass particles. Nevertheless ethanol productivities as high as 68g/L can be reached without biomass retention problems.

Ethanol fermentation broth produced by an aggregated form of *S uvarum* strain contained invertase when sucrose based raw materials was used. 75% recovery yield of invertase with 9-fold purification and 30-fold concentration could be achieved [Chen *et al* 1994].

Production of ethanol and invertase by *S cerevisiae* grown in Blackstrap molasses was reported by Vitolo in 1996. Ethanol production using fed-batch culture of free and immobilized cells of *S cerevisiae* from beet molasses was reported by Roukas in1996.

Takeshige *et al* in1995 studied the factors affecting ethanol productivity on yeast in molasses and also the effect of yeast invertase on ethanol production.

Ethanol production efficiency was increased by 50% with supplementation of additives to high gravity cane molasses [Bajaj *et al* 2005].

Invert Syrup:

Invert sugar is sweeter than sucrose due to the high degree of sweetness of fructose, which is 1.2 to 1.8 times sweeter than sucrose. It has wide applications in the food and pharmaceutical industries because of its functionally more desirable properties like high solubility and hygroscopic nature. It is a natural preservative, having a long shelf life. It caramelizes at lower temperature, thereby reducing the baking temperature and giving a rapid crust color. It assimilates faster in the stomach than ordinary sugar and maintains the calcium balance in the body. Due to its hygroscopic nature it is used as humectants in the manufacture of chocolate-coated soft centered sweeteners, candy products, fondants and after dinner mints. Its use in confectionary ensures that the product remains fresh and soft even when kept for a long time. It is also used in the production of non-crystallizing ice-creams, condensed milk, infant foods, jams, jellies and as a substitute for honey in the production of sugar syrup in pharmaceutical industry. The low viscosity characteristic of invert sugar helps reduce the viscosity of fondant creams. It is also used as honeybee feed (Macha et *al* 1988).Honey bees fed with invert solution had 30% longer life spans than honeybees fed with sucrose.

High-fructose syrups (HFS) comprise fructose, dextrose, and minor amounts of oligosaccharides such as 1-kestose, nystose, and fructosyl nystose. The predominant

syrups contain 42% and 55% fructose. HFS production was made possible by concurrent developments in refining, isomerization, and separation technologies in the 1960s. Fructose contributes many useful physical and functional attributes to food and beverage applications, including sweetness, flavor enhancement, humectancy, color and flavor development, freezing- point depression, osmotic stability etc. HFS is used extensively in carbonated beverages, baked goods, canned fruits, jams and jellies, and dairy products. D Fructose is a low calorie, noncariogenic sweetener which is well tolerated by diabetics, improves iron absorption in children and favors the removal of ethanol from the blood of alcoholics[Pessoaa and Vitolo 1999].The importance of fructose and fructose oligosaccharides in human nutrition has increased significantly and gained tremendous commercial importance owing to their favorable functionalities such as improving the intestinal microflora, relieving constipation, decreasing total cholesterol and lipid in serum, enhancing local and systemic immune response[Gill *et al* 2004]

Production of lactic acid:

Canned pineapple syrup, a food processing waste was utilized as a substrate for lactic acid production by *Lactococcus lactis* along with grape invertase. The highest lactic acid concentrations achieved were 20 and 90 $gL^{-1}$ from 20 to 100g total sugars $1^{-1}$ respectively without a lag period for sucrose consumption [Ueno *et al* 2003].

Another report [Aksu and Kutsal 1986] gave a product yield of 83% when molasses was used as the substrate along with the enzyme invertase and does not cause any appreciable increase in the production cost when compared with other possible substrates.

Production of glycerol:

Glucose repression and the osmotic stress response system regulate glycerol-3-phosphate dehydrogenase synthesis independently. Specific control mechanisms sense the osmotic situation of the cell and induce responses such as the production and retention of glycerol [Albertyn *et al* 1994].

High fermentable sugar concentrations cause physiological conditions that allow for enhanced glycerol production and retention, the degree of which is strain dependant. (Myers *et al* 1997)

Nevoigt and Stahl in 1998 have investigated the factors affecting the production of glycerol in *S cerevisiae*. The substantial increase in glycerol yield was associated with a

reduction in ethanol yield and a higher by-product formation. Reduced pyruvate decarboxylase and increased glycerol-phosphate dehydrogenase {$NAD^+$} levels enhance glycerol production in *S cerevisiae*.

**1.10.2: Other Applications:**

Honey freshness:

Invertase activity is a good parameter for evaluating honey freshness. All honeys were stored in darkness at room temperature for up to 24 months and analyzed each 6 months so as to determine the invertase activity evolution tendency [Sanchez *et al* 2001].

Hydroxymethylfurfurale (HMF) diastase and invertase are used as a measure of honey freshness [Oddo et *al* 1999]. Invertase is used in the preparation of artificial honey.

Invertase as anchor protein:

B lactamase a secretory protein in *E. coli* is efficiently secreted in *S. cerevisiae* under the control of the invertase signal sequence [Bielefeld and Hollenberg 1992]. The presence of invertase signal peptide led to B-lactamase secretion up to 75%. Localization of target proteins in yeast periplasm using invertase has been reported. C or N terminal of the target protein was fused to the invertase and the fusion protein expressed under the control of constitutive glyceraldehyde dehydrogenase promoter [Tanino 2004].

Nicaud *et al* in 1989 constructed a gene fusion where the *Saccharomyces cerevisiae* SUC2 gene was placed under the control of the promoter and signal sequence of the *Yarrowia lipolytica* XPR2 gene, which encodes alkaline extracellular protease. Strains bearing this fusion express invertase activity and grow on sucrose as the carbon source. The chimeric gene could be used as a dominant marker for transformation in a one step procedure.

Traditional Medicine:

Woodfordia fructicosa flowers had an impact on the immunomodulatory activity, alcohol and sugar content of the ayurvedic drug 'Nimba arishta'. An invertase activity exhibited by the flowers may be causative of this effect [Kroes *et al* 1993].

Immunological detection:

β- fructofuranosidase activity was shown to be stimulated in grape berries after infection with Botrytis cinerea. The importance of careful immunogen preparation in the

production of specific antibodies and the potential for the immunological detection of B cinerea was discussed by Ruiz and Ruffner in 2002.

Protection of wine from Haze:
Yeast invertase, a mannoprotein with haze protective activity, was used as a model substrate to investigate the mechanism of haze protection. Invertase was found to reduce the turbidity, due to (HPM) the haze protective mannoprotein material [Isabelle *et al* 2000].

Digestive tablets:
Omegazyme tablets/powder (Garden of Life Products) is a digestive blend comprising of twenty digestive enzymes (including invertase) which aids in digestion of proteins, fats, carbohydrates. Invertase helps to prevent gastrointestinal problems, flatulence and discomfort.

### 1.10.3: Immobilization

In industry, the bulk sucrose hydrolysis for obtaining the invert sugar syrup is carried out at 75˚C in the presence of HCl. However, the product attained has several types of by-products(derived from glucose and fructose cyclization) that must be removed otherwise, it is unsuitable either as a sweetener or as a raw material for producing more valuable commodities such as fructose and gluconic acid. The use of invertase constitutes an alternative to acid hydrolysis, because the enzyme acts under mild pH (4.0-5.0) and temperature (35-50˚C) conditions.
The immobilization of invertase in an inert matrix is a fundamental approach. However, the immobilization procedure must not be hazardous to the enzyme, and the support preferably a food-grade product. Inspite of the fact that invertase has been immobilized on a great variety of supports by different immobilization methods, only a few fulfill the requirements appointed. Among these methods, hydrogel entrapment is cheap and easy to manufacture and handle.
The newer technological developments in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploitation of biocatalysts in industry, waste treatment, medicine, and in the development of bioprocess monitoring devices like the biosensor.

Immobilization thus allows by essence, to decouple the enzyme location from the flow of the liquid carrying the reagents and products. Immobilization helps in the development of continuous processes allowing more economic organization of the operations, automation and decrease of labor. Immobilized biocatalysts offer several other advantages; notable among them is the availability of the product in greater purity. The other major advantages include greater control over enzymatic reactions as well as high volumetric productivity with lower residence time.

Permeabilized cells often referred to as non viable cells can be exploited in an immobilized form as a very economical source of intracellular enzyme.

An advantage of using protoplasts is that the products are released freely into the broth with the double consequences of increasing overall productivity and facilitating downstream processing. High molecular weight substances which are otherwise not accessible to the cells can directly access the cell membrane in protoplasts. When *Saccharomyces cerevisiae* protoplasts were provided with Strontium alginate gel as an artificial cell wall, invertase was secreted in the broth [Aoyagi and Tanaka 1999]

Immobilized cell technologies have widely developed since early 1980. A large number of techniques and support are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application.

The industrial importance of invert syrup/alcohol has led to intensive efforts to develop immobilized systems for invertase and whole cell immobilization. The main objectives have been to achieve high immobilization efficiencies with retention of activity and to enhance catalyst half life of the immobilized system through improvements in enzyme stability and reduction of losses through leakage. Historically, invertase is perhaps the first reported enzyme in an immobilized form.

Different immobilization techniques are used to maintain cells and their viability, in particular their use in the development of high-throughput and chip based systems. Whole cell based biosensors find application in different environmental media, such as water, soil and atmospheric monitoring (Gu *et al* 2004).

Silica sol gel were used for entrapping invertase and glucose dehydrogenase to develop a process for the enzymatic production of hydrogen from environmental carbohydrate sources[O'Neil *et al* 2002].The feasibility of using eggshells, a waste product of the

poultry industry and zeolites as carriers for immobilization of invertase has been examined by Sunitha and Sai Prakash in 1994.

Invertase from *S cerevisiae* has been immobilized by ionic adsorption on polyethylene coated Sepabeads [Torres 2003]. Invertase has been immobilized on natural supports, polyethleneimine coated rice husk [D'Souza and Godbole 2002], waste cotton thread [Godbole *et al* 1990] and glutaraldehyde treated wool [Krastanov 1997]. Jafri and Saleemuddin in 1977 constructed specific immunoaffinity supports for the immobilization of invertase by coupling the affinity purified glycoprotein antibodies to cyanogen bromide activated sepharose.

**Table 1.8 and Table 1.9** shows different matrices and the method used for immobilization of invertase active whole cells and invertase.

**Table 1.8**

**Immobilization of Invertase active whole cells**

| | Matrix | Method | Reference |
|---|---|---|---|
| 1 | Jute fabric using polyethylene imine | adhesion | D'souza *et al* 2001 |
| 2 | Gelatin hydrogels | Cross linking with chromium salts | Singur *et al* 2006 |
| 3 | Nylon treated with glutaraldehyde | adsorption | EL – Refai H *et al* 2004 |
| 4 | Acryl amide | entrapment | D'Souza *et al* 1980 |
| 5 | Polyethyleneimine and glutaraldehyde | Covalent bonding | Hasal *et al* 1992 |
| 6 | Alginate | entrapment | Johansen, Flink 1986 |
| 7 | SiO2 gel deposited on glass sheets | Sol gel | Carturan *et al* 1989 |
| 8 | Grape skins | adsorption | Mallouchos *et al* 2002 |
| 9 | Natural Zeolite | adsorption | Shindo *et al* 2001 |
| 10 | Hydroxyethyl methaacrylate gels | entrapment | Cantarella *et al* 1984 |
| 11 | Strontium alginate | protoplasts | Tanaka *et al* 2000 |

**Table 1.9**

**Matrices used recently for Immobilization of Invertase**

| MATRIX | METHOD | REFERENCE |
|---|---|---|
| Celite and polyacrylamide | adsorption | Mansour and Dawood 2003 |
| Strontium barium alginates | entrapment | Meena and Raja 2004 |
| Montmorillonite | adsorption/covalent | Sanjay and Sugunan 2005 |
| Polypyrrole | electrochemical | Isik *et al* 2003 |
| Sepiolite | adsorption | Prodanovic *et al* 2003 |
| Copolymer electrodes | entrapment | Yildiz *et al* 2005 |
| Activated cellulose membrane | covalent | Bora *et al* 2005 |
| Dowex anion exchange resins | adsorption | Tomotani and Vitolo 2004 |
| Granulated carbon containing supports of sapropel | adsorption on yeast membrane | Kovalenko *et al* 2005 |
| Concanavalin A immobilized affinity adsorbents | adsorption | Handan *et al* 2004 |
| Vinyl imidazole carrying metal-chelated beads | adsorption | Bilgen *et al* 2005 |
| Dimer acid-co-alkyl polyamine | covalent | Tumturk and Tufan 2004 |
| Acrylamide/ maleic acid hydrogels | adsorption | Arslan *et al* 2000 |
| Methacrylate | covalent | Bayramoglu *et al* 2003 |
| Concanavalin PCMS beads | affinity | Bahar and Tunkal 2004 |
| Carbon containing ceramic macrostructured carriers | adsorption | Kovalenko *et al* 2003 |
| Chitosan | covalent | Chang *et al* 2005 |
| Composite gel fiber by Sol gel process | entrapment | Nakane *et al* 2000 |
| Lectin | Adsorption | Ahmed *et al* 2001 |

### 1.11 Glycosidase Inhibitors:

The significant role that carbohydrates play in a variety of biological processes of pharmaceutical relevance has stimulated the interest in compounds that could interfere in carbohydrate metabolism and in carbohydrate based recognition phenomena. In this context, great efforts have been devoted recently to the synthesis of glycomimetics, such as imino sugars and C-glycosides that can act as inhibitors of carbohydrate processing enzymes and/or as stable analogues of glycosidic entities. The chemotherapeutic potential of sugar-mimic glycosidase inhibitors as anti diabetic [Watson *et al* 2001], anti-cancer [Barchi 2000], anti-viral agents [Elbein 1991] has been recognized and has stimulated the demand for more compounds. The clinical applications of several such compounds have been reported [Zou 2005].

Inhibition of these enzymes affords various opportunities to manipulate the rates of glycosidase reactions, either for enzymology studies or for therapeutic purposes. C-linked oligosaccharides are now in demand for studying sugar metabolism as they act as enzyme inhibitors by virtue of their resistance to chemical and enzymatic hydrolysis of the glycosidic linkage and their ability to interact with protein receptors similar to their O linked counterparts. These structures are not only found in natural products but can serve as interesting chiral building blocks for the construction of complex polycyclic or acyclic molecules.

D-glucose and insulin levels of plasma are usually high in diabetics especially after food ingestion, and reducing intestinal carbohydrate absorption, such as monosaccharides which are hydrolyzed by glycosidase is one way to control disorders of carbohydrate metabolism

**Table1.10**

**Glycosidase Inhibitors as Therapeutics**

| Inhibitor | Glycosidase | Treatment |
|---|---|---|
| Acarbose | α glucosidase, amylase | Diabetes-Type II |
| Pepstatin A | xylanase | Allergies |
| Myglitol | α glucosidase | Diabetes- Type II |
| N butyl nojirimycin (Miglustat) | glucosidase I,II, glucosyl transferase | Gaucher's disease |
| Rolenza | sialidase | Influenza |
| Salacinol | α amylase | Anti diabetic |
| Voglibiose | pig intestinal sucrase, maltase | Anti diabetic |

The present study dealt with the evaluation of the activity of β fructofuranosidase in the presence of the synthesized pseudo-C-disaccharides or C (4)-C (5) linked sugars shown in CHAPTER 4 (Fig 4.1)

The THESIS embodies work on a thermotolerant yeast strain *Kluyveromyces marxianus* NCYC 2675.The investigations include optimization of fermentation parameters for production of the enzyme, its purification and characterization, inhibitor studies using sucrose analogues and immobilization using different matrices for enzyme and whole cells.

# CHAPTER 2

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# ISOLATION, CHARACTERIZATION AND OPTIMIZATION OF FERMENTATION PARAMETERS

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## 2.1 SUMMARY

Several isolates of yeasts and fungi from soil, ripened fruits, and high sugar sources like jaggery and standard type cultures of microorganisms were screened for invertase activity in three sequential stages. The initial stage comprised screening by 2:3:5-triphenyltetrazolium chloride (TPTZ) technique, which forms a red complex with liberated fructose. The second stage comprised assay of reducing sugar produced by whole cells on cleavage of sucrose and the third stage comprised the spectrophotometric assay of reducing sugar from sucrose.

On second stage screening, the following cultures were found to produce whole cell activity towards invertase:

*S cerevisiae* NCIM 3209- NCYC 93, *Saccharomyces uvarum* NCIM 3332–PRL 186, *Kluyveromyces marxianus* NCIM 3217-IFO 0288, *Kluyveromyces marxianus* NCIM 3231 NCYC 111, *Kluyveromyces marxianus* NCIM 3232 NCYC 243 and our isolate *Kluyveromyces marxianus* NCYC 2675. Out of these cultures, *K marxianus* NCYC 2675 was shown to have high levels of both intracellular invertase and inulinase activity when grown in the presence of sucrose as an inducer.

Extracts of homogenized cells of *K marxianus* NCYC 2675 showed similar levels of both invertase as well as inulinase activity when grown in the presence of sucrose as an inducer (S/I = 1.3). On the basis of these results *K marxianus* NCYC 2675 was used as the source material for the isolation and characterization of the enzyme. The effect of different cultural conditions like pH, temperature, inoculum size, dispensing volume, and rate of invertase production were investigated under shake flask conditions. The effect of sucrose concentration, metal ions, organic and inorganic nitrogen on invertase production was investigated. For cost effective/and accelerated production of this enzyme/invert syrup, the effect of cheap carbon sources and synthetic zeolites as additives were studied. The increase in enzyme yield obtained after optimization was approximately 45%.

## 2.2 INTRODUCTION

As described in the GENERAL INTRODUCTION several bacteria, yeast and fungi have been shown to produce invertase activity. A preliminary survey was made in the present studies to identify a thermotolerant strain producing high levels of enzyme activity. Isolates and standard type cultures were screened preliminarily for invertase production before attempting the isolation and characterization of its kinetic and molecular properties. Also the effects of different growth and cultural conditions were optimized for invertase production.

We report here the production of intracellular invertase (E C 3.2.1.26) from our isolate *K marxianus* NCYC (National Collection of Yeast Cultures) 2675. The yeast strain was isolated from over ripe grapes identified and deposited to NCYC, USA. There are many reports of production of inulinase (EC 3.2.1.7) from strains of *K marxianus* [Bazaraa et *al* 1999, Pessoa *et al* 1999, and Guerrero *et al* 2006]; however there are scanty reports of production of intracellular invertase from this yeast species hitherto [Zherebtsov 2003]. Earlier both inulin and sucrose hydrolyzing activities have been detected in culture supernatants of *Kluyveromyces marxianus* var *marxianus* [Rouwenhorst *et al* 1990].Optimization of inulinase from K*marxianus* NRRL y- 7571 has been reported [Bender *et al* 2006]. Our yeast shows similar levels of intracellular inulinase as well as invertase activity when grown in the presence of 2g% sucrose. Further this yeast shows thermotolerance, which is of advantage as regards the commercial importance. Since there are no reports of intracellular invertase production from *K marxianus* NCYC 2675 we decided to optimize fermentation parameters for invertase production. The effect of different cultural conditions like pH, temperature, inoculum size, dispensing volume, and rate of invertase production were investigated under shake flask conditions. An appropriate incubation period is of critical importance for invertase synthesis, as longer incubation can cause feedback repression of the enzyme [Vrabel *et al* 1997; Gomez *et al* 2000]. The effect of sucrose concentration, metal ions, organic and inorganic nitrogen on invertase production was investigated. Cheap and renewable carbon sources and synthetic zeolites as additives were also studied to see their effect on invertase activity. Since the enzyme is produced intracellularly different permeabilization agents like CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide), toluene which facilitate release of periplasmic invertase were used. The increase in enzyme yield obtained after optimization was 45%.

## 2.3 MATERIALS AND METHODS:

### 2.3.1 Materials:

Yeast extract, peptone, malt extract, inulin were obtained from HIMEDIA India. 3,5-dinitrosalicylic acid and sucrose were obtained from MERCK India. TPTZ (triphenyl tetrazolium chloride) was obtained from BDH Chemicals UK. All other chemicals were commercially available high purity or analytical grade compounds. All media were prepared in distilled water and buffers in glass distilled water. Synthetic zeolites ZE1-ZE10 were prepared by Division of Catalysis, NCL, Pune 411008.

### 2.3.2 Microorganisms:

The cultures *Saccharomyces cerevisiae* NCYC 93, *Saccharomyces uvarum* PRL186, *Kluyveromyces marxianus* IFO 0288, *Klyuveromyces marxianus* NCYC 111, and *Kluyveromyces marxianus* NCYC 243 were obtained from NCIM (National Collection of Industrial Microorganisms) NCL, Pune. *Kluyveromyces marxianus* NCYC 2675 was isolated from over ripe grapes (out of 7-8 different yeast) by selective pressure method and sent to NCYC, USA for culture identification and species typing.

### 2.3.3 Methods:

#### 2.3.3.1 Preparation of DNSA reagent:

5g of 3,5 - dinitrosalycylic acid was added slowly and dissolved in approximately 100 ml of water. 8.0g of NaOH dissolved in 75.oml of water was added drop wise to the above reagent under continuous stirring, and if necessary gently heated on water bath until clear solution is obtained.150.0g of sodium potassium tartarate (mixed in water) was added in small portions. Water was then added to a final volume of 500 ml. The solution was filtered through Whatman filter no 1 and stored at room temperature in a brown bottle in the dark.

#### 2.3.3.2 Method of Isolation

High sucrose content sources (ripe mango, dates, banana, over ripe grapes, and jaggery) were chosen for the source of the organisms and a selective pressure of high sucrose was given while isolating the same. The following media were used for isolation.

a) Medium A: MSYP [$gl^{-1}$] peptone, 5; yeast extract, 3; malt extract, 3; sucrose, 50; agar, 20; pH 6.0.

b) Medium B: Czapek Dox [$gl^{-1}$] glucose, 20; $KH_2PO_4$, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.05; $NaNO_3$, 1; agar, 20; containing 5g% sucrose; pH 6.0.

c) Medium C: Jaggery medium [$gl^{-1}$] yeast extract, 20; Jaggery, 50; agar, 20; pH 6.0.

d) Medium D: PDA [$gl^{-1}$] potatoes, 200; dextrose, 20; yeast extract, 0.1; agar, 20; containing 5g% sucrose; pH 4.5.

For isolation, the over ripe grape was carefully crushed in laminar flow and 1g of pulp was suspended in sterile, distilled water (2ml), thoroughly mixed and left to settle. A loopful of the supernatant was streaked on solid medium in petri plates. The petri plate was incubated at 30ºC, 40ºC and 50ºC for 48 hours. Isolated colonies of microorganisms obtained on petri plate growing at high temperature were picked up, sub cultured on slants, and these isolates were then screened individually for invertase production.

**2.3.3.3 Screening of Microorganisms**

Screening was performed in four successive stages.

**Stage 1:**

*Activity Staining*: The preliminary screening of samples was done by TPTZ overlay technique according to the method described by Yamanaka in 1975. In a typical procedure, the isolates were spot inoculated in specific sectors of the basal medium plate containing 2 -5g% sucrose. After incubation for 48 hours at 40ºC and 50ºC the plates were overlaid with 0.1%(w/v) solution of 2:3:5-triphenyltetrazolium chloride in 1N NaOH at room temperature in the dark for 1 -2 min. TPTZ reduction with ketose occurs at alkaline pH to form a water-insoluble deep red pigment, triphenyl formazan. The diameter of the red zone was used as an approximate measure of activity. Washing the plate with 1N HCl followed by distilled water can prevent the darkening of the background. This was used as a primary screen for the isolates used from various sources.

**Stage 2:**

*Cell bound activity- qualitative test*

Cultures that showed significant red zones were grown in liquid MSYP medium.12 ml of liquid medium was dispensed in a boiling tube, a loopful of the isolate was inoculated in the sterile medium respectively and was incubated for 24 hours at 40ºC on rotary shaker at 160 rpm. 4 ml of sample was withdrawn and microfuged for 3 minute at 5000 rpm. The pellet, containing approximately 20 mg of cells was washed

with acetate buffer 50 mM pH 4.5, weighed, and whole cell activity was estimated as described by Gascon and Lampen [1968]. Reducing sugar released by hydrolysis was measured as described by Miller [1959] using known concentration of glucose as standard. The typical procedure was as follows. 2g% sucrose in 500µl acetate buffer (50mM) pH 4.5 was added to the cell pellet and mixed. The slurry was incubated at 50?C for 10 minutes in a water-bath. 500 µl of 0.2M $K_2HPO_4$ was added and thoroughly mixed to quench the reaction. The mixture was centrifuged for 2 minute at 5000 rpm. 500 µl of the supernatant was added to 500 µl of distilled water. 1.0 ml of DNSA reagent was added and the mixture boiled for 10 minutes in a boiling water-bath. 10 ml of distilled water was added to each tube. The invertase producers are assessed on the visual gradation of the intensity (0 to ++++) of the red color formed. Cells without sucrose served as the control and buffer without cells served as the DNSA Blank.

**Stage 3:**

*Extracellular activity-Qualitative test*

The supernatant broth from **Stage 2** was used to determine the invertase activity. 200 µl of sucrose (2g%) was added to 500 µl of the broth and the mixture was incubated at 50ºC for 10 minutes in a water bath. 500 µl of 0.2M $K_2HPO_4$ was added and mixed thoroughly to quench the reaction. 500µl of this reaction mixture was added to 500 µl of distilled water. 1.0 ml of DNSA reagent was added and the mixture boiled for 10 minutes in a boiling water-bath.10 ml of distilled water was added to each tube. The invertase producers are assessed on the visual gradation of the intensity (0 to ++++) of the red color formed. Blank and Control tubes were similar to those in **Stage 2.**

**Stage 4:**

*Cell bound activity-Quantitative test*

The cultures that showed high (++++) activity were assayed for production of invertase.

The inoculum was developed in the boiling tube containing MSYP liquid medium by transferring a loopful of the isolate to be tested. The tubes were then incubated at 40ºC for 24 hours on a rotary shaker at 160 rpm. The inoculum was transferred to 250 ml flask containing 50 ml medium and incubated for 24-48 hours at 40ºC on rotary shaker. Cells were harvested by centrifugation using Kubota 7780 refrigerated centrifuge at 5000 rpm, 10ºC for 10 minutes. The cells were washed twice with acetate buffer (50 mM) pH 4.5. 0.1g of packed cells were suspended in acetate buffer and invertase activity per gram of cells determined as in **stage 2** after

measuring the absorbance at 540 nm. Blank and Control tubes were similar to those in **Stage 2**.

**Stage 5:**

*Activity of cell free extracts:*

Cells of cultures that showed high invertase activities in **stage 4** were disrupted by sonication. 5.0 g cells were suspended in 10 ml of 50 mM acetate buffer pH 4.5 in standard homogenization glass bottles. Approximately 20 g of glass beads were added and the mixture kept on ice. Sonication was performed using Brauns' homogenizer with 8 cycles (1 minute intervals) each of 15 seconds. Homogenized cells were checked microscopically to determine the complete lysis. Cell debris was removed by centrifugation at 4000 rpm for 20 minutes at 4ºC. Aliquots of clear supernatant were assayed for invertase activity.

One unit of enzyme is defined as the amount of enzyme required to liberate 1 µmole of reducing sugar/min/ml under the assay conditions.

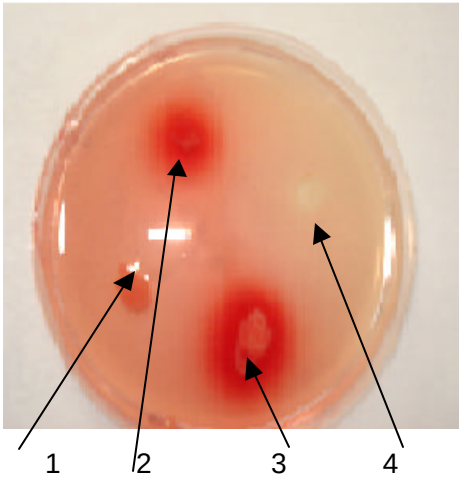
Protein was determined by the procedure of Lowry *et al* [1951] using bovine serum albumin as the standard and the specific activity (units/mg) calculated.

*Inulinase cell assay:* The assay is similar to the above assay except that substrate inulin (5mgm %) was used.

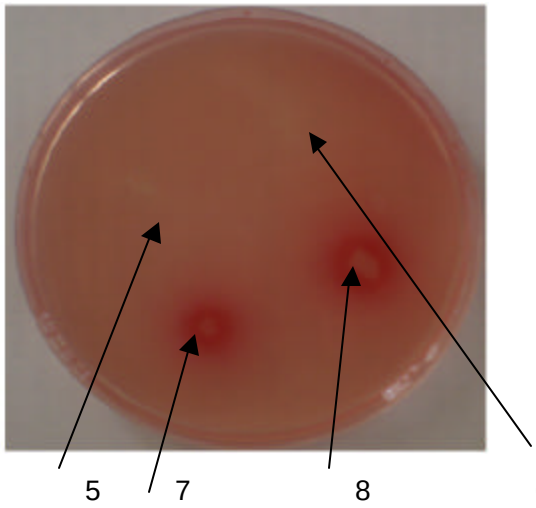
One unit of enzyme is defined as the amount of enzyme catalyzing the hydrolysis of 1 µmole of inulin/min/g under the assay conditions.

**Photographs**

**2.1 Activity staining of isolates**




**2.3 Growth of yeast cells on 5% MSYP medium**

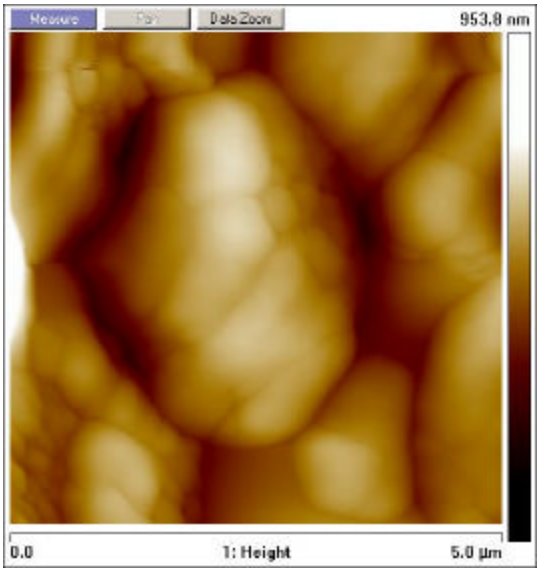


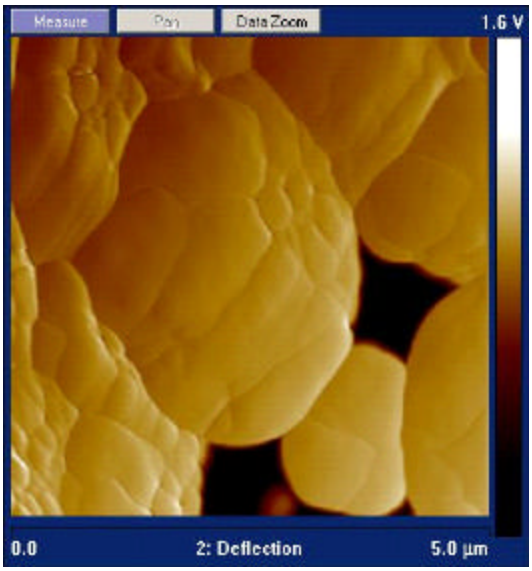

**2.2 Activity staining of isolates**

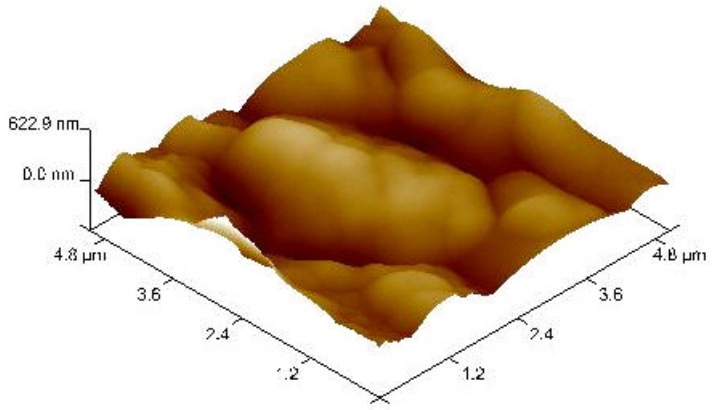
1. *K marxianus* NCIM 3232
2. MA – 2 Isolate
3. *K marxianus* NCYC 2675
4. *Candida bonbicola*
5. *Candida rugosa*
6. *Rhodotorula aurantica*
7. *Saccharomyces uvarum* NCIM 3332
8. *Saccharomyces cerevisiae* NCIM 3209

**Photograph 2.4**

**AFM images of *Kluyveromyces marxianus* NCYC 2675**

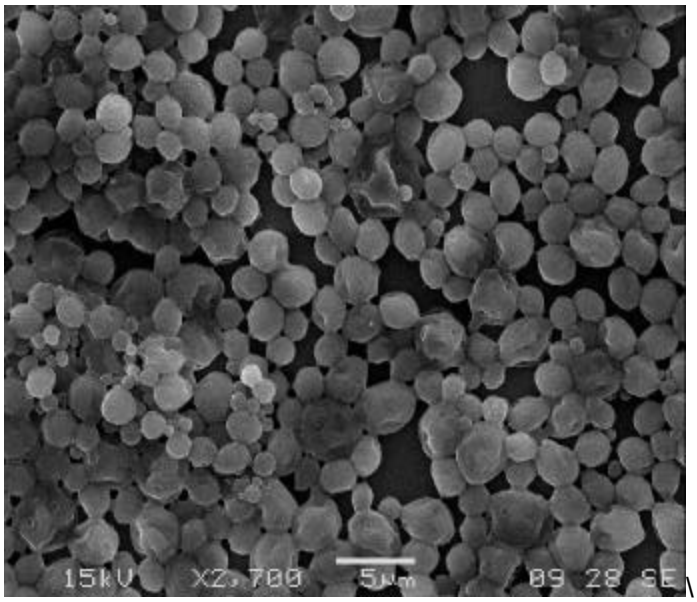


**Photograph 2.5**

**SEM Images of *Kluyveromyces marxianus* NCYC 2675**




**2.3.3.4 Maintenance:**

The yeasts with measurable invertase activity were routinely maintained on MSYP slants/plates containing 2g% sucrose at 4?C by periodic transfers, and also in glycerol at -20 °C.

**2.3.3.5 Inoculum preparation:**

Cell suspension was prepared from 2 to 3 days old slant culture of *K marxianus* NCYC 2675.The culture was precultivated in 50 ml MSYP liquid medium at 40ºC and shaken at 160 rpm for 24hours.

**2.3.3.6 Fermentation:**

Production of invertase was carried out at shake flask level. 10 ml of cells from the late log phase (24 hours) were inoculated into 250 ml flask containing 90 ml MSYP liquid medium and grown at 40 ?C on shaker (160rpm) for 48 hours. Different growth and fermentation parameters on the cell level were studied as follows. All cell growth tests were performed in triplicates.

*Inoculum size:* Cells were grown in MSYP medium for 48 hours using different concentrations of inoculums ranging from 2% to 20%. Same amount of ingredients were added in all the flasks so as to prevent nutrient depletion. Invertase activity/g cells and cell mass as g% were estimated.

*Effect of dispensing volume*: Cells were grown in MSYP medium (volume 25ml-150 ml) in 250 ml flasks containing 5g% sucrose and invertase activity/g cells determined after 24 hours, 48 hours and 72 hours.

*Effect of initial pH*: Cells were grown in 50 ml of MSYP medium for 48 hours on a rotary shaker at 160 rpm where the initial pH was adjusted ranging from pH 3.0-pH 10.0. Samples were withdrawn after 48 hours and invertase activity/g and cell mass as g% was determined.

*Effect of incubation temperature*: Cells were grown in MSYP medium containing 5g% sucrose for 48 hours on a shaker (160 rpm) at different incubation temperatures ranging from 10ºC to 70?C. Invertase activity/g and cell mass, as g % was determined

*Time kinetics profile*: Cells were grown in MSYP medium containing 5g% sucrose at 40?C on a shaker at 160 rpm. 2 ml samples were withdrawn at intervals of 12 hours. However the first sample was removed after 6 hours. Invertase activity/g cells and cell mass as g% was determined. The experiment was terminated after 7 days.

*Effect of metal ions:* Effect of different metals was checked after growing the cells in minimal medium containing 5g% sucrose of the following composition [$gl^{-1}$] $Na_2HPO_4.7H_2O$, 12.8; $KH_2PO_4$, 3.1; NaCl, 0.5; $NH_4Cl$, 1.0; $MgSO_4.7H_2O$, 0.5; Yeast extract, 2.0; along with 1mM concentration of the respective metal salt which was added separately at the time of inoculation to avoid precipitation. (100 mM stocks were prepared in 10 ml sterile water). The cells were grown for 72 hours on shaker at 40?C after addition of metal salt. The flasks were checked for cell mass and invertase activity after 24 hours, 48 hours and 72 hours and compared with control flask (without any metal salts).

*Effect of Substrate concentration*: Cells were grown in minimal medium with different concentrations of sucrose ranging from 1g % to 16g % on shaker at 160 rpm at 40?C for 48 hours and invertase activity/ g of cells determined. Samples were withdrawn for cell mass and invertase activity.

*Effect of Nitrogen source*: Nitrogenous compounds both organic and inorganic were added to 5g% minimal medium, individually and in combinations in final concentrations of 0.5g% and cells were allowed to grow in this medium for 48 hours at 40?C on a rotary shaker. Invertase activity/ g of cells and cell mass as g% was determined as described earlier.

*Effect of Synthetic zeolites:*

10 different zeolites ZE 1 to ZE 10 of pore size ranging from 3Aº to 8Aº were calcinated by heating at 160ºC for two hours. Concentrations of $1gl^{-1}$ were added to growing cells as well as resting cells, and invertase activity/g estimated.

Two sets of experiments A and B were performed.

*Experiment A (using growing cells*)

30 ml of MSYP medium was inoculated with culture and kept on a shaker at 40?C overnight. 5 ml of the overnight culture was added to 45.0 ml of sterile medium in 250 ml flasks. Activated zeolites were added to each flask respectively to obtain a final concentration of $1gl^{-1}$. The flasks were incubated on shaker at 40?C for 48 hours. 5 ml samples were withdrawn, centrifuged and washed with acetate buffer. Invertase activity was assayed using DNSA method as described earlier.

*Experiment B (using resting cells)*

Cells were grown as described earlier without adding zeolite during growth. The cells were harvested, washed, resuspended in acetate buffer and invertase activity was checked after incubating with the respective zeolites for one hour.

*Effect of cheap and renewable carbon sources as additives:*

Different cheap and renewable carbon sources were selected on the basis of their sugar content and added to minimal medium in a concentration of 5g%. The additives used were wheat bran, tea leaves, fenugreek seeds powder, soya bean seeds powder, banana peels, soya bean casein extract, cottonseeds powder, groundnut seeds powder, corn husk, and corn paste. 2.0 g of the additive was mixed with 4 ml of water and boiled for one hour and the entire extract added. The invertase activity per gram of cells was determined respectively. The control flask contained 5g% sucrose in minimal medium

*Effect of permeabilizing agents:*

Cells were suspended in 50 mM acetate buffer, pH 4.5 (7ml g-1packed cells) containing 0.05% CTAB and gently stirred for 40 minutes at room temperature. Treated cells were recovered by centrifugation at 6000 rpm for 20 minutes at 40°C. These conditions were established after preliminary trials with CTAB concentrations in the range of 0.01%-0.2 % and periods of treatment extending up to one hour. Similarly cells were exposed to hot toluene for 10 minutes. Treated cells were recovered by centrifugation for 20 minutes at room temperature. Invertase activity per gram of cells was determined respectively and compared with untreated cells.

## 2.4 RESULTS

**Table 2.1a**

Table 2.1a

NCYC no. 2675 Species Kluyveromyces marxianus

Source Over ripe grape

Information Ferments at 40°c with a yield of 5-6% v/v ethanol after 18 hrs from 15% reducing sugar

Morphology on MSYP medium

| | |
|---|---|
| Broth: Cells@48h | Oval to long multipolar budding cells found singly, in pairs and groups |
| Culture@21d | White flocculent deposit, no ring or pellicle |
| Agar: Cells@48h | Cells found singly, in pairs and groups |
| Culture@21d | White / cream, smooth culture |

Biochemical Characteristics are as per NCYC as depicted in **Table 2.1a** and **Table 2.1b**

**Table 2.1b**

| | Negative | Positive |
|---|---|---|
| **Fermentation** | | |
| D Glucose | ☐ | ☑ |
| D Galactose | ☐ | ☑ |
| Sucrose | ☐ | ☑ |
| Maltose | ☑ | ☐ |
| Cellobiose | ☑ | ☐ |
| α, α-Trehalose | ☑ | ☐ |
| Lactose | ☐ | ☑ |
| Melibiose | ☑ | ☐ |
| Raffinose | ☐ | ☑ |
| Melezitose | ☑ | ☐ |
| Inulin | ☐ | ☑ |
| Soluble starch | ☑ | ☐ |
| Methyl α-D glucopyranoside | ☑ | ☐ |
| **Assimilation** | | |
| D Glucose | ☐ | ☑ |
| D Galactose | ☐ | ☑ |
| L Sorbose | ☐ | ☑ |
| Sucrose | ☐ | ☑ |
| Maltose | ☑ | ☐ |
| Cellobiose | ☐ | ☑ |
| Trehalose | ☑ | ☐ |
| Lactose | ☐ | ☑ |
| Melibiose | ☑ | ☐ |
| Raffinose | ☐ | ☑ |
| Melezitose | ☑ | ☐ |
| Inulin | ☐ | ☑ |
| Soluble starch | ☑ | ☐ |
| Xylose | ☐ | ☑ |
| L-Arabinose | ☐ | ☑ |
| D-Arabinose | ☑ | ☐ |
| D-Ribose | ☑ | ☐ |
| L-Rhamnose | ☑ | ☐ |
| Ethanol | ☐ | ☑ |
| Glycerol | ☐ | ☑ |

| | Negative | Positive |
|---|---|---|
| Ribitol | ☐ | ☑ |
| Galactitol | ☑ | ☐ |
| D-Mannitol | ☐ | ☑ |
| D-Glucitol | ☐ | ☑ |
| Methyl α-D-glucopyranoside | ☑ | ☐ |
| Salicin | ☐ | ☑ |
| Lactic acid | ☐ | ☑ |
| Succinic acid | ☐ | ☑ |
| Citric acid | ☐ | ☑ |
| myo-inositol | ☑ | ☐ |
| D-Glucono-1, 5-lactone | ☐ | ☑ |
| D-Glucosamine | ☑ | ☐ |
| Methanol | ☑ | ☐ |
| Xylitol | ☐ | ☑ |
| Ammonium sulphate | ☐ | ☑ |
| Potassium nitrate | ☑ | ☐ |
| Ethylamine | ☐ | ☑ |
| Cadaverine | ☐ | ☑ |
| L-Lysine | ☐ | ☑ |
| **Miscellaneous** | | |
| Vitamin free growth | ☐ | ☐ |
| 0.01% cycloheximide growth | ☐ | ☑ |
| 0.1% cycloheximide growth | ☐ | ☑ |
| 50% glucose growth | ☐ | ☑ |
| 60% glucose growth | ☑ | ☐ |
| 1% Acetic acid growth | ☑ | ☐ |
| Lipolytic activity | ☑ | ☐ |
| Acid Production | ☑ | ☐ |
| Growth at 37 C | ☐ | ☑ |
| Arbutin hydrolysis | ☐ | ☑ |
| Urease activity | ☑ | ☐ |
| Starch production | ☑ | ☐ |
| Salt Tolerance | ☐ | 10% ☑ |
| Growth at 40°C temperature | | ✓ |

The results of screening for Invertase production are summarized under three stages described in **MATERIALS AND METHODS**, followed by fermentation parameters for maximum invertase production.

**Stage 1:**

Among the several yeast isolates, 7 yeast cultures showed significant activity in **Stage 1** of screening. Among randomly chosen standard type cultures, the following showed activity at this stage of screening. These include 5 yeast cultures as shown in **Table 2.2**.As described in **MATERIALS AND METHODS**; formation of pink formazan was taken as a measure of positive activity and further taken up for **Stage 2**. As seen in photograph 2.1-2.2, dark pink/red color indicated formation of complex which was the result of invertase activity.

**Stage 2 and Stage 3:**

At this stage activity of invertase was checked using DNSA method and visual gradation was made as shown in **Table 2.2.** The activities in all cases were cell-bound, the supernatants being inactive.

As it can be seen from **Table 2.2**, out of 5 standard type cultures, obtained from NCIM, and the 7 isolates, two typed cultures and an isolate showed promising activity when grown at high temperature. These were taken up to **Stage 4** of screening. Our isolate was sent to NCYC for biochemical characterization and was identified as *K marxianus* NCYC 2675 as shown in **Table 1a** and **Table 1b**.

**Stage 4**

The cultures selected from **Stage 1** and **Stage 2** were further assayed quantitatively as shown in **Table 2.3.**

**Table 2.2**

**Invertase Visual Assay of Standard Type Cultures and Isolates:**

| Yeast | Source | Invertase activity |
|---|---|---|
| GR 2 | Isolate | + |
| *S uvarum* NCIM 3332 | NCIM | +++ |
| GR 4 | Isolate | + |
| BA 4 | Isolate | + |
| MA 5 | Isolate | + |
| MA 2 | Isolate | ++ |
| JA 5 | Isolate | + |
| *K marxianus* NCYC 2675 | Isolate | +++ |
| *S cerevisiae* | NCIM 3209 | +++ |
| *K marxianus* | NCIM 3232 | ++ |
| *K marxianus* | NCIM 3231 | + |
| *K marxianus* | NCIM 3217 | ++ |

**Table 2.3**

**Invertase Activity of Whole Cells By Spectrophotometric Assay**

| Yeast | Source | Invertase activity (µmoles/min/g) |
|---|---|---|
| *K marxianus* NCYC 2675 | Isolate | 834 |
| *S cerevisiae* | NCIM 3209 | 687 |
| *S uvarum* | NCIM 3332 | 789 |

As is evident from **Table 2.3** , *Kluyveromyces* species isolated from over ripe grapes (*K marxianus* NCYC 2675) showed the highest invertase activity of 834 µmoles/min/g wet cells compared to the corresponding value of 687 and 789 *of S cerevisiae* NCIM 3209 and S *uvarum* NCIM 3332 respectively when grown in minimal medium with 5g% sucrose. In all the cases sucrose was used as an inducer. Although whole cells of *S cerevisiae* and *S uvarum* were less active than *K marxianus* NCYC 2675, sonicates of all the cultures were assayed for activity to determine whether the whole cell activity difference were due only to difference in the cell permeability.

**Stage 5:**

The sonic extracts were assayed for invertase activity and fractionated by ammonium sulphate precipitation after pilot runs for determining the ammonium sulphate saturation for precipitation of the activity. The results are shown in **Table 2.3.**

**Table 2.4**

**Invertase specific activities (µmole/min/g) of Sonicates and Ammonium Sulphate Fractions of Sonic Extracts**

| Yeast | *S cerevisiae* NCIM 3209 | *K marxianus* NCYC 2675 | *S uvarum* NCIM 3332 |
|---|---|---|---|
| Sonicate | 0.16 | 0.14 | 0.15 |
| Supernatant from 40% $(NH_4)_2SO_4$ Fraction | 0.17 | 0.36 | 0.23 |

It can be seen from **Table 2.3** that *K marxianus* NCYC 2675 and standard yeast cultures were almost comparable in their intracellular enzyme levels. Although the sonicates showed almost comparable specific activities in these three cases, the supernatant of the ammonium sulphate fraction obtained from *K marxianus* NCYC 2675 was significantly higher than the rest of the two yeasts species. Based on these results *K marxianus* NCYC 2675 invertase producer was chosen as the source material for further studies. The control flasks (medium without the inducer sucrose) showed negligible cell bound and cell free extract activity of invertase. Hence these values are not included in further **Figures** and **Tables.**

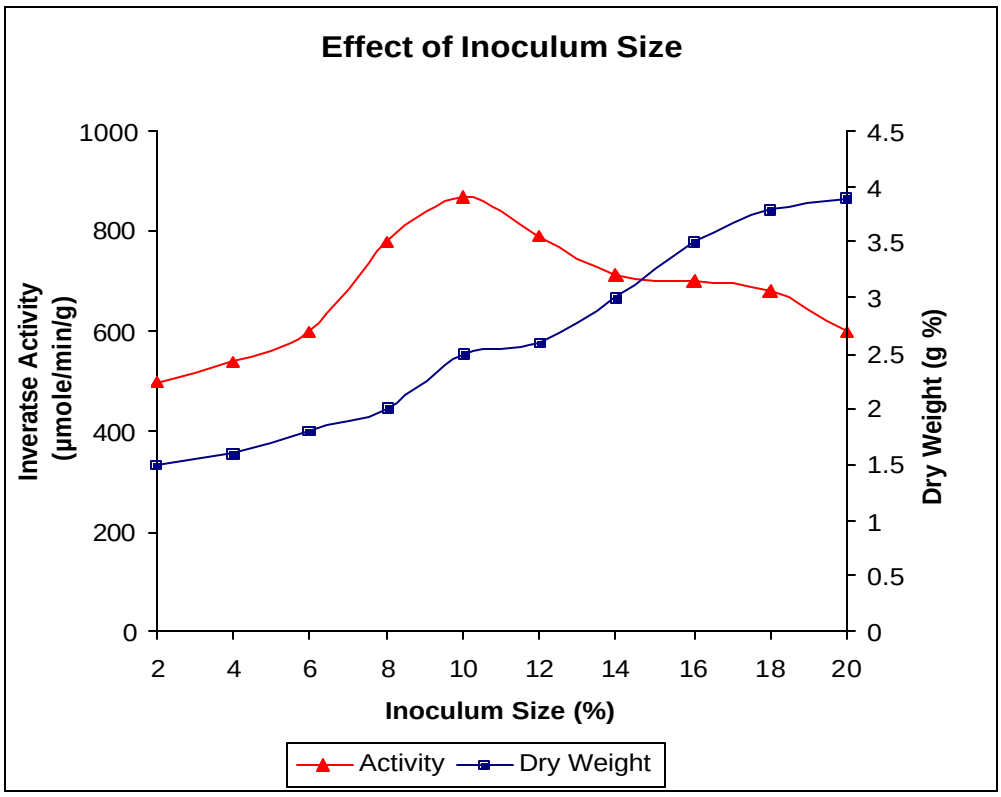



**FIG 2.1: Effect of Inoculum size on cell bound invertase activity**

Inoculum level was an important factor for the production of invertase. Higher enzyme production 870 µ moles /min/g was obtained at 10% (v/v) inoculum level as compared to low or high inoculum levels as seen in **FIG 2.1** Dry wt increases with increase in inoculum size.

**Table 2.5**

**Effect of dispensing volume on enzyme production by *K marxianus***

| Dispensing volume | 1st day (24hrs) | 2nd day (48hrs) | 3rd day (72hrs) |
|---|---|---|---|
| | Invertase activity per g of cells | Invertase activity per g of cells | Invertase activity per g of cells |
| 25ml | 570 | 600 | 580 |
| 50ml | 600 | 620 | 610 |
| 70ml | 685 | 730 | 710 |
| 100ml | 700 | 870 | 750 |
| 125ml | 693 | 810 | 720 |
| 150ml | 660 | 800 | 700 |

The effect of aeration on enzyme production was studied by changing the volumes of medium in flasks [volume of the medium to volume of the flask ratio (v/v): 1:10, 1:5, 1:2.5, 1:2, 1.5:2.5]. As seen in **Table 2.5** maximum invertase activity (870 units) was observed with 100 ml medium in 250 ml flask (1:2.5 v/v) after 48 hours of incubation at 40ºC using shake flask conditions. Increase in the medium volume decreased the activity. Like inoculum size, dispensing volume plays an important role in the invertase production.

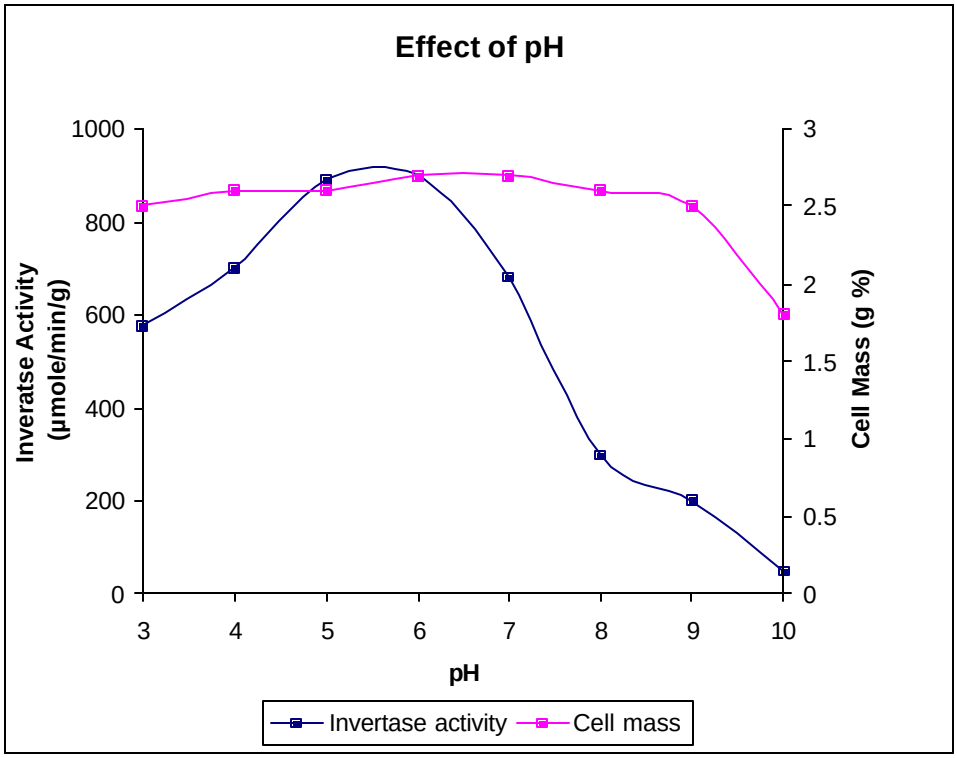



**FIG 2.2: Effect of initial pH on cell bound enzyme activity**

**FIG 2.2** shows effect of initial pH on enzyme production. Maximum invertase activity was observed at pH 6.0 as shown in **FIG 2.2**. Similarly dry cell mass was almost similar at all pH values. *K marxianus* NCYC 2675 can grow well in unbuffered media at different initial pH values, without significant differences.

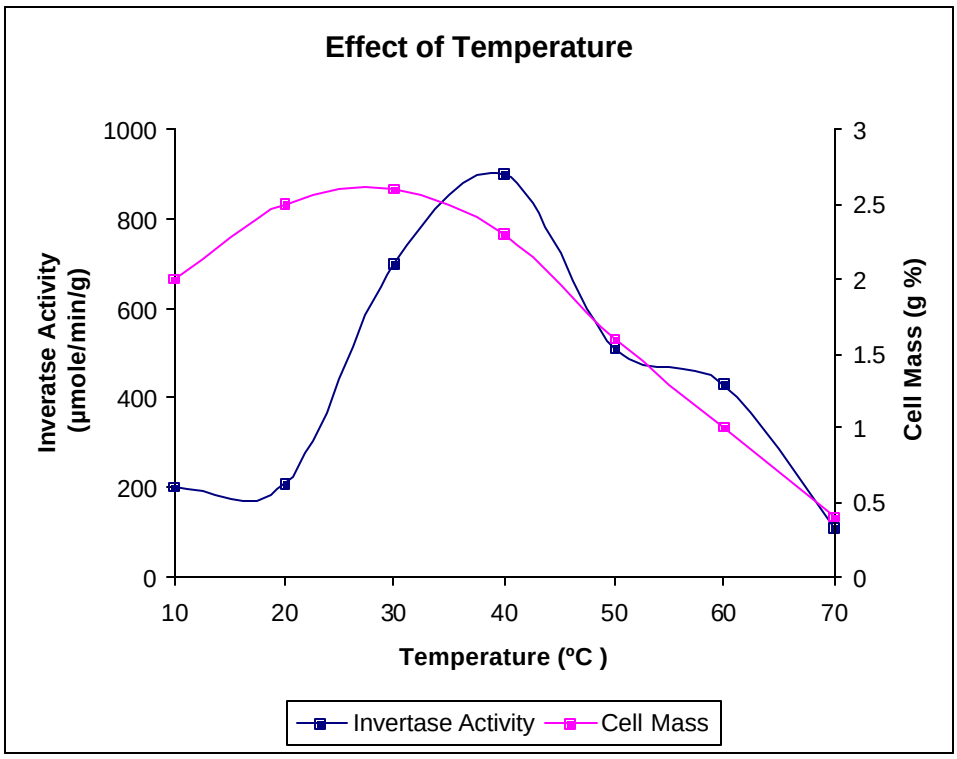



**FIG 2.3: Effect of temperature on cell bound invertase activity**

Maximum enzyme activity was observed at 40ºC, which was not seen in the other yeasts mentioned in **FIG 2.3**. However maximum cell mass was obtained at 30ºC .At 50ºC and 60ºC the cells do show invertase activity however at higher temperatures there is a decline in the activity.

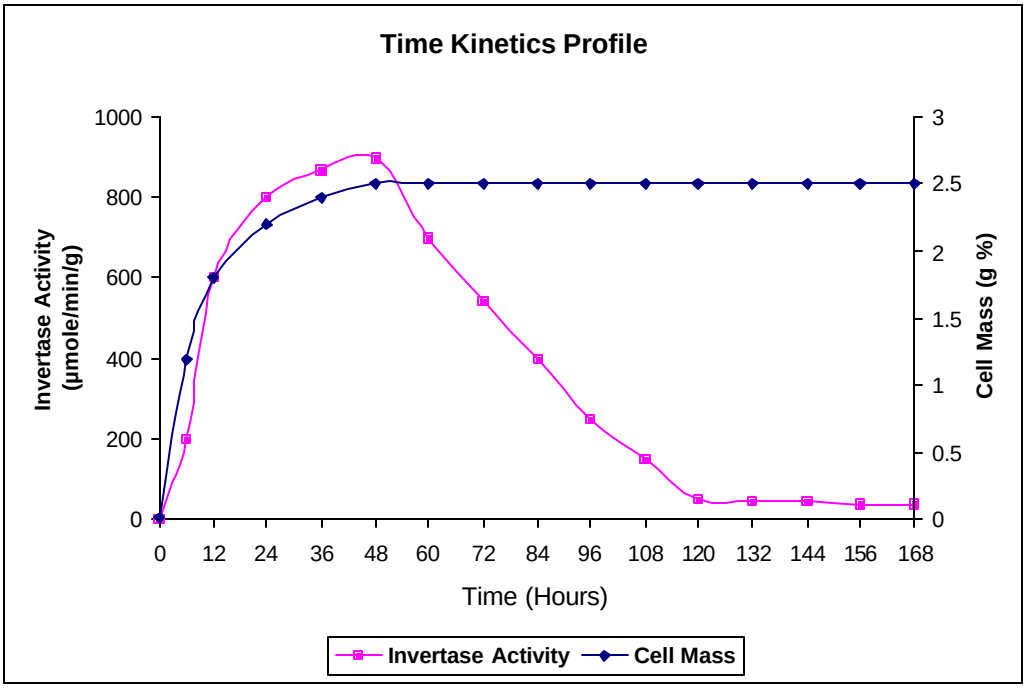



**FIG 2.4: Time kinetics profile of invertase production by *K marxianus***

**FIG 2.4** shows the rate of invertase production by *K marxianus* NCYC 2675. In a batch wise fermentation, enzyme production started after a lag phase of 8 hours and reached a maximum at the onset of the stationary phase. Later, enzyme activity declined due to a decrease in nutrient availability in the medium, or carbon catabolite repression. The expression of invertase in *Saccharomyces* was checked in the presence of monosaccharides like glucose and fructose in earlier reports [Herwig *et al* 2001]. Thus proper incubation time was very important and critical for maximum invertase production. Maximum invertase activity was observed after 48 hours, although cell mass remains constant till 7 days. Further increase in incubation period did not enhance invertase production. This might be due to a decrease in the amount of available nitrogen source in the fermentation medium, the age of the organism, inhibitors produced by the yeast itself and the protease production characteristic of the decline phase. Other workers have reported invertase production by *Saccharomyces cerevisiae* in similar cultural medium incubated for 24-48 hours [Dworschack and Wickerham 1960].

**Table 2.6**

**Effect of metal ions on growth and enzyme production.**

| Compound | 24 hours | | 48 hours | | 72 hours | |
|---|---|---|---|---|---|---|
| | Activity | Cell mass | Activity | Cell mass | Activity | Cell mass |
| $HgCl_2$ | 209 | 1.0 | 237 | 1.3 | 212 | 1.39 |
| EDTA | 543 | 1.2 | 589 | 1.4 | 562 | 1.54 |
| $MnCl_2$ | 659 | 1.9 | 678 | 2.0 | 632 | 2.19 |
| $CuSO_4$ | 569 | 1.98 | 692 | 2.3 | 619 | 2.37 |
| $CoCl_2$ | 766 | 2.1 | 789 | 2.2 | 708 | 2.25 |
| $NH_4$molybdate | 623 | 2.4 | 695 | 2.9 | 654 | 2.9 |
| $FeCl_3$ | 878 | 2.3 | 1013 | 2.5 | 905 | 2.81 |
| $FeSO_4$ | 798 | 2.4 | 958 | 2.6 | 916 | 2.76 |
| $ZnSO_4$ | 798 | 2.3 | 776 | 2.86 | 773 | 2.89 |
| $CaCl_2$ | 890 | 2.4 | 1038 | 2.5 | 940 | 2.7 |
| Control | 643 | 2.4 | 799 | 2.5 | 765 | 2.51 |

Residual activity is expressed as µmoles/min/g and cell mass as g %. 30% 25% and 20% increase in invertase activity was observed with $CaCl_2$, $FeCl_3$ and $FeSO_4$ respectively after 48 hours incubation. However there was no increase in cell mass as compared to control cells. Negligible activity and cell mass was observed with $HgCl_2$. $ZnSO_4$ and ammonium molybdate supported growth as can be seen by increase in cell mass but there was no increase in invertase activity.

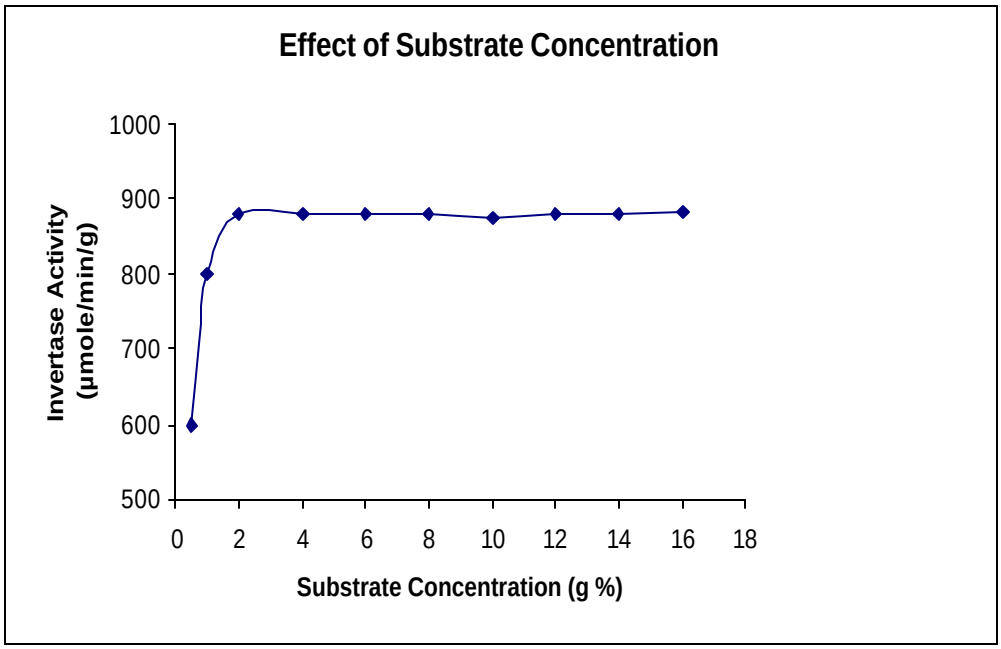



**FIG 2.5: Effect of substrate concentration on cell bound invertase activity.**

As seen in **FIG 2.5** maximum enzyme activity was obtained at a sucrose concentration of 2g%. Sucrose concentrations of more than 2g% caused an increase in dry cell mass, however there was no increase in invertase production The reason may be the generation of a high concentration of invert sugar in the medium resulting in glucose induced repression of invertase [Elorza *et al* 1997, Vitolo *et al* 1995]. At concentration of sucrose less than 2g%, enzyme production was less than the optimum level. As sucrose was the carbon source in the medium, lower concentrations limited the proper growth of yeast, resulting in a lower yield of invertase [Myers *et al* 1997].

**Table 2.7**

**Effect of organic and inorganic nitrogen source:**

| Nitrogen source | Invertase | |
|---|---|---|
| | Cell mass (g%) | Activity (µmoles/min/g) |
| $NaNO_3$ | 2.2 | 800 |
| Peptone | 2.0 | 805 |
| Yeast extract | 2.3 | 810 |
| Urea | 1.8 | 773 |
| $(NH_4SO_4)$ | 1.98 | 820 |
| $NaNO_3$? peptone | 3.0 | 1150 |
| $NaNO_3$? $NH_4SO_4$ | 3.3 | 1213 |
| $KNO_3$ | 2.3 | 740 |
| $NH_4Cl$ | 2.4 | 775 |
| Tryptone | 2.31 | 769 |
| Control | 2.5 | 780 |

Application of an appropriate nitrogen source was very important for optimal production of invertase as shown in **Table 2.7**. In the following study, considerable invertase activity and dry cell mass was obtained when sodium nitrate and ammonium sulphate were used together as the nitrogen source. A mixture of sodium nitrate and peptone gave a high cell mass and activity. Least cell mass was obtained when urea was used as the nitrogen source; however enzyme production was similar to that of the control.

**Table 2.8**

**Effect of synthetic zeolites**

| Zeolite | Activity of growing cells | Activity of resting cells | % increase in activity of growing cells | % increase in activity of resting cells |
|---|---|---|---|---|
| ZE 1 | 1315 | 1105 | 142 | 121 |
| ZE 2 | 1138 | 945 | 110 | 89 |
| ZE 3 | 1001 | 850 | 85 | 70 |
| ZE 4 | 580 | 520 | 07 | 04 |
| ZE 5 | 1071 | 800 | 98 | 60 |
| ZE 6 | 590 | 510 | 29 | 02 |
| ZE 7 | 580 | 520 | 07 | 04 |
| ZE 8 | 570 | 580 | 05 | 03 |
| ZE 9 | 580 | 520 | 07 | 04 |
| ZE 10 | 738.4 | 580 | 36 | 16 |
| Control | 540.7 | 500 | - | - |

Enhancement of invertase activity was observed with zeolites that were microporous, having high aluminium content and with ion exchange capacity as shown in **Table 2.8**. Synthetic zeolites such as silicalite have been shown to act as accelerators of fermentation of sugarcane molasses to ethanol by *Saccharomyces cerevisiae* as cited by Prabhune *et al*, 1996. We observed similar properties when ZE 1 and ZE 2 were used during the fermentation. $Ca^{+2}$ ions was shown to increase the invertase activity (CHAPTER 4). Cation containing zeolites in a concentration of 0.5g% stimulated invertase production in *Penicillium purpurogenum* [Dhake and Patil 2006). Similar increase is seen after addition of calcium containing zeolites. These zeolites may be enhancing cell membrane activities such as sucrose uptake. They also affect changes in floc size, thereby increasing the mass transfer within the aggregates. Silicalite particle size possibly precludes any entrapment of yeast cells. Among the other possible modes of zeolites are the removal of inhibitory substances which enhance the enzyme activity or a conformational change in the active site of the enzyme.

A change in floc size was observed on microscopic examination as shown in photograph 2.6 which probably resulted in a more efficient mass transfer. Zeolites used were acidic, basic silicalite of pore size ranging from 3A? to 8A?. The 5 zeolites ZE 1, ZE 2, ZE 3, ZE 5 and ZE 10 that showed marked increase in invertase activity as compared to control cells (not containing zeolites) were basic in nature.

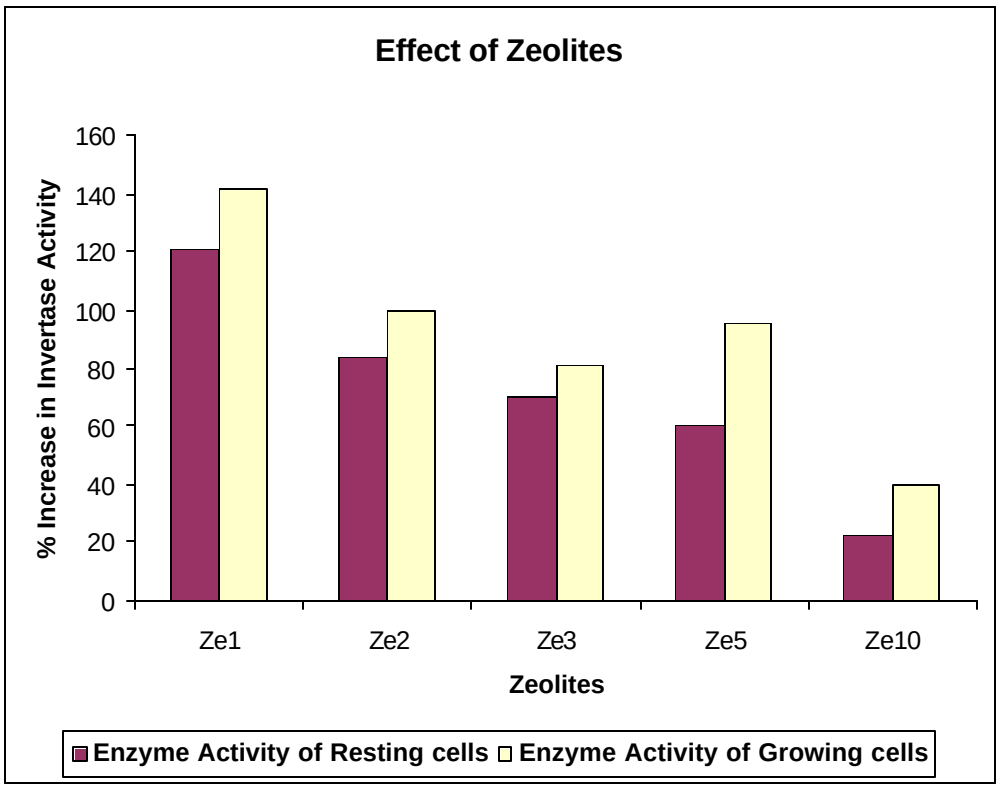



**FIG 2.6:Comparison of the enzyme activity of resting and growing cells using five zeolites.**

As seen in **FIG 2.6** addition of zeolites ZE 1, ZE 2, ZE 3, ZE 5 and ZE 10 to growing cells showed almost 10 to 30% increase in the activity when compared with resting cells and zeolites.

**Photograph 2.6**

**Floc size of the yeast before and after treatment with zeolite ZE 1**

**Before** **After**

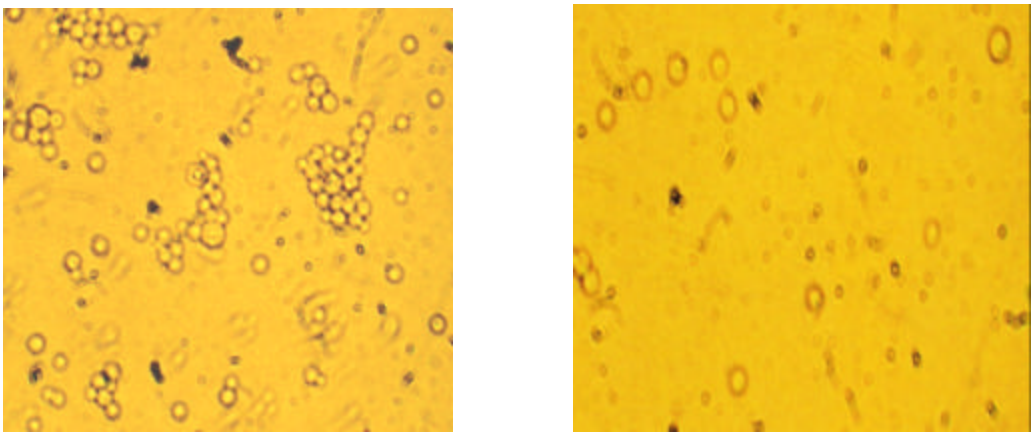


**Table 2.9**

**Effect of cheap carbon source as additives**

| Additive | Invertase activity µmole/min/g | % increase |
|---|---|---|
| Wheat Bran | 1153 | 31 |
| Tea leaves | 990 | 13 |
| Fenugreek seeds | 970 | 10 |
| Soya bean seeds | 920 | 5 |
| Banana peels | 930 | 6 |
| Soya bean casein extract | 1104 | 26 |
| Cotton seeds | 890 | 1 |
| Ground nut seeds | 840 | - |
| Corn husk | 870 | - |
| Corn paste | 1008 | 15 |
| Control(sucrose) | 875 | - |

Among the different cheap and renewable carbon sources which are used as additives soya bean casein extract and wheat bran showed significant increase in invertase activity (**Table 2.9**). However the increase in invertase activity is significantly lower than that obtained with synthetic zeolites ZE 1, ZE 2, ZE 3, ZE 5 and ZE 10 (**Table 2.8)**.

**Table 2.10**

**Effect of CTAB and Toluene on Invertase activity at 40ºC**

| Treatment | Invertase activity(µ moles /min /g) |
|---|---|
| None | 875 |
| CTAB | 1108 |
| Toluene | 1125 |

**Table 2.10** summarizes the data of the effect on CTAB and toluene treatment of *K marxianus* cells on invertase activity at pH 4.5 and 40°C.Treatment of the cells with CTAB (0.05%) or with toluene resulted in enhanced hydrolytic rates. Permeabilization of *E coli* cells with penicillin G activity has been reported earlier by Joshi *et al* 1987; Prabhune *et al* 1992.

**2.5 DISCUSSION:**

Standardization of fermentation parameters is a prerequisite for any cost effective development of a process. Invertase is an industrially important enzyme and its demand is increasing in line with the global markets for processed food, especially the confectionary industry. The use of invertase is somewhat limited due to its high price. Hence optimization of the production process is very important so as to make it more economical and feasible. The fermentative production of invertase has been studied. Both qualitative and quantitative aspects of the fermentation were determined by optimizing cultural conditions and medium design to improve the production of invertase. A pH value of 6.0, a temperature of 40?C, a dispensing volume of 100 ml, a level of inoculum of 10% and a fermentation period of 48 hours were found to be optimum. Among the nitrogen sources a combination of $NaNO_3$ and peptone resulted in a significant increase in enzyme activity. Wheat bran was found to be the best carbon additive followed by soya bean casein extract when added without sucrose. Five of the zeolites ZE 1, ZE 2 ZE 3 ZE 4, and ZE 5 showed increase in the invertase activity for growing and resting cells. The activity of growing cells was observed to be higher than that of resting cells. Toluene and CTAB (0.05%) enhanced the catalytic activity by 26 and 28% respectively.

The above-mentioned exercise increased the yields of whole cell invertase activity by 45 % for the strain *K marxianus* NCYC 2675, the invertase activity under optimized conditions being 1209 µ moles/min/g

*K marxianus* NCYC 2675 showed both invertase and inulinase activity induced by sucrose. In vivo hydrolysis of inulin and sucrose has been examined in selected yeasts of the genus *Kluyveromyces*. In yeast *K marxianus* CBS 6556 two distinct ß frutosidases exist, namely, invertase and inulinase. However cell suspensions of *K marxianus* var. drosophilarum, *K marxianus* var vanudenii, and *Saccharomyces kluyveri* rapidly utilized sucrose but not inulin. Supernatants of cultures grown at pH 5.5 did not catalyze the hydrolysis of inulin and sucrose. This suggested that these yeasts contained a strictly cell bound invertase, an enzyme not capable of inulin hydrolysis. However washing of the cells or treatment with sulfhydryls removed the permeability barrier for inulin that does not exist for sucrose. Non denaturing polyacrylamide gel electrophoresis and determination of the S/I ratio proved that in these yeasts, as in *K marxianus* var marxianus, hydrolysis of sucrose and inulin is catalyzed by the same enzyme namely inulinase [Rouwenhorst *et al* 1990].

Most of the reports are on inulinase production from *K marxianus*. Invertase production has been reported from *Saccharomyces cerevisiae*, *Candida utilis* and *Pichia anomala*.

# CHAPTER 3

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# PURIFICATION, CHARACTERIZATION AND CHEMICAL MODIFICATION

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## 3.1 SUMMARY:

β fructofuranosidase from *K marxianus* NCYC 2675 was purified using hydrophobic/ affinity column chromatography, octyl Sepharose. Molecular weight was determined using gel filtration and SDS-PAGE. This was further confirmed by MALDI-TOF. $K_m$, $V_{max}$ and pI (iso electric point) of the enzyme were determined. Inhibition was studied using products glucose and fructose in the presence of substrate sucrose. Phenol sulphuric method was used to determine if the enzyme is a glycoprotein. The protein was found to be a non glycosylated monomer of molecular mass 67 kDa.

The homogenized enzyme preparation was subjected to pH profile, temperature profile, pH stability, temperature stability and substrate inhibition studies. Similarly, various metal ions, EDTA, and urea were used to test their effect on enzyme activity. Effects of amino acid specific modifying agents were studied to determine the amino acid residue at the catalytic site. NBS and HNBBr, chemical modifiers of tryptophan showed concentration and time dependant linear inactivation of the enzyme. Modification with 60 μM of NBS and 40 mM of HNBBr resulted in 75% and 70% inactivation respectively. Substrate sucrose and products glucose and fructose significantly lowered the extent of inactivation by the tryptophan modifying reagents. The reactions followed pseudo first-order kinetics and the inactivation kinetics indicated the essentiality of a single tryptophan residue near or at the catalytic site.

## 3.2 INTRODUCTION:

The genus *Kluyveromyces* represents one of the most extensively studied inulinase producers [Wang *et al* 2000, Guerrero *et al* 2006, Wan et *al* 2003]. There are also reports of *Kluyveromyces marxianus* species producing high levels of inulinase EC 3.2.1.7 [Kushi *et al* 2000, Ongen-Baysal *et a*l 1996], but there are only a few reports on invertase [β fructofuranosidase EC 3.2.1.26] production from the same [Rouwenhorst *et al* 1990]. In contrast to the limited data available for β fructofuranosidase from *K marxianus*, there are several reports on purification and extensive characterization from other yeast sources. Invertase has been obtained in homogenous/purified form and characterized for their molecular properties from the source *Schizosaccharomyces pombe* [Moreno *et al* 1990, Tanaka *et al* 1998]. Intracellular invertase was isolated from Saccharomyces *cerevisiae* and purified by ion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G–200 [Matulatite *et al* 1980]. Invertase has been purified and characterized in several non conventional yeast including *Candida utilis* [Belcarz *et al* 2002], *Schwanniomyces occidentalis* [Costaglioli *et a*l 1997], *Rhodotorula glutinis* [Rubio *et*

*al* 2002], *Pichia anomala* [Rodriguez *et al* 1995]. Purification and properties of β fructofuranosidase from a strain of *Kluyveromyces fragilis* has been reported by Workman and Day in 1983. Gupta et al have reported Inulinase studies from a strain of *K fragilis* NCIM 3217 in 2004

Extracellular and total invertase has been produced by industrially important species other than *Candida utilis* and *Saccharomyces*. High yields were given by *Hansenula jadinii*, *Torulopsis colliculosa* and *Saccharomyces ashbyi* [Dworschack and Wickerham1961].To exploit new industrial potentials of invertase, it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this important enzyme. The organism *K marxianus* NCYC 2675, a thermotolerant yeast was therefore selected for enzyme production required for studies on some basic and applied aspects of this industrially and pharmaceutically important enzyme. Standardization of a procedure for obtaining a homogenous preparation of pure β fructofuranosidase from the organism and some kinetic and molecular properties of the enzyme are described in this CHAPTER.

Scanty information is available in the literature on the reaction mechanism and the active centers of invertase (β fructofuranosidase) from *K marxianus.*

Leskovac *et al* in 1975 have shown the role of tryptophanyl residues in catalysis of a commercial yeast β fructofuranosidase. Complete inhibition of invertase correlates with the oxidation of 5.5 tryptophans per molecule of enzyme. Little else is known regarding the active site residues. Another report by Pons *at al* 2003 had indicated a direct implication of acidic residues in the glycoside hydrolase families in substrate binding and catalysis.

Reddy and Maley in 1996 have proposed a mechanism for the hydrolysis of sucrose which involved Asp 23 as a nucleophile and Glutamate 204 as an acid / base catalyst through the procedure of affinity labeling and site-directed mutagensis. Role of cysteine and the kinetic analysis has been studied by Waheed and Shall in 1970. However no reports are available on the active site residues of β fructofuranosidase, particularly from *K marxianus*. In the present work, chemical modification studies were carried out to probe essential amino acid residue in β fructofuranosidase from *K marxianus* NCYC 2675.

## 3.3 MATERIALS AND METHODS:

### 3.3.1 Materials:

Sucrose, glucose and fructose were obtained from HIMEDIA, India. 2 deoxyglucose from Sisco research laboratories India, HNBBr (2-hydroxy-5-nitrobenzyl bromide),

PMSF (phenyl methyl sulfonyl fluoride), DEP (Diethylpyrocarbonate) were obtained from ICN Biochemicals USA, NBS (N bromosuccinimide) was procured from SRL India, DTNB (2 2'dithiobisnitrobenzoic acid), NAI( N acetyl imidazole) were obtained from Sigma USA, woodwards reagent K(2 ethyl-5-phenyl isoxazolium 3'-sulphonate), phenyl glyoxal were obtained from Fluka, Germany.

Ampholine buffer (pH range 3-10), octyl Sepharose CL-4B, Gel filtration molecular weight markers were obtained from Sigma USA. SDS-PAGE molecular weight markers were obtained from Bangalore Genei INDIA. 30 kDa centricons were obtained from Millipore Corporation, UK.

All buffers were prepared in glass distilled water.

All other chemicals used were commercially available high purity or analytical grade compounds.

**3.3.2 Methods:**

**3.3.2.1 Purification of β fructofuranosidase from *K marxianus* NCYC 2675**

**Growth of cells:**

*K marxianus* cells were grown in MSYP medium as described in CHAPTER 2 of the THESIS. The cells were harvested after 48 hours of growth by centrifugation at 6000 rpm for 30 minutes in a Sorvall RC-5B refrigerated centrifuge at 10˚C. Freshly harvested cells were washed with 50 mM acetate buffer, pH 4.5 and kept at 4˚C till further required.

**Cell-free extract:**

Batches of about 25g packed cells (obtained from 1 liter medium) were worked up at a time. Cells were suspended in 50 mM acetate buffer and homogenized using Braun's homogenizer as described in CHAPTER 2 of the THESIS. The clear supernatant obtained after removal of cell debris was adjusted to a protein concentration of 12 mg/ml and treated with slow addition of streptomycin sulphate (1%w/v) under stirring on ice. The stirring was continued for a period of an hour, after which the precipitated nucleic acids were removed by centrifugation at 10,000 rpm for 30 minutes.

**Ammonium sulphate fractionation**

The supernatant from the earlier step which contained the activity was processed further. It was subjected to fractional precipitation by the slow addition of finely ground ammonium sulphate under stirring on ice. The fraction which precipitated at 40% saturation was removed by centrifugation at 10,000 rpm for 30 minutes, checked for activity, and discarded. The remaining supernatant showed activity and was loaded on octyl column as described below.

**Octyl-Sepharose column chromatography:**

A 2.0 cm X 10.5 cm glass column was packed with octyl Sepharose matrix using conventional method. The packed octyl column was washed with distilled water and later with five bed volumes acetate buffer 50 mM, pH 4.5. The column was further equilibrated with ten bed volumes of 50 mM acetate buffer, pH 4.5 containing 20% ammonium sulphate. The supernatant (containing crude enzyme) was treated with solid ammonium sulphate to a final concentration of 20%w/v and loaded on the octyl column. One bed volume buffer (containing 20% ammonium sulphate) wash was given to remove the unbound protein. This was followed by elution with 50 mM acetate buffer pH 4.5. The flow rate of the column was maintained at 1.5ml/min and fractions of 3.0 ml quantity were collected. The enzyme eluted between 50-60 ml and the fractions showing enzyme activity were pooled (approximately 12 ml).

Pooled fractions showing activity in the previous run were loaded on a successive octyl column washed and equilibrated as described above. The enzyme eluted in fractions between 40-46 ml. The fractions were concentrated using 30 kDa centricons at 4˚C and later by lyophilization using speedvac SVC 100. Protein and enzyme activity was determined and Native and SDS-PAGE was performed using 7.5% gels to check the homogeneity of the enzyme. Protein bands were visualized by silver staining and Commassie blue staining.

The purified enzyme was stored at 4˚C till further use.

**Enzyme Assay**

Activity measurements were done using DNSA by the spectrophotometric method described by Miller [1959]. Protein was estimated as described earlier by the method of Lowry et *al* [1951], using bovine serum albumin as a standard.

### 3.3.2.2 Properties of β fructofuranosidase from *K marxianus* NCYC 2675

**pH profile:**

The purified enzyme (100 µg) was added to 0.5 ml different buffers (50 mM) in the range of pH 4.0 to pH 10.0 and kept for 10 minutes at 50˚C in the presence of 2 g% sucrose and enzyme assay was performed for each aliquot. Buffers used were acetate buffer pH 4.0 pH 5.0, phosphate buffer pH 6.0, pH 7.0, Tris buffer pH 8.0, carbonate bicarbonate buffer pH 9.0, pH 10.0.

**pH stability:**

The purified enzyme (150µg) was added to 0.5 ml of various buffers (50mM) ranging from pH 4.0 to pH 10.0 and incubated for one hour at room temperature

after thorough mixing. Aliquots were removed and invertase activity of each aliquot was assayed under standard assay conditions.

**Temperature Profile:**

The purified enzyme (150 µg) was taken in 0.5 ml acetate buffer [50 mM, pH 4.5] and incubated at different temperatures ranging from 30°C to 80°C for 10 minutes in the presence of 2g% sucrose. Enzyme assay was performed for each aliquot as described in enzyme assay.

**Temperature stability:**

To investigate the effect of temperature on stability, the purified enzyme (150 µg) in 50mM acetate buffer was incubated for an hour at temperatures ranging from 30ºC to 80ºC.Aliquots were removed and Invertase activity was checked under standard assay conditions.

**Substrate stability studies:**

The purified enzyme (150 µg) was incubated in various sucrose concentrations in the range of 2g% to 34g% and enzyme activity determined under standard assay conditions, keeping appropriate substrate blanks.

**Effect of metal ions and compounds:**

Metal salts $AgNO_3$; $HgCl_2$; $ZnSO_4.7H_2O$; $BaCl_2.2H_2O$; $CoCl_2.6H_2O$; $FeSO_4.7H_2O$; NiSO4.6H2O; $MgSO_4.7H_2O$; $MnSO_4.H_2O$; $CuSO_4.5H2O$; and $CaCl_2.2H_2O$ in 1 mM concentrations were used to assess their effect on invertase activity. The effect of compounds sodium azide, urea and EDTA- sodium salt.$2H_2O$ in 1mM concentrations was also determined. 150 µg of enzyme was used for all the experiments. Since $Co^{+2}$ $Ni^{+2}$ $Mg^{+2}$ $Mn^{+2}$ $Ca^{+2}$ions enhanced invertase activity, different concentrations (0.5mM-10mM) of some of the above metal ions were used to see their effect on enzyme activity.

**Determination of $K_m$, $V_{max}$and $K_{cat}$**

The purified enzyme (100 µg) was made up to 300 µl with acetate buffer and varying substrate concentrations (sucrose) in the range of 2 mM-100 mM were added. The invertase activity was determined under standard assay conditions. The $V_{max}$ and $K_m$ values were estimated from the Lineweaver-Burk plots. $K_{cat}$ values were calculated from the equation

$$V_{max} = k_{cat} \{E\} t$$

Effect of products glucose (50mM), fructose (50mM) and a substrate analog of glucose (2 deoxy glucose) were experimented to see their effect on enzyme activity in the presence of 2 mM-100 mM sucrose respectively. Inhibitor blank and enzyme blank served as controls.

### 3.3.2.3 Determination of the carbohydrate content

The carbohydrate content of β fructofuranosidase from *K marxianus* NCYC 2675 was determined by phenol-sulphuric acid method described by Dubois *et al* 1956.Enzyme solution (750µg protein measured by Lowry method) was diluted to 200 µl using distilled water.200 µl of 5% phenol reagent was added and the mixture was kept for 10 minutes at 28˚C, followed by the addition of 200 µl of concentrated sulphuric acid. The mixture was cooled to room temperature before reading the absorbance at 490 nm. The carbohydrate content was calculated using a standard curve prepared with mannose (10µg-100µg/ml)

### 3.3.2.4 Molecular Weight Determination

**A) Gel Filtration**

Molecular weight determination of native enzyme was carried out according to the method of Andrews (1965) by gel filtration on a Sephadex G-200 column of bed volume 85ml. Molecular weight markers used were β amylase (Mr 200,000), alcohol dehydrogenase (Mr 150.000), cytochrome c (Mr 12,400), bovine serum albumin (Mr 66,000) and carbonic anhydrase (Mr 29,000).

Glass distilled water was used for elution of the proteins.

Marker proteins (2mg each) were loaded separately on the column. Fractions of 2 ml each were collected at a flow rate of 8ml/hour. The void volume (Vo) of the column was determined with Blue Dextran (Mr 2,000,000) and locating it by recording the midpoint of the absorbance maximum at 280 nm. The elution volume (Ve) of each marker protein was determined by measuring the buffer volume eluted up to the midpoint of the elution peak of the particular protein. The concentrated purified enzyme was loaded separately. A standard curve was plotted of Ve/Vo *versus* log molecular weight of the standard proteins. The molecular weight of the enzyme was determined from the standard curve obtained with the marker proteins.

**B) SDS-PAGE.**

The molecular weight of the enzyme was determined by running SDS-PAGE slab gel electrophoresis [Laemmli 1970] using a mixture of marker proteins comprising Phophorylase b (97,000), bovine serum albumin (66,000), Ovalalbumin (43,000),

carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000) and lysozyme (14,300). The enzyme sample (300 µg) and marker sample (4 µl) were separately mixed with 5 µl SDS-PAGE denaturing dye of the following composition: (5x) 1M Tris. HCl 0.6ml, 50% glycerol 5ml,10% SDS 2ml,1% BPB 1ml, $H_2O$ 0.9 ml, 2 mercaptoethanol 0.5ml.The volume was made to 20 µl using 50 mM acetate buffer pH 4.5.Enzyme sample and marker sample were heated in a boiling water bath for 2 minutes and loaded in adjacent wells in a 7.5%SDS-PAGE gel. Protein bands were visualized by Coomassie blue staining method [Bollag 1996].

Composition of Coomassie stain: [$gl^{-1}$] Coomassie blue R-250 1.0g, methanol 450 ml, water 450 ml, glacial acetic acid 100 ml.

Composition of Destainer: methanol: acetic acid: distilled water 10:10:80.

**C) MALDI-TOF (Matrix assisted laser desorption/ ionization time of flight mass spectrometry)**

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337nm) was used for desorption and ionization. Spectra were acquired in the range of 10 to 100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4500Da.The analysis was performed in four replications. The instrument was calibrated with myoglobulin and bovine serum albumin.

5µl of enzyme (500µg/ml) was mixed with 25µl of Sinapinic acid (10mg/ml) prepared in 30% ACN containing 0.2% TFA, by the dried-droplet method.

### 3.3.2.5 Chemical Modification

Preliminary experiments to test the effects of some amino acid specific reagents on β fructofuranosidase from *K marxianus* NCYC 2675 were performed [Means and Feeney 1971]. The purified enzyme (150 µg) was incubated at 25°C for 20 minutes with various chemical modifiers as mentioned below. Aliquots were withdrawn for assay of enzyme activity. Enzyme incubated with buffers in absence of the modifying reagent, served as control. Treatment with diethyl pyrocarbonate, Woodwards reagent K, phenyl glyoxal, N acetyl imidazole, PMSF, DTNB, and TNBS showed no effect on enzyme activity. However inactivation was observed when the enzyme was treated with NBS and HNBBr.

**Treatment with Tryptophan –modifying reagents:**

NBS and HNBBr were used as modifying reagents for tryptophan. NBS is a useful reagent for selectively cleaving tryptophanyl peptide bonds in peptides and proteins. It also cleaves tyrosyl peptide bonds at different pH, but more slowly than those of tryptophan [Ramachandran and Witkop 1967]. Histidine residues undergo a similar reaction, but very much slowly. NBS is a potent oxidizing agent oxidizing –SH groups more rapidly than its reaction with tryptophan. Methionine and cysteine can also be oxidized. This compound is a highly reactive source of very electrophilic bromonium ions $Br^+$. The great reactivity of HNBBr appears due to resonance stabilization of its incipient carbonium ion. Only tryptophan and to a lesser extent cysteine are able to effectively compete with water for this reactive intermediate. Purified β fructofuranosidase(150µg) from *K marxianus* NCYC 2675 was incubated at 28˚C with different concentrations of NBS (10µM-60µM) in 50mM sodium acetate buffer pH 4.5, or HNBBr (10mM-40mM) in 50 mM sodium acetate buffer at pH 4.5 from freshly prepared stock solutions of NBS(1mM) and HNBBr (500mM). Aliquots were withdrawn at regular intervals of time for the assay of enzymatic activity. Enzyme incubated in the buffer in absence of the modifying reagents served as control.

**Kinetics of Inactivation:**

In the chemical modification of an enzyme, the dependence of the apparent first-order rate constant of inactivation $K_{app}$ on the concentration of the modifier is expressed by the relationship:

$$K_{app} = K\,(M)^n$$

OR

$$\log K_{app} = \log K + n \log (M)$$

Where K is the second-order rate constant, (M) the molar concentration of the modifier and n is the average order of the reaction with respect to the concentration of the modifier. $K_{app}$ can be calculated from a semi-logarithmic plot of the enzyme activity remaining as a function of time.

The order of the reaction (n) can be obtained experimentally by determining $k_{app}$ at a number of concentrations of the modifier. A plot of log $k_{app}$ *versus* log (M) should give a straight line with a slope equal to n, where n is the number of molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme-inhibitor complex [Ramkrishna and Benjamin 1981]

**Titration of accessible Tryptophan Residues by NBS:**

The tryptophan-modifying reagent is a potent oxidizing agent which oxidizes the indole moiety of the amino acid tryptophan a chromophore absorbing at 280 nm to oxyindole, a much weaker chromophore at this wavelength. The titration of accessible tryptophan residues in β fructofuranosidase with NBS was followed spectrophotometrically at 280 nm. The control cuvette had 50 mM sodium acetate buffer pH 4.5 and the experimental had β fructofuranosidase (500 ug protein) in a total volume of 1ml of 50 mM sodium acetate buffer. Successive 10 µl aliquots of 20 µM NBS were added to both control and experimental cuvettes at 2 minutes interval and changes in absorbance at 280 nm was recorded till no further change in absorbance was observed. After each addition, an aliquot of 10 µl was removed and the reaction arrested by the addition of 50 mM L tryptophan. The residual activity was determined under standard assay conditions. NBS mediated inactivation was monitored by measuring the decrease in absorbance at 280 nm. Tryptophan was determined by titration with NBS as described by Spande and Witkop in1967. The number of tryptophan residues oxidized (n) per mole enzyme was calculated from the relationship:

$n = (1.31 \times \Delta_{280}) / (5500 \times \text{molarity of enzyme})$

where $\Delta_{280}$ is the decrease in absorbance at 280nm, $5500 M^{-1} cm^{-1}$ is the molar extinction co-efficient of tryptophan at pH 4.5 and 280 nm and 1.31 is an empirical factor based on oxidation of model tryptophan peptides [Patchornik *et al* 1958].

$M_r$=67 kDa was used for calculating molarity of the enzyme β fructofuranosidase from *K marxianus* NCYC 2675.

**Protection against Inactivation:**

Protection of the enzyme against inactivation by NBS (20µM) and HNBBr (40mM) was determined by incubating the enzyme with varying concentrations of substrate sucrose or products glucose or fructose prior to treatment with modifying reagents under the reaction conditions.

**3.3.2.6 Isoelectric focusing (IEF)**

Isoelectric focusing in polyacryamide gel was performed according to the method of Vesterberg [1972]. The tube gels were prepared in duplicate of the following composition: 835µl of 30% acrylamide,125 µl ampholine,150 µl of 10% APS, Glycerol 290µl. Approximately 100 µg of protein was loaded and the volume made to 2.5 ml using glass distilled water. The upper tank of the electrophoresis unit contained 1N NaOH (300ml) and the lower tank had 1N $H_3 PO_4$ (300ml).The electrophoresis was

run initially at 200 volts and 40 mA for one hour and then at 300 volts and 15 mA for 16 hours at 10ºC. At the end of the run, the gels were removed, one gel was stained for protein using Coomassie Brilliant Blue R-250 and subsequently destained using a mixture of methanol:acetic acid:water(10:10:80).The other gel was cut into small pieces which were subsequently put into individual tubes for elution with glass distilled water. The eluates of the gel slices were checked for pH and enzyme activity.

## 3.4 RESULTS

**Table 3.1**

Summary of purification of β fructofuranosidase from *K marxianus* NCYC 2675

| Fraction | Total activity µmoles/min /ml | Total protein mgms | Specific activity U/mg | Recovery % | Purification fold |
|---|---|---|---|---|---|
| Sonicate | 35000 | 4000 | 8.75 | 100 | --- |
| Supernatant from 40% $(NH_4)_2SO_4$ fraction | 22009 | 1570 | 14 | 62 | 1.6 |
| Octyl Sepharose 1 | 9995 | 15 | 666.3 | 45 | 76 |
| Octyl sepharose 2 | 6500 | 3 | 2166 | 65 | 247 |

### 3.4.1 Properties of Purified β fructofuranosidase from *K marxianus*

The homogenous enzyme preparation with specific activity of 2166 units/mg was used in further studies.

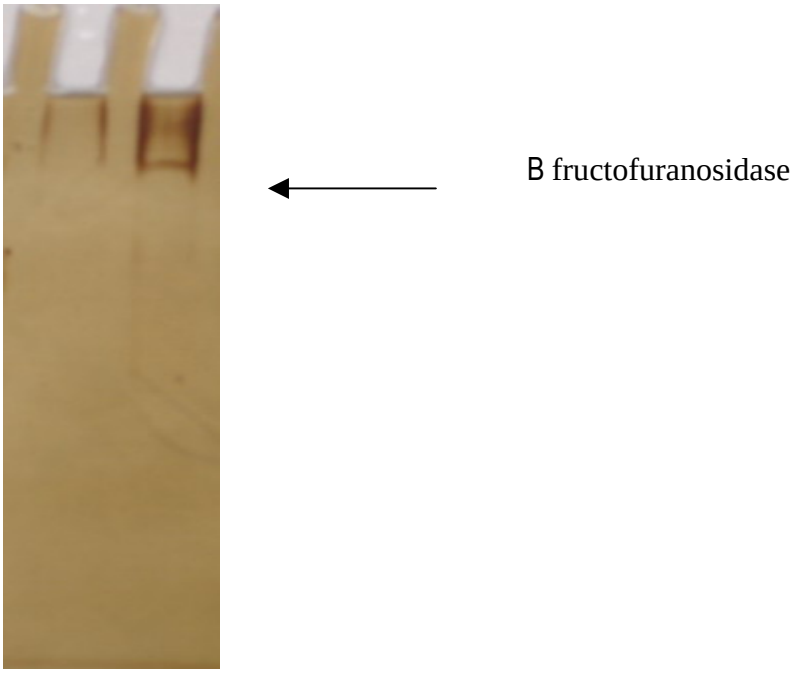



**Fig 3.1a**: Native PAGE

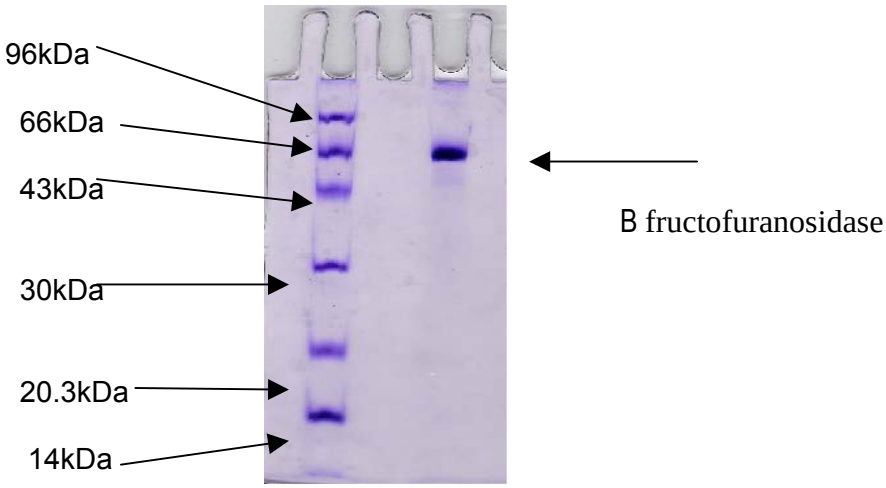



**Fig 3.1 b:** SDS-PAGE

The purified enzyme β fructofuranosidase from *K marxianus* NCYC 2675 moved as a single band on 7.5% native PAGE (**Fig 3.1a**) and SDS-PAGE (**Fig 3.1b**). The SDS-PAGE run of the purified enzyme showed a molecular weight of 67kDa (**Fig 3.1b**)

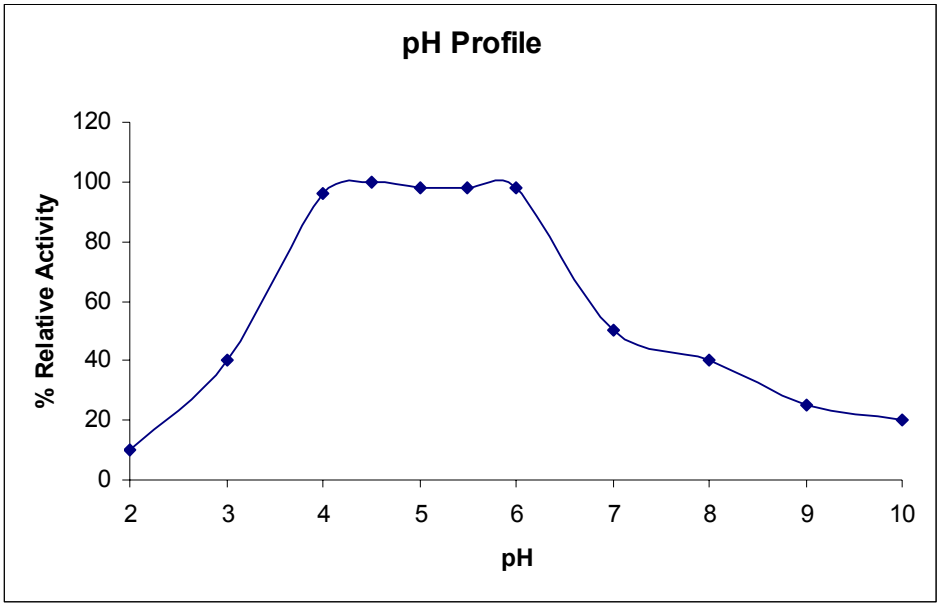



**Fig 3. 2: pH profile of purified enzyme**

**Fig 3.2** shows the pH profile of the purified enzyme. At pH 2.0, there was negligible activity which was subsequently increased with higher pH values. However there was a steady decline/drop in the activity when the pH was adjusted to 7, 8, and 9. Almost 90% loss of activity was observed at pH 10.The optimum pH was 4.5.Sucrose blanks were kept for all the pH conditions to avoid error due to natural chemical hydrolysis of sucrose.

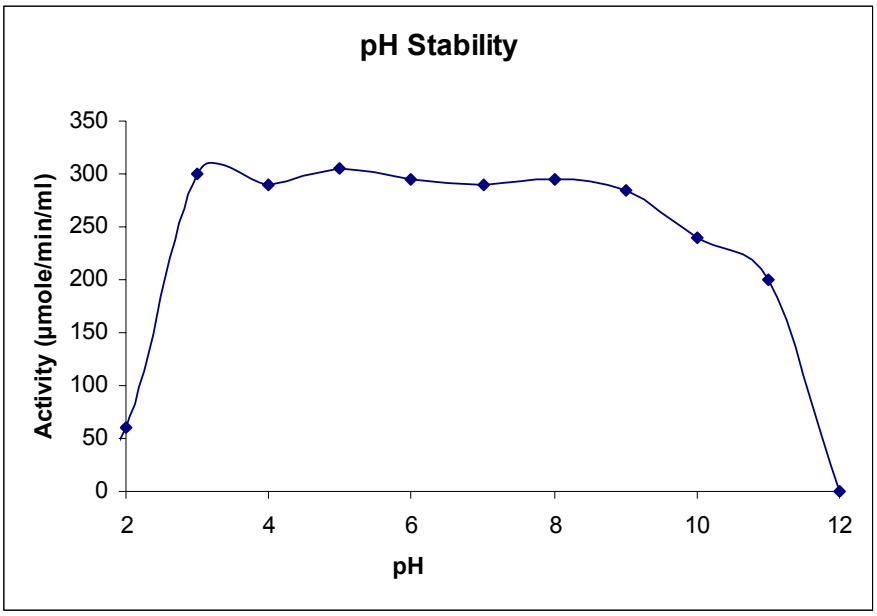



**Fig 3.3: pH stability of purified enzyme**

The enzyme was stable in the pH range 3-9 exhibiting a plateau at these pH values. This is of advantage from the commercial point of view especially in the food industry. After pH 11.0 the stability was reduced and dropped to zero at pH 12.0.The enzyme lost 80% of its stability at pH 2.0.

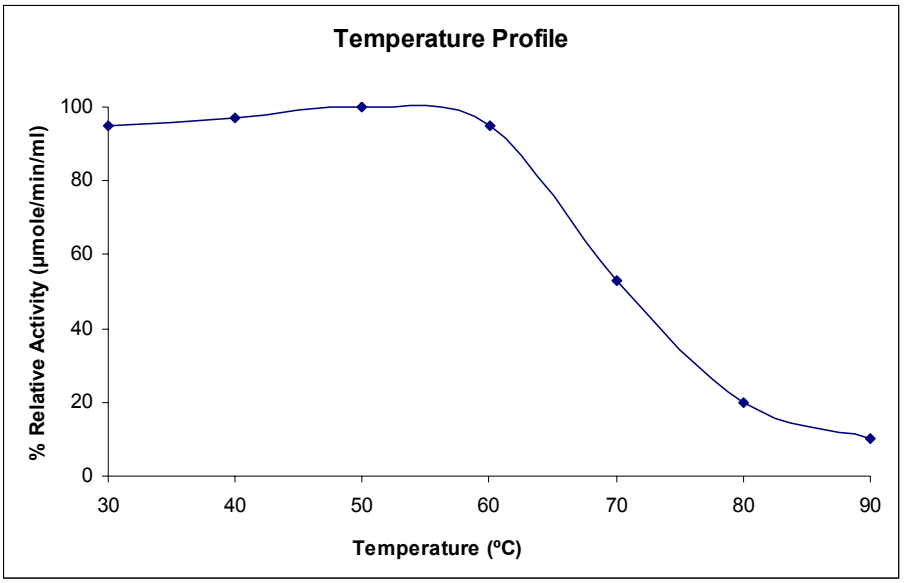



**Fig 3.4**: **Temperature profile of purified enzyme**

As shown in **Fig 3.4** good activity was observed at 50˚C and 60˚C after which the activity dropped and reduced by 90% at 90˚C. At 30˚C and 40˚C the enzyme activity was 80% and 85% of the relative activity respectively.

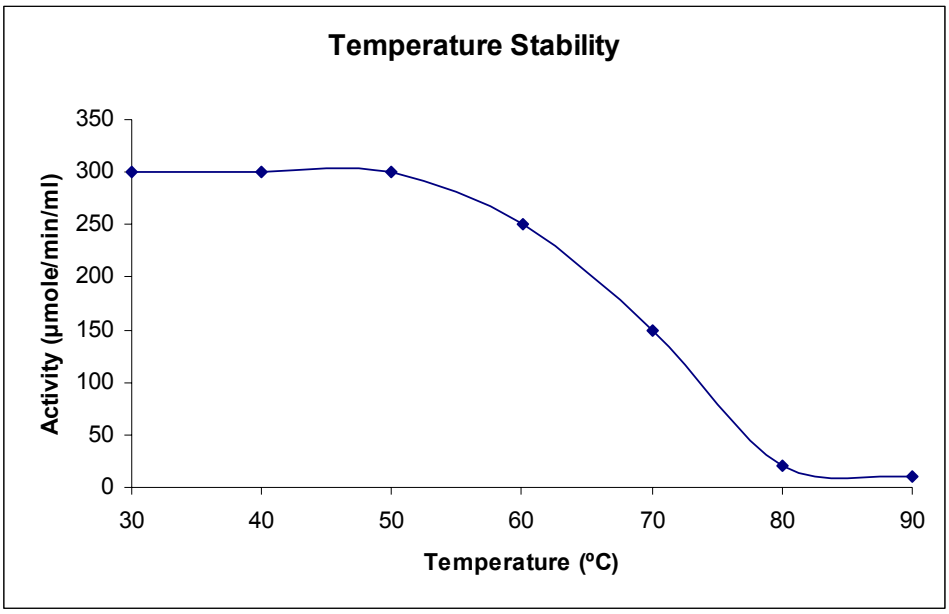



**Fig 3.5**: **Temperature stability of purified enzyme**

**Fig 3.5** shows the effect of temperature on the stability of invertase. Maximum enzyme activity was observed at 50˚C. An increase in temperature beyond the optimum caused a rapid inactivation of the enzyme with 95% loss of activity observed above 80˚C.

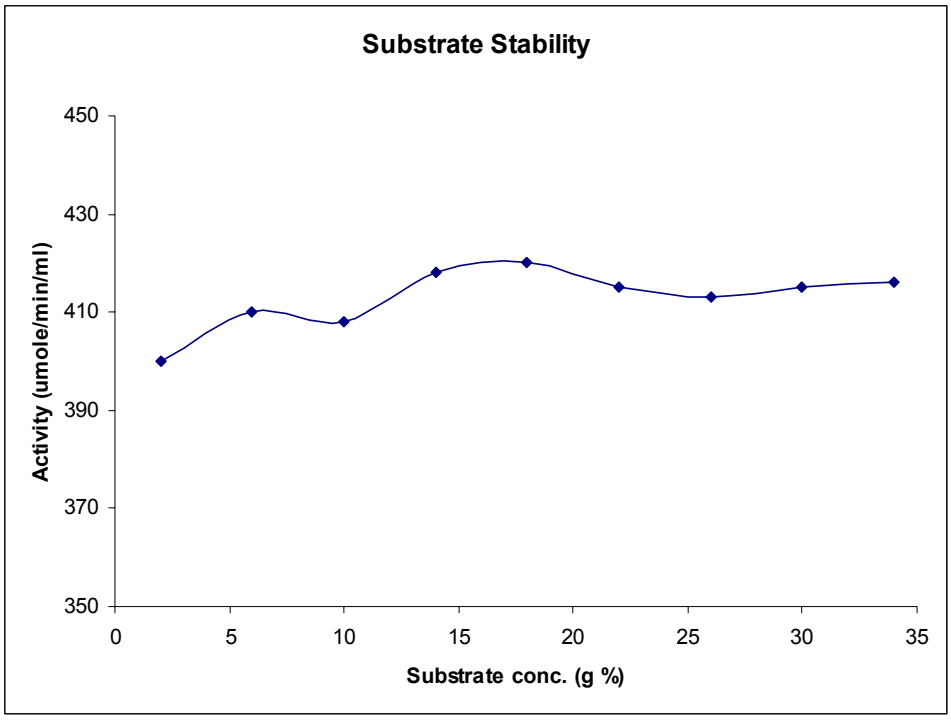



**Fig 3.6: Substrate stability of purified enzyme**

As shown in **Fig 3.6** concentration of substrate ranging from 2.0 g% to 34 g% showed no inhibitory effects on enzyme activity. This profile is especially useful when concentrated sucrose/syrups are to be used in confectionary and in the production of invert syrup. Also the high hydrolytic activity would make the enzymatic hydrolysis process economically efficient for syrup production using by-products with high sugar content such as sugar cane molasses.

**Table 3.2**: **Effect of Metal ions, EDTA and Urea on purified enzyme**

| Compound 1 mM | Activity (%) |
|---|---|
| Control | 100 |
| $AgNO_3$ | 40 |
| $HgCl_2$ | 35 |
| $ZnSO_4$ .$7H_2O$ | 106 |
| $BaCl_2$ .$2H_2O$ | 101 |
| $NaN_3$ | 100 |
| $CoCl_2$.$6H_2O$ | 114 |
| $FeSO_4 7H_2O$ | 100 |
| $NiSO_4$.$6H_2O$ | 118 |
| $MgSO_4 7H_2O$ | 140 |
| $MnSO_4$.$H_2O$ | 125 |
| Urea | 112 |
| EDTA | 100 |
| $CuSO_4 5H_2O$ | 100 |
| $CaCl_2$.$2H_2O$ | 135 |

Control activity (without any metal ions)was assumed as 100%

All experiments were performed in triplicates.

The results summarized in **Table 3.2** showed the effect of metal ions, EDTA and urea on enzyme activity. $Hg^{+2}$ and $Ag^{+1}$ions inhibited enzyme activity by 70% and 80% respectively. EDTA, copper sulphate, ferrous sulphate, sodium azide, and barium chloride showed no effect. Since cobalt chloride, nickel sulphate, magnesium sulphate, manganese sulphate, calcium chloride stimulated invertase activity as compared to the control, further concentrations of the above metal ions (0.5 mM-10 mM) were used to see their effect on enzyme activity. Maximum activity was seen in the presence of 0.5 mM concentration of $Ca^{+2}$ and $Mg^{+2}$ions and 1mM concentration of $Mn^{+2}$ and $Ni^{+2}$ ions.

A similar effect was observed when zeolites containing calcium were used to see their effect on enzyme production as mentioned in CHAPTER 2. Inhibition by $Hg^{+2}$

ions has been reported for other invertases and has application in designing biosensors [Mohammadi et *al* 2005].

There are reports of $Hg^{+2}$ and $Ag^{+1}$ ions exhibiting a marked inhibition of the enzyme in *Kluyveromyces fragilis* (Workman and Dey 1983) and $Mg^{+2}$ and $Ca^{+2}$ stimulating invertase activity 3 fold in *Rhodotorula glutinis*[Rubio *et al* 2002].

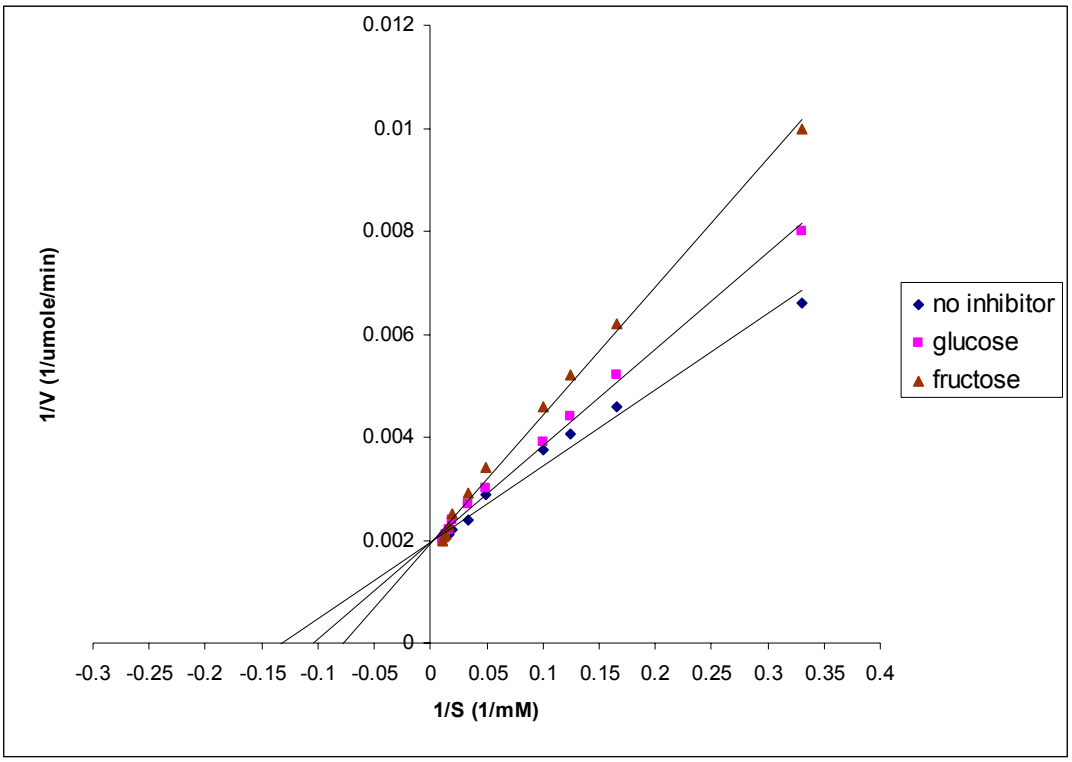



**Fig 3.7**: **Lineweaver Burk plot**

As shown in **Fig 3.7** the kinetic parameters of the enzymatic reaction were estimated by the direct linear method of the Lineweaver-Burk plot of the initial sucrose hydrolysis rates from the experimental data. The apparent $K_m$ was 8 mM and $V_{max}$ was found to be 450 uM/min. Both showed competitive inhibition. No inhibition was observed with glucose analog 2-deoxy glucose. $K_{cat}$ was calculated as 219 $sec^{-1}$.

### 3.4.2: Carbohydrate content:

Sugar was determined from the standard graph and the percentage of sugar in the protein was calculated. No significant level of sugar was obtained in the protein. The standard graph was prepared using mannose since a majority of the glycosylated invertases have a high content of mannose oligosaccharides [Gascon and Lampen

1968]. The enzyme from *K marxianus* NCYC 2675 was found to be a non glycosylated enzyme.

### 3.4.3: Determination of Molecular weight

The molecular weight has been determined by gel filtration **Fig 3.8**, SDS-PAGE **Fig 1b** and MALDI-TOF **Fig 3.9**.All the three experiments estimated the molecular mass as 67kDa.Most of the non glycosylated invertases fall in this range of the molecular weight.

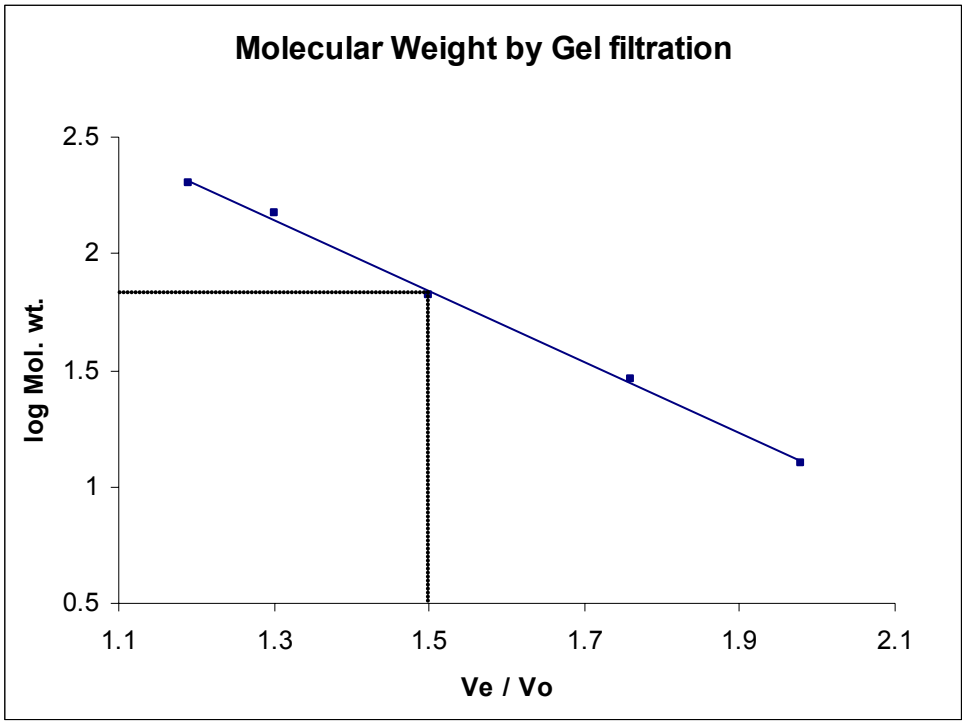



**Fig 3.8**: **Determination of molecular weight by Gel Filtration**

The result of determination of molecular weight of the native β fructofuranosidase from *K marxianus* NCYC 2675 by gel filtration has been shown in **Fig 3.8**. A value of Ve/Vo of 1.5 was obtained which corresponds to 1.83 on the y axis. The value estimated from the above calibration curve obtained with standard molecular weight markers was found to be 67 kDa.

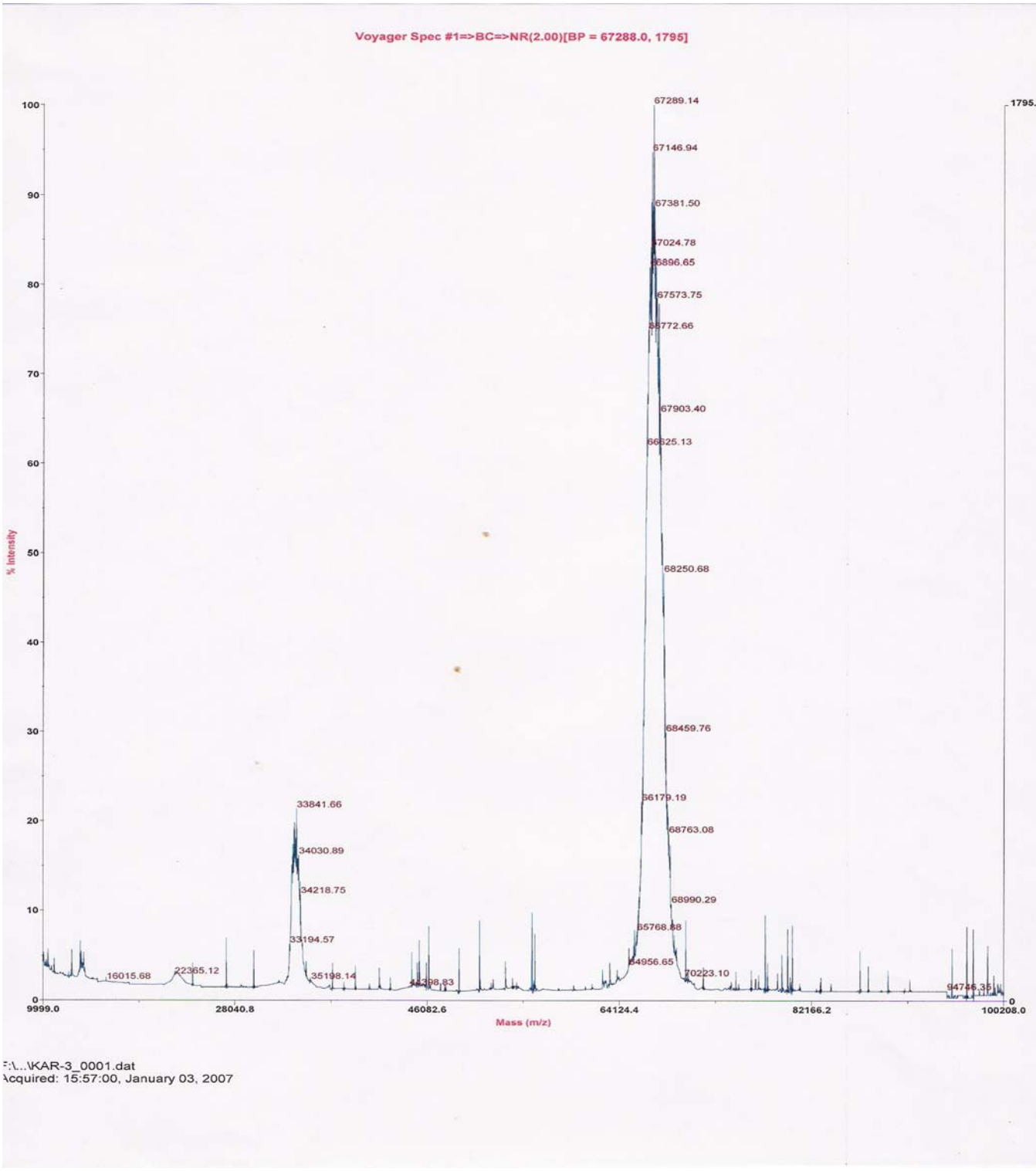



**Fig 3.9: MALDI TOF**

MALDI analysis of the purified protein from *K marxianus* NCYC 2675 showing molecular mass of 67 kDa

**Table 3.3 Effect of amino acid specific modifying reagents on β fructofuranosidase from *K marxianus***

| Reagent | Concentration | Possible residue modified | Residual activity (%) | Buffer |
|---|---|---|---|---|
| none | ---- | ---- | 100 | acetate pH 4.5 |
| TNBS | 1mM | Lysine | 100 | Tris pH 8.0 |
| DEP | 1mM | Histidine | 100 | Phosphate pH 7.0 |
| PMSF | 1mM | Serine | 100 | phosphate pH 7.0 |
| DTNB | 5mM | cysteine | 100 | phosphate pH 6.8 |
| Citraconic anhydride | 5mM | Lysine | 100 | Tris pH 8.0 |
| Woodwards reagent K | 2mM | Aspartic, glutamic | 100 | Phosphate pH 6.0 |
| Phenyl glyoxal | 10 mM | Arginine | 100 | Tris pH 8.0 |
| Nacetyl imidazole | 10mM | Histidine, tyrosine, cysteine | 100 | Phosphate pH 7.0 |
| NBS | 60μM | Tryptophan | 30 | Acetate pH 4.5 |
| HNBBr | 40mM | Tryptophan | 25 | Acetate pH 4.5 |

All the buffers were used in 50 mM concentrations

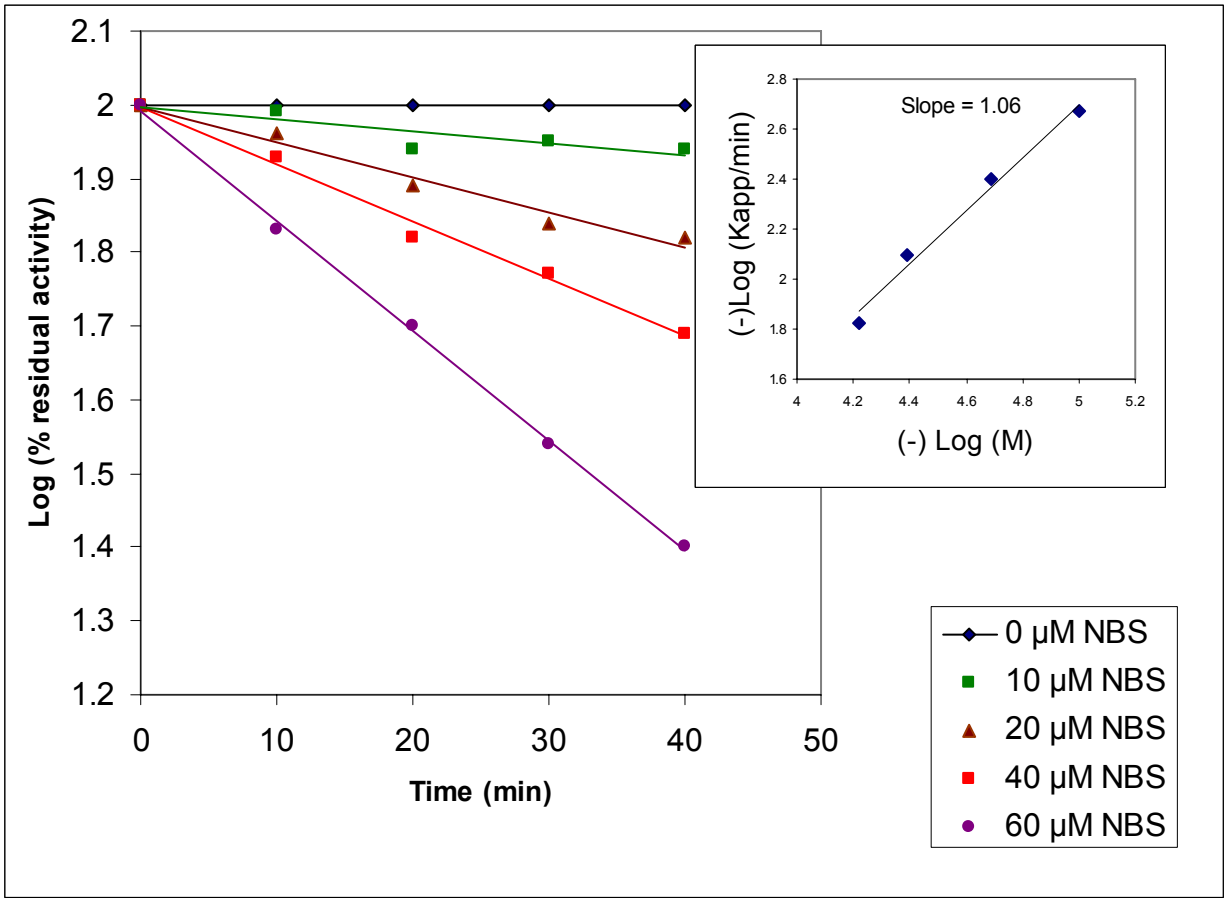



**Fig 3.10a**: **Inactivation of β fructofuranosidase from *K marxianus* NCYC 2675 by NBS**

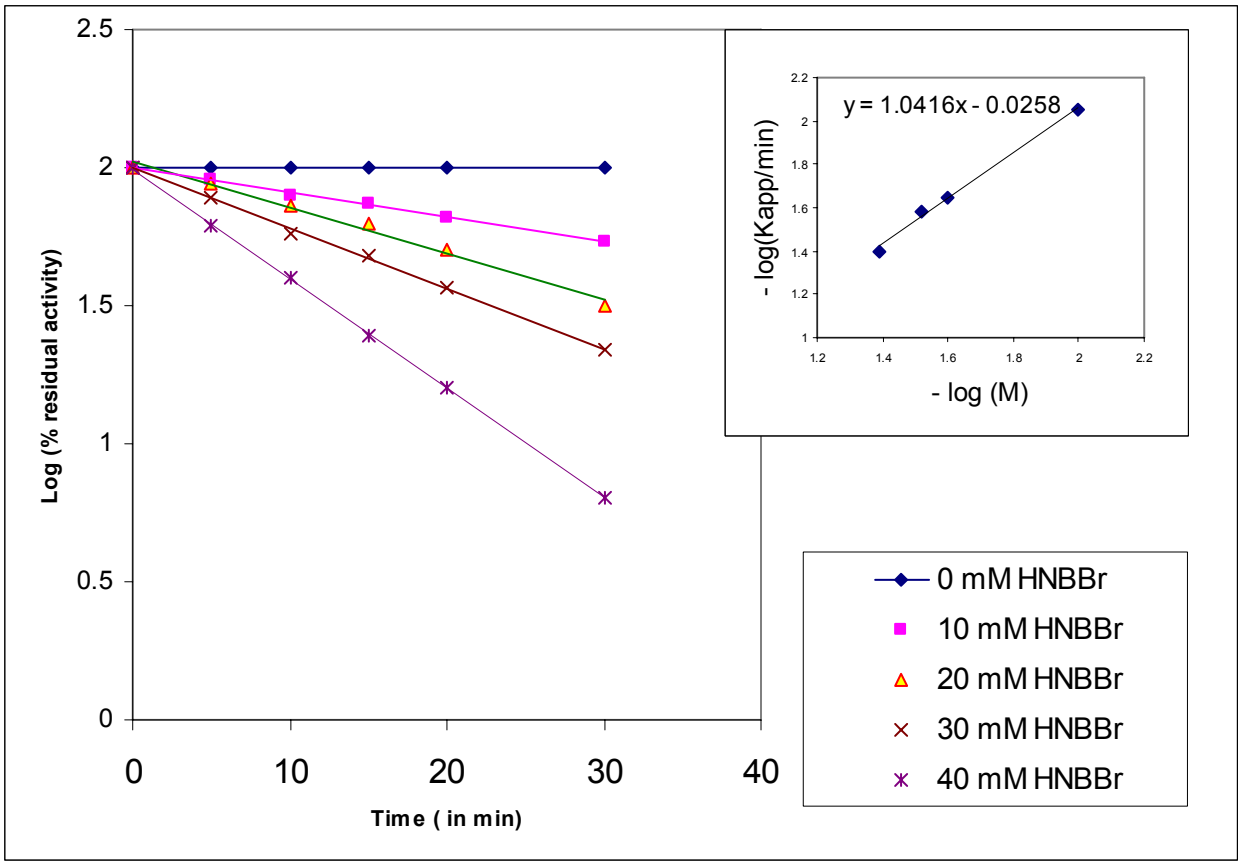



**Fig 3.10b**: **Inactivation of β fructofuranosidase from *K marxianus* NCYC 2675 by HNBBr**

### 3.4.4 Chemical Modification of Tryptophan using NBS and HNBBr

Both NBS and HNBBr inactivated the enzyme β fructofuranosidase rapidly at pH 4.5 as shown in **Fig 3.10a** and **Fig 3.10b**. Treatment with other chemical modifiers obviates ambiguity about the modified amino acid. NBS inactivated the enzyme more rapidly. The enzyme activity in the presence of 60 μM NBS was 25% at the end of 40 minutes test period, while residual activity in the presence of 40 mM HNBBr was 40% at the end of 30 minutes of incubation.

The plots of the logarithm of residual activity versus time of incubation with the NBS and HNBBr were linear throughout the test period of 40 minutes and 30 minutes respectively at all the concentrations of the modifier that were used, indicating pseudo first–order kinetics of inactivation by both reagents. In absence of modifiers, no loss in enzyme activity was observed. The individual slopes of the plots were calculated to determine the respective first-order rate constant Kapp. The reaction order (n)with respect to the tryptophan modifying reagents determined from the plots of log[Kapp] versus log reagent concentration[M] gave value of n=1.06 for NBS and n=1.04 for HNBBr.

These values indicate that the modification of a single tryptophan residue results in the inactivation of a mole of the enzyme (inset of **Fig 3.10a** and **Fig 3.10b**).

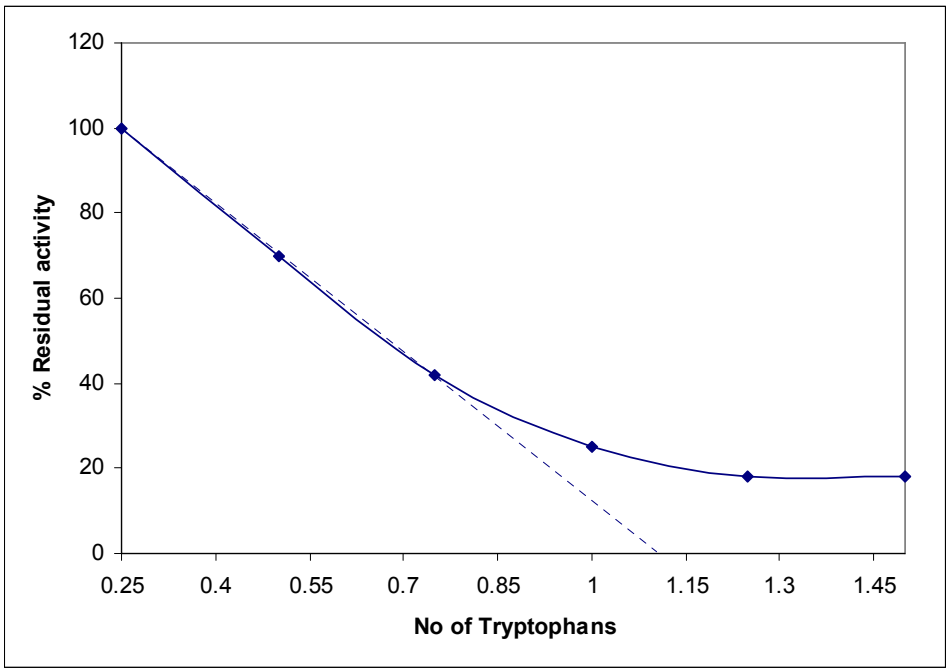



**Fig 3.11: Titration with NBS of accessible tryptophan residues in *K marxianus* β fructofuranosidase**

**Table 3.4:**

**Protection of β fructufuranosidase against inactivation by tryptophan-specific reagent**

| **Treatment** | **Enzyme activity (%f the initial activity)** |
|---|---|
| None | 100 |
| N-Bromosuccinamide (60μM) | 25±1 |
| Sucrose(60mM)+N-bromosuccinimide (60μM) | 75±2 |
| Glucose (60mM) +N-bromosuccinamide (60μM) | 63±3 |
| Fructose (60mM) +NBS | 60±2 |
| HNBBr (40mM) | 30±1 |
| Sucrose (60mM) +HNBBr (40mM) | 81±2 |
| Glucose (60mM) +HNBBr (40mM) | 80±4 |
| Fructose (60mM) +HNBBr (40mM) | 83±3 |

The protective action of substrate sucrose and products of invertase namely glucose and fructose on the inactivation of the enzyme by NBS and HNBBr is shown in **Table 3.4**. In the presence of sucrose (50mM) residual activity was 75% of the initial activity after 30 min incubation with NBS (60μM) and 81% after incubation with HNBBr (40μM), compared with 25% and 30% respectively in the absence of substrate.

Glucose (50mM) gave corresponding values of 63% and 80% activity for NBS and HNBBr respectively. Fructose protected the enzyme almost 60% against inactivation of the enzyme by NBS (60μM) in 30 min. Under the conditions of the experiment fructose protected the enzyme 83% against inactivation by HNBBr(40mM).

The kinetic evidence for the involvement of a single tryptophan residue and the protective action of the substrate and products against inactivation of β fructofuranosidase from *K marxianus* NCYC 2675 by tryptophan modifying reagents are indicative of the presence of the tryptophan moiety at or near the active site of the enzyme.

### 3.4.5: Titration with NBS of accessible residues in *K marxianus* β fructofuranosidase:

The tryptophan residues in *K marxianus* NCYC 2675 β fructofuranosidase were oxidized with stepwise addition of NBS. The number of tryptophan residues oxidized

was determined from $A_{280}$nm and the molar ratio calculated using $M_r$ value of 67,000 for the enzyme as described in **MATERIALS AND METHODS**. **Fig 3.11** shows the effect of NBS on enzyme activity after each addition of NBS. There was a progressive decrease in absorption at 280 nm as well as increase in extent of inactivation. By extrapolating the initial linear portion of the plot to zero activity, the number of tryptophan moieties oxidized per mole of enzyme was found to be1.0. This usually gives the number of residues modified when the enzyme is completely inactivated.

### 3.4.6: Iso electric focusing:

The enzyme showed maximum activity corresponding to a pH of 3.7. Highly purified invertase from baker's yeast has an IEP of about pH 4.0. [Wiseman 1978]. The isoelectric point for *Candida utilis* has been reported as 3.35 by Belcarz *et al* in 2002**.** Intracellular invertase isolated from *Saccharomyces cerevisiae* was found to be heterogeneous. It's molecular forms had isoelectric points at 3.0, 4.0, and 4.4. [Matulatite *et al* 1980].

### 3.4.7: Invertase substrate specificity:

The purified enzyme from *K marxianus* NCYC 2675 was tested for substrate specificity against oligosaccharides raffinose, stachyose and inulin. The enzymatic hydrolysis of potential invertase substrates was monitored by measuring the release of sugars. When checked against raffinose and stachyose as substrates invertase showed specific activity of 2330 µmoles/min/ml and 2520 µmoles/min/ml respectively which was 10% and 19% higher than that exhibited in the presence of sucrose as a substrate. The enzyme did not hydrolyze inulin.

## 3.5: DISCUSSION:

In the present work, an intracellular invertase from the yeast *K marxianus* NCYC 2675 was purified to homogeneity by ammonium sulphate fractionation and two successive octyl sepharose affinity column chromatography. The final step of purification yielded an enzyme with specific activity of 2116 µmoles /min/mg. The activity yield was 65%. The homogeneity of the purified enzyme had been established by PAGE, SDS-PAGE and IEF. Single protein bands were obtained in all the procedures. $Ca^{+2}$ and $Mg^{+2}$ ions enhanced the reactivity of the purified enzyme markedly. $Ag^{+}$ and $Hg^{+2}$ inhibited invertase activity. The optimum temperature and pH for the enzyme was 50˚C and pH 4.5 respectively. The molecular weight was

estimated to be 67 kDa as determined by gel chromatography, SDS-PAGE and MALDI-TOF. The $K_m$ and $V_{max}$ values were 8 mM and 450 µmoles/min respectively. The $k_m$ with sucrose for bakers' yeast invertase was 26 mM [Gascon and Lampen 1968] and *Candida utilis* showed an apparent km of 11mM [Chavez *et al* 1997]. However the $K_m$ and $V_{max}$ for invertase from a strain of *Rhodotorula glutinis* is 227mM and 0.096µM/min.

Products glucose and fructose showed competitive inhibition. Competitive inhibition was also obtained with 2, 5-anhydro-D-mannitol an analogue of fructose (Workmen and Dey 1983).D-fructose acts as a competitive inhibitor, in agreement with the mechanism proposed by Myrback and Boyer in 1961 which involves the formation of a covalent D-fructosyl-enzyme intermediate. D-glucose was shown to be a partial, non-competitive inhibitor [Combes and Monson 1983]. Glycoprotein estimation indicated non-glycoprotein nature of the enzyme. This observation is agreeable with earlier reports of Gascon and Lampen in1968, and Kern *et al* in1992.

The mechanism of action and the nature of the essential amino acid residues at or near the active site of *K marxianus* NCYC 2675 have been reported for the first time. The present studies indicate the involvement of a single tryptophan residue in the active site. The evidences are the kinetics of inactivation by NBS and HNBBr and protection from NBS inactivation by substrate.

# CHAPTER 4

---

# SYNTHETIC SUCROSE MIMETICS AND THEIR ROLE AS INVERTASE INHIBITORS

---

## 4.1 SUMMARY:

The alteration of glycosidase activity is of great interest because of the involvement of glycosidases in a wide range of catabolic and anabolic processes. This principle is the basis for the potential use of glycosidase inhibitors in viral infection, cancer, and genetic disorders. The synthesis of sucrose mimetics/ stable analogues of sucrose, in which a carbon atom substitutes the interglycosidic oxygen, are challenging synthetic targets, both the anomeric centers of the disaccharides being involved in the C-glycosidic linkage. They are particularly attractive due to the serious and widespread sucrose metabolism disorder, Diabetes Mellitus. Four such synthesized deoxy pseudo C- disaccharides A-D, which resemble sucrose, were tested with the enzyme invertase, which was purified from *K marxianus* as reported in CHAPTER 3 of the THESIS. Deoxy pseudo C- disaccharides A and D of the four sucrose mimetics showed greater than 50% inhibition of invertase in the presence of substrate sucrose. Compound A was chosen for all further studies as it was available in sufficient amounts. Effect of variable exposure time, variable incubation time and temperature were studied. Different concentrations of A were used to reveal the inhibition pattern. Substrate protection studies were performed using monosaccharides and disaccharides to understand the type of inhibition.

## 4.2 INTRODUCTION:

The role of carbohydrate molecules in various biological functions has stimulated tremendous interest in the synthesis of carbohydrate analogues with therapeutic potential Glycosidases are involved in a wide range of anabolic and catabolic processes such as digestion, lysosomal catabolism of glycoconjugates, biosynthesis, ER quality control, and ER-associated degradation of glycoproteins. Glycosidase inhibitors could have many kinds of beneficial effects as agrochemicals, and therapeutic agents, such as anti fungal agents, insecticides, anti diabetic, anti obesity, antiviral and therapeutic agents for some genetic disorders [Asano 2003].

More than hundred polyhydroxylated alkaloids have been isolated from plants and microorganisms that mimic carbohydrate substrates [Watson *et al* 2001]. Among the different families of inhibitors, much attention has been paid in recent years to imino sugars-polyhydroxylated piperidine, pyrrolizidines, indolizidines as well as their bicyclic analogues. Due to their numerous biological implications a multitude of methods have been designed

by synthetic chemists in order to achieve their construction [Ayad T *et al* 2004].C-glycosides, aza-C-glycosides, and aza-C-disaccharides are potential glycosidase and glycosyl transferase inhibitors [Zou 2005].Nitrogen analogues of Salacinol have been synthesized and evaluated as glycosidase inhibitors.

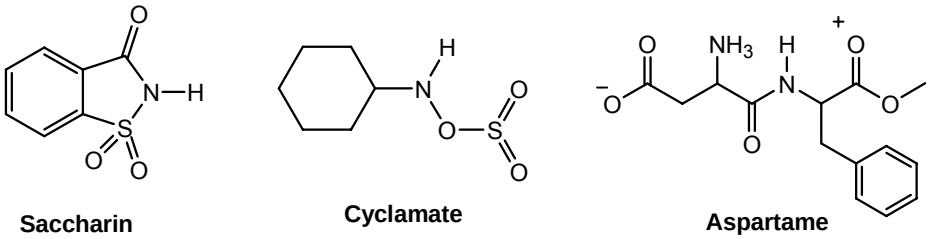
C-saccharides are stable mimics of the naturally occurring *O*-saccharides in which a methylene group has replaced the interglycosidic oxygen. Pseudo-C-saccharides are compounds in which the two sugar rings are bonded directly to one another or are linked by three or more carbon atoms.

C-linked saccharides are now in demand for studying sugar metabolism as they act as enzyme inhibitors by virtue of their resistance to chemical and enzymatic hydrolysis of the glycosidic linkage and their ability to interact with protein receptors similar to their O-linked counterparts. The synthesis of imino C-glycosidic analogues of sucrose has been reported by Ferla *et al* in 2001.These analogues show no inhibition of invertase and very weak inhibition of α glucosidase from yeast.

A few pseudo oligosaccharides that exhibit excellent inhibitory effect on intestinal α glucosidase have been reported, including voglibiose, myglitol and acarbose. They have the chemical structures similar to that of oligosaccharides derived from the digestion of starch [Chen *et al* 2006]. However there are no reports of β glucosidase inhibitors. Our compounds A and D showed an inhibitory effect on β fructofuranosidase. Thus, compounds which can lead to the inhibition of invertase or sucrase have tremendous potential in the treatment of diabetes. They act by delaying the digestion and absorption of carbohydrates, thereby inhibiting postprandial hyperglycemia.

Another way in which diabetes is controlled is by the introduction of artificial sweeteners in regular diet. Artificial sweeteners are non-metabolizable and therefore non-caloric. Some of them are shown in **Chart 4.1**. The more popular sucralose is known to be about 600 times sweeter than sucrose and is the only artificial sweetener which is synthesized from sucrose. Structurally, it is synthesized by replacing three of the hydroxyls of sucrose by chlorine atoms and can be named as 1, 6-dichloro-1, 6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-alpha-D-galactopyranoside.

**Chart 2: ARTIFICIAL SWEETNERS**



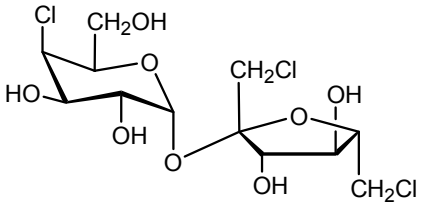
**Saccharin**



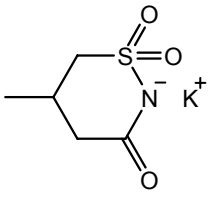
**Cyclamate**



**Aspartame**



**Sucralose**



**Acesulfame-K**

Another important application of these compounds could be to increase the shelf-life of food products where invertase activity might lead to spoilage

Thus, sugar mimetics play a very important role in either themselves acting as drugs or as 'leads' for the synthesis of a plethora of such molecules. Their role as enzyme inhibitors can also be used for purely academic reasons, for the study of the enzymes that they inhibit and to study the structure function relationship of the protein.

## 4.3 MATERIALS AND METHODS:

### 4.3.1 Materials:

Sucrose and 3, 5- Dinitrosalicylic acid was obtained from MERCK, India. Mannose, Galactose, Fructose, Xylose, Glucose, 2 Deoxyglucose, Lactose were obtained from HIMEDIA.

Deoxy pseudo C-disaccharides A-D were synthesized from glucose by sequential hydroxylation followed by ring closing metathesis and were kind gifts from Organic Chemistry Division, University of Pune.

### Microorganisms:

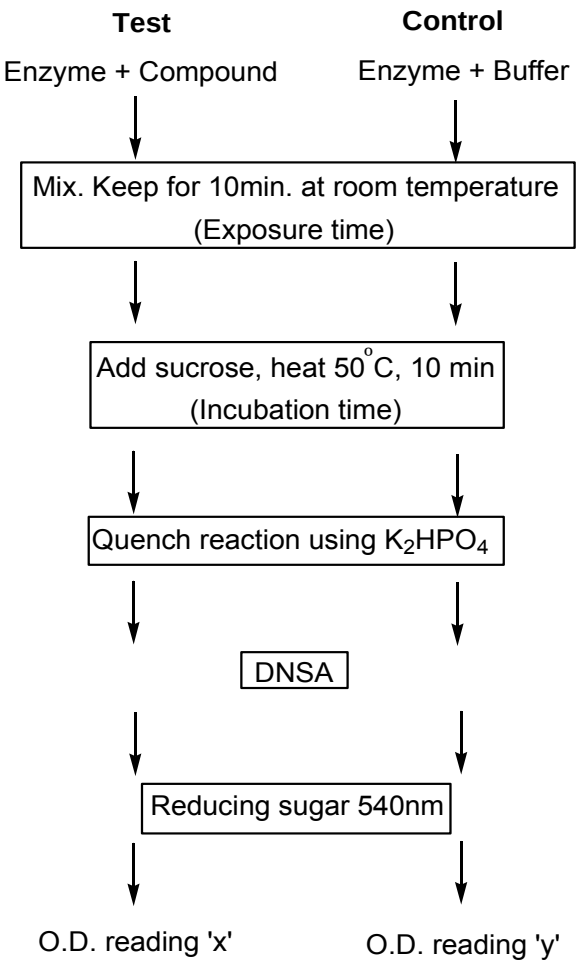
The culture *Kluyveromyces marxianus* NCYC 2675 was isolated from over ripe grapes as described in CHAPTER 2 of the THESIS and the enzyme β fructofuranosidase was purified as described in CHAPTER 3 of the THESIS.

### 4.3.2 Methods:

#### 4.3.2.1 Invertase assay:

The invertase assay was typically carried out in a reaction mixture containing sucrose as described in CHAPTER 2 of the thesis.

A flow diagram depiction of the assay is shown below




The % inhibition was calculated by

(i) Correlating the absorbance at 540 nm (‘x’ of test and ‘y’ of control) with µgs of glucose released in the test and blank (control)

(ii) The µgs of glucose released in presence of the inhibitor (test) if in absence of the inhibitor (control) enzyme releases 100 µgs of glucose

(iii) Subtraction of the above from 100 would give the % inhibition

Experiments were carried out to study the effects of the compounds A, B, C and D.

#### 4.3.2.2 Experiment 1:

**Effect of compounds A to D:**

20µl of purified enzyme in 260 µl of 50 mM acetate buffer pH 4.5 was mixed with 20µl of compound A and incubated for 10 minutes at room temperature.200µl of sucrose(2g%) was added and invertase activity was checked under standard assay conditions, keeping appropriate inhibitor and sucrose blanks as controls. The effective concentration of the compound and enzyme used was 4.0 mg/ml and 300 µg/ml respectively.

Similar experiments were performed using compounds B, C and D.

#### 4.3.2.3 Experiment 2:

**Effect of variable exposure time using compound A:**

The enzyme was suitably diluted with 50 mM acetate buffer and was mixed with the inhibitor and kept on ice. Aliquots were removed at intervals of 5 minutes, keeping a maximum exposure time of 50 minutes. To each aliquot 2g% of sucrose was added in 50mM acetate buffer. Invertase activity was determined using standard assay procedure keeping appropriate controls.

#### 4.3.2.4 Experiment 3:

**Effect of variable incubation time and temperature using compound A:**

The inhibitor A (250µg) was added to the enzyme (300µg) in 50mM acetate buffer at pH 4.5. Three different temperatures were selected for 10 minutes and 30 minutes exposure time. In the first experiment, the enzyme was incubated with inhibitor at 30˚C for 10 minutes and aliquot was removed and assayed for invertase activity under standard assay conditions. Another aliquot was removed after 30 minutes. Similar experiment was performed at 40˚C and 50˚C and corresponding samples were removed as explained above.

#### 4.3.2.5 Experiment 4:

**Effect of variable inhibitor concentration using compound A:**

The enzyme (300µg) in buffer was mixed with various concentrations of inhibitor and kept at room temperature for 10 minutes. 2g% sucrose in 50 mM acetate buffer was added to each aliquot and invertase activity determined. The concentration of inhibitor was varied from 0.5mg to 6.0 mg per ml of the total test volume.

#### 4.3.2.6 Experiment 5:

**Substrate protection studies with Compound A:**

The enzyme assay was performed using monosacchrides such as xylose, glucose, 2-deoxy glucose, mannose, galactose fructose and disaccharides sucrose and lactose. Sugar stocks used were 0.5g%. 50µl of the enzyme was added to 200µl of substrate (sugar to be tested) and to 200µl of the inhibitor respectively. The mixture was kept on ice for 10 minutes.100µl aliquots were removed and checked for invertase activity under standard assay conditions.100µl sample removed at zero time served as the blank reading.

## 4.4 RESULTS

### 4.4.1 Effect of compounds A-D on invertase activity.

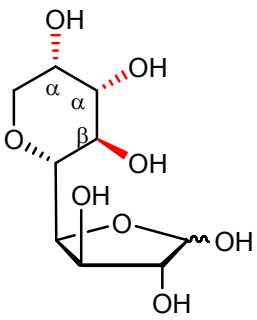
**Table 4.1: Effect of compounds A-D on invertase activity**

| Compound | % inhibition |
|---|---|
| **A** | 54 ± 0.5 |
| **B** | 10 ± 0. 09 |
| **C** | 13 ± 0.1 |
| **D** | 54 ± 0.9 |

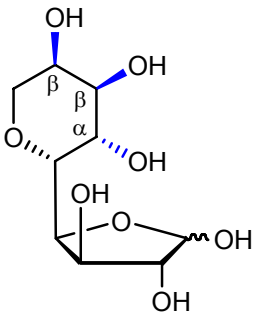
As shown in **Table 4.1** none of the compounds affected an increase in the activity

However the compounds **A** and **D** showed significant inhibition (av. 54%) of invertase activity while **B** and **C** affected the enzyme activity negligibly. Since two (**A** and **D**) out of the four compounds showed a significant inhibition of enzyme activity, it could be inferred that the furanose moiety does not contribute to the activity of the enzyme and the pyranyl ring is probably responsible for the inhibition.

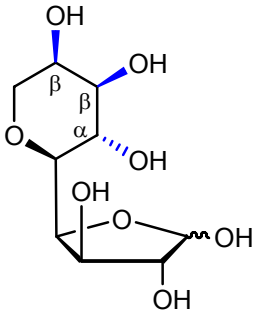
The ‘preference’ of the enzyme for **A** and **D** over **B** and **C** was very interesting since the stereochemistry of the hydroxyls at C-6, C-7 and C-8 (6β, 7α, 8α) in **A** and **D** are identical. They differ at the configuration of only the C-5 oxygen, which is 5α in **A** and 5β in **D**, which does not seem to play any significant role.



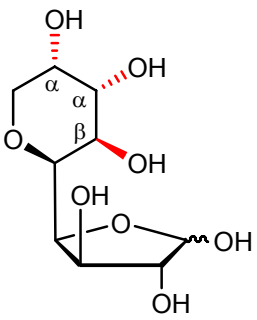

**160a**




**160b**




**160c**




**160d**

The % inhibition obtained was noteworthy in the light of a recent report by Nicotra and co-workers in 2001. Inhibitor **A** was chosen for all further study due to its availability.

### 4.4.2 Effect of variable exposure time:

The exposure time' is the time given for the binding of the enzyme to the inhibitor or substrate without the enzyme acting on it. Thus, the enzyme was mixed with the inhibitor and kept on ice. This ensures enzyme-substrate interactions without the enzyme acting on the substrate. It was necessary to find the optimum time required for the enzyme to bind with the substrate or inhibitor and the effect of varying this time.

It was confirmed that exposure time does not result in significant changes in the % inhibition. Therefore, the binding of the inhibitor (**I**)/compound A to the enzyme (**E**), which would form the **EI** complex, seems to be more or less instantaneous.

**Table 4.2: Effect of Variable exposure time on % inhibition of enzyme**

| Time in minutes | % Inhibition[#] |
|---|---|
| 5 | 54.23 |
| 10 | 52.15 |
| 20 | 51.07 |
| 30 | 53.31 |
| 40 | 53.98 |
| 50 | 55.41 |

*# Average of five*

### 4.4.3 Effect of variable incubation time and temperature

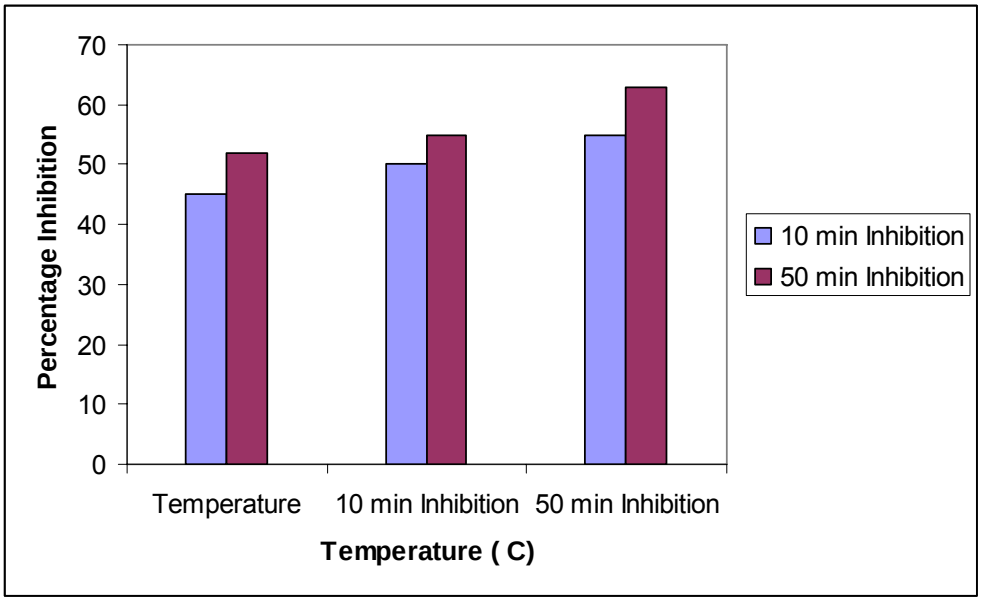



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**Fig. 4.1:Effect of variable incubation time and temperature on % inhibition of enzyme**

As shown in **Fig 4.1** maximum inhibition was observed at 50ºC when the time of incubation was 50 minutes. It was speculated that, an enzyme held in a particular conformation by weak forces of attraction could break on increasing temperature and time. This might result in the 'opening up' of the enzyme permitting an easier approach for the inhibitor to bind to the enzyme, which probably explains the % increase in inhibition.

### 4.3.5 Effect of variable inhibitor concentration:

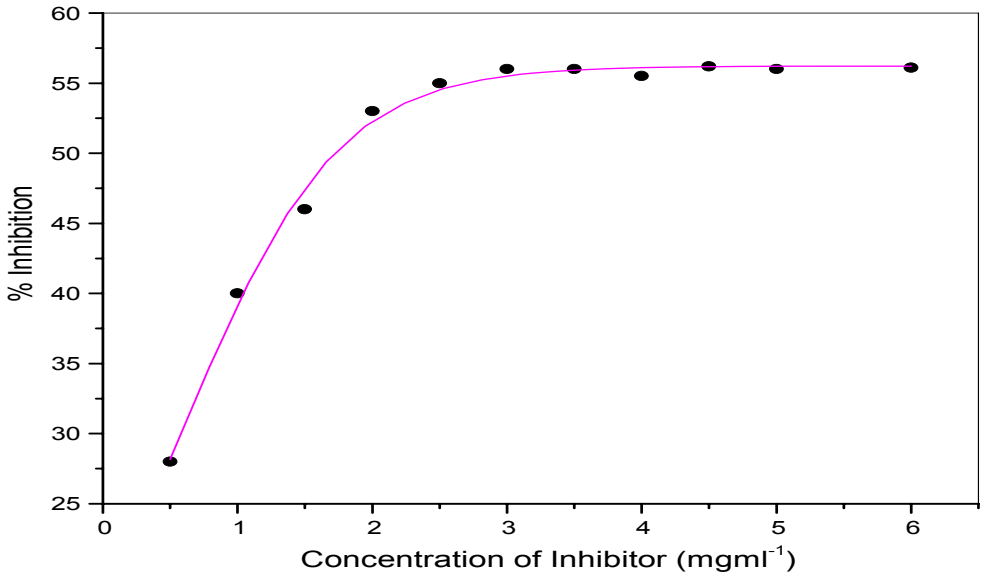



**Fig. 4.2: Variable inhibitor concentration on % inhibition of enzyme activity:**

The graph shows a steady increase in inhibition till a concentration (2.5mgs / ml) above which saturation was observed.

### 4.3.6: Substrate protection studies:

The binding affinities of the inhibitor **A** for the enzyme in presence of several monosaccharides like xylose, glucose, 2-deoxyglucose, mannose, galactose, fructose and disaccharides like sucrose and lactose were studied. The sugars compete with the inhibitor for the active site of the enzyme and the 'preference' of the enzyme can be determined.

The enzyme assay was performed using each one of the sugars. It was observed that the sugars mannose, galactose, fructose and sucrose show 100% protection of the enzyme which confirms that the affinity of the enzyme for these sugars is more than the affinity of the enzyme for the inhibitor **A.** On the other hand, xylose, glucose, 2-deoxyglucose and lactose did not 'protect' the enzyme when co-incubated with the inhibitor **A** as depicted in **Table 4.3**.

**Table 4.3: Substrate protection studies**

| Sugars which protect the enzyme | Sugars which do not protect the enzyme |
|---|---|
| Mannose, Galactose, Fructose,<br>Sucrose | Xylose, Glucose, 2-deoxyglucose<br>Lactose |

The pyranyl moiety of the inhibitor **A** displays a β, α, α configuration which is the D-arabino configuration containing two axial hydroxyls (at C-2 and C-3). The sugars which protect the enzyme (**Chart 4.2**) from the inhibitor have at least one axial hydroxyl group whereas the sugars which do not protect have no axial hydroxyls. Although no clear explanation in terms of the stereochemistry of the –OH functions can be offered for the above observation, it can be speculated that the axial hydroxyls might play some role in binding of the substrate / inhibitor with the enzyme. To determine exactly the position and group on the pyranose ring involved or essential in binding of the active site requires further study of the kinetic parameters.

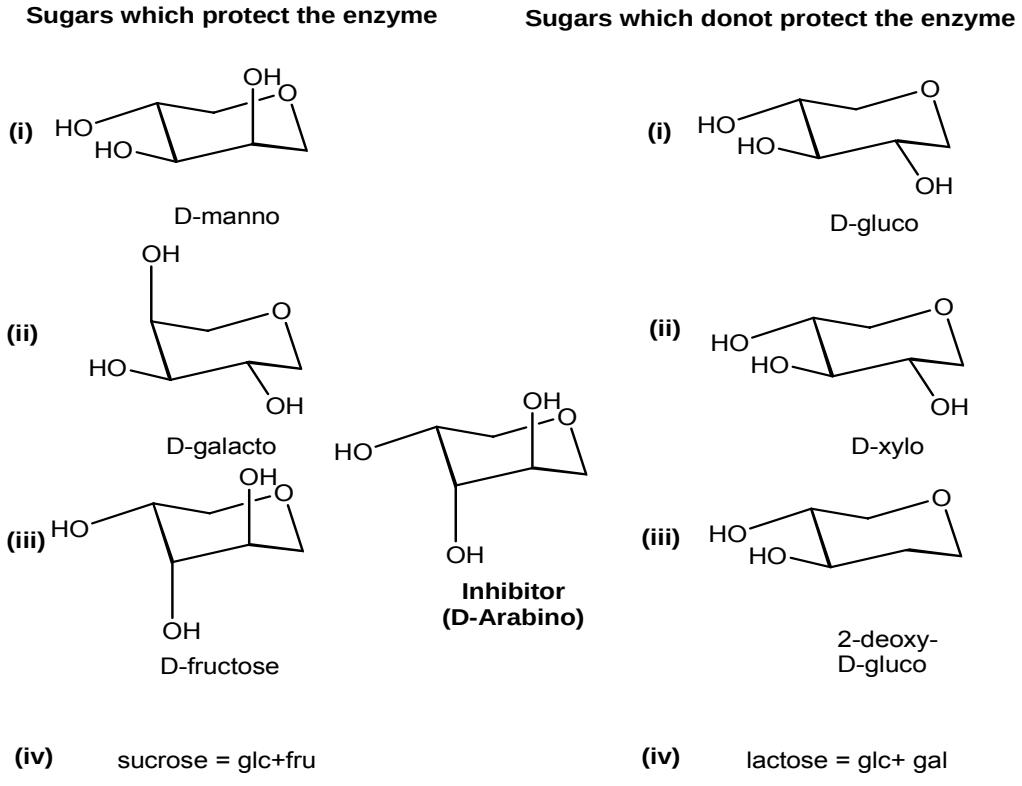
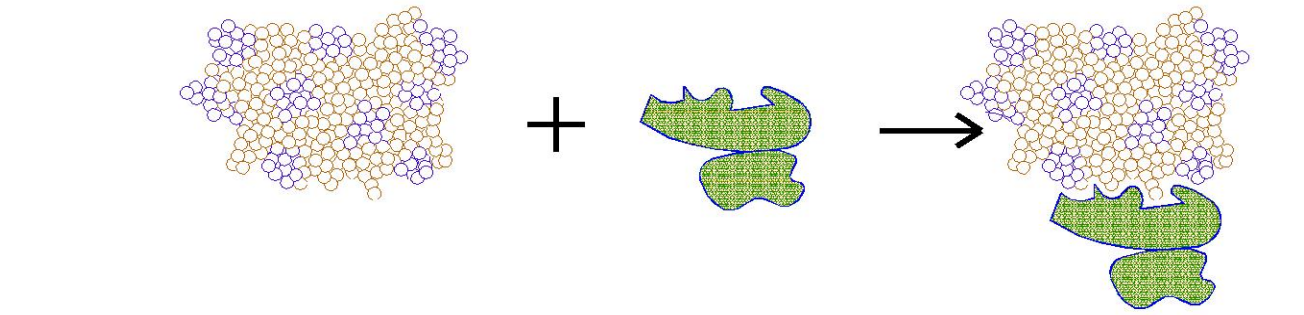
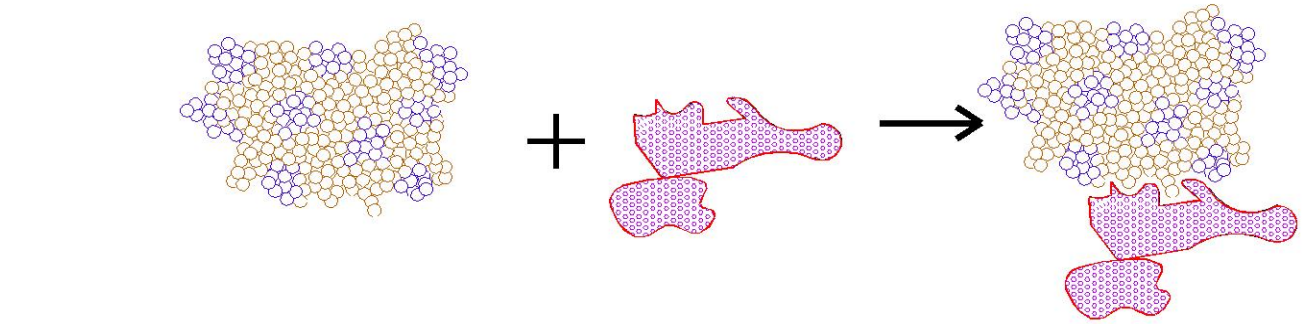



**Chart 4.2 Substrate protection studies**

## Binding of Enzyme to Substrate and Sugar mimetic



Beta- fructo furanosidase (E) Sucrose (S) E+S Complex



Beta- fructo furanosidase (E) Sugar mimetic (I) E+I Complex

**Chart 4.3**

**4.5 DISCUSSION:**

The present study dealt with the evaluation of the activity of the enzyme β fructofuranosidase isolated from *Kluyveromyces marxianus* NCYC 2675 in the presence of four pseudo-disaccharides **A-D**. The compounds **A** and **D** show significant inhibition (above 50%) of invertase activity. The effect of variable exposure time, incubation time and temperature were studied using compound A and the optima for each were determined. The effect of variable substrate concentration was also analyzed. Protection studies with several sugars were carried out and the relative affinities were examined.

The natural substrate of invertase is sucrose, which is comprised of a glucopyranose and a fructofuranose moiety linked by an O-glycosidic bond. The synthetic compounds **A** to **D** also have a furanose-pyranyl linkage, although without any spacer (direct-linked) and their structural configurations allow them to inhibit invertase. Hence the study of invertase activity in their presence was the most logical course of action. For this purpose, a homogeneous preparation of β-fructofuranosidase enzyme from *Kluyveromyces marxianus* NCYC 2675 was used.

The inhibitors **A** and **D** are promising in the treatment of diabetes, either directly or may act as 'leads' for the synthesis of such drugs. They could be used as monotherapy or adjunct therapy for poorly controlled non insulin dependant Diabetes Mellitus (NIDDM). They also might prove to be useful as artificial sweeteners. However the application of the inhibitors for the above requires further study.

The determination of active-site residues of the enzyme invertase with the use of inhibitors is another area to be explored.

# CHAPTER 5

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# IMMOBILIZATION OF K MARXIANUS CELLS ON GELLAN GUM, AEROGEL AND β FRUCTOFURANOSIDASE ON MESOPOROUS SILICA

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## 5.1 INTRODUCTION:

Immobilization means associating the biocatalyst with an insoluble matrix, so that it can be retained in proper reaction geometry for its economic reuse under stabilized conditions [D'Souza 1989]. Immobilization of cells or enzymes helps in their economic reuse, and development of continuous bioprocesses. Enzyme immobilization is a technique that aims at biocatalyst separation from the product and localization within a reactor. Immobilization can also provide a better microenvironment for enzyme activities enhancing better thermal and operational stability.

Immobilization on an insoluble support converts an enzyme to a recoverable solid catalyst, which may offer several advantages such as enhanced stability, activity retention, easier product recovery and purification, possibility of continuous processes over extended periods of time, increased enzyme activity and repetitive enzyme use [Mattiasson 1983]. Although in most cases, only successful enzyme immobilization data are published, it has become increasingly apparent that a universal method for enzyme immobilization is currently not feasible. This is because of the nature of the structure-function relationship of enzyme activity. In effect, small changes at the surface of an enzyme, resulting in conformational changes in the polypeptide backbone, can greatly impact the catalytic activity of the immobilized enzyme.

Immobilization of whole cells has been shown to be a better alternative to immobilization of enzymes [Junter and Jouenne 2004]. Immobilized cells have been used in a variety of applications such as biotransformation [Phadtare *et al* 2004], biosensors [Gu *et al* 2004], production of ethanol [Oztop *et al* 2003], degradation of phenol [Mordocca *et al* 1999), waste water treatment (Hamedaani *et al* 2003) etc. The need of using immobilized cells is mainly for obvious economic benefits of continuous production or repeated use in continuous as well as batch mode. The advantage of an immobilized system of whole cells with cell bound activity as opposed to the immobilized enzyme is that the costly and tedious process of enzyme concentration and purification are avoided. It obviates the need for the removal of unwanted macromolecules released during the extraction steps. Whole cells are more stable and easier to prepare than cellular components and are capable of detecting and responding to a wide range of biological active compounds. It preserves the natural environment of the enzyme thus protecting it from inactivation either during immobilization or its subsequent use in a continuous system. It may also provide a multipurpose catalyst especially when the process requires the participation of number of enzymes in sequence. Microorganisms like yeast cells, bacteria, fungi etc. have been immobilized on a variety of matrices such as agar, cellulose, calcium alginate, carrageenan, polyacrylamide, agarose etc. (Chibata

1989, Bucke 1987, Skryabin and Koscheenko 1987). The major limitations which may need to be addressed while using such cells are dispersion of cells, flow of nutrients away from cells, diffusion of substrate and products through the cell wall, and unwanted side reactions due to the presence of other enzymes. The cells can be immobilized either in a viable or non-viable form. The main disadvantage however is of relatively lower activities that are generally present in whole cells compared to the purified enzyme preparation entailing extended conversion period or greater catalyst loading. This disadvantage is partially overcome with mutants or constructs with higher enzyme activities.

The main advantage of viable immobilized cell cultures over conventional ones is:

a) Higher reaction rates due to increased cell densities

b) Possibilities of regenerating the biocatalytic activity of immobilized cell structures

c) Ability to conduct continuous operations at high dilution rates without washout

d) Easier control of the fermentation process

e) Long term stabilization of the fermentation process

f) Reusability of the biocatalyst

g) Higher specific product yields.

## 5 A: WHOLE CELL IMMOBILIZATION USING GELLAN GUM:

### 5A.1: SUMMARY:

The microbial extracellular polysaccharide gellan gum has been explored for its application in whole cell immobilization. Preliminary evaluation of the physicochemical properties of gellan gum in the presence of monovalent cation tetramethyl ammonium chloride (TMACl) was carried out. Attempts have been made to reveal the evaluation of gellan gum as a matrix to immobilize a thermotolerant yeast *Kluyveromyces marxianus* NCYC 2675. A $3^2$ factorial design was used to study the simultaneous effect of two variables. The effect of the polymer concentration and TMACl concentration on various dependent variables like gelling temperature, mean particle size and enzyme activity of the yeast cells were studied. A statistical model with a significant interaction term was obtained to predict the results. The optimized immobilized system showed enhanced reusability with maximum conversion of > 90 % when cross-linked with glutaraldehyde. Gellan gum immobilized cells demonstrated better rheological properties than gellan gum.

### 5 A.2: INTRODUCTION:

For decades, microbial exopolysaccharides have been used as important materials in the food industry, cosmetics, pharmaceuticals and related biomedical industries [Nishinari and Takahashi]. Gellan gum an anionic heteropolysaccharide first discovered in 1978 is produced by strains of the species *Sphingomonas elodea* formerly known as *Pseudomonas elodea* [Kang et al 1982]. It consists of tetrasaccharide repeating units of β-D-Glucose, β-D-Glucuronic acid, β-D-Glucose and α-L-Rhamnose (Figure 5.1). Commercially available gellan gums are: high acyl (HA) and low acyl (LA) gellan. Among the two, high acyl gellan gum has glycerate residues, which forms weak and rubbery gels. Deesterification of native gellan gum by alkali treatment yields low acyl gellan gum, which forms hard and brittle gels [Chandrasekaran and Radha 1995]. This polysaccharide is widely used in the food industry and biotechnology because it forms a transparent gel which is resistant to heat and acid when compared to other polysaccharides. The precise gelation properties depend on the cation present.

In the present study gellan beads were formed by ionotropic gelation method. Effect of monovalent cation on the gelling property of gellan gum was studied to explore the use of gellan gum as an immobilization matrix for immobilizing catalytically active microbial cells.

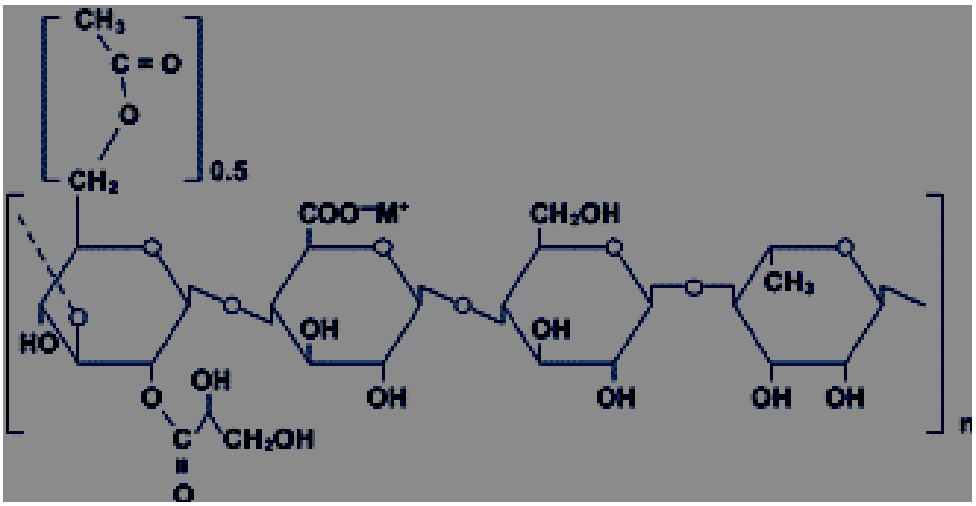
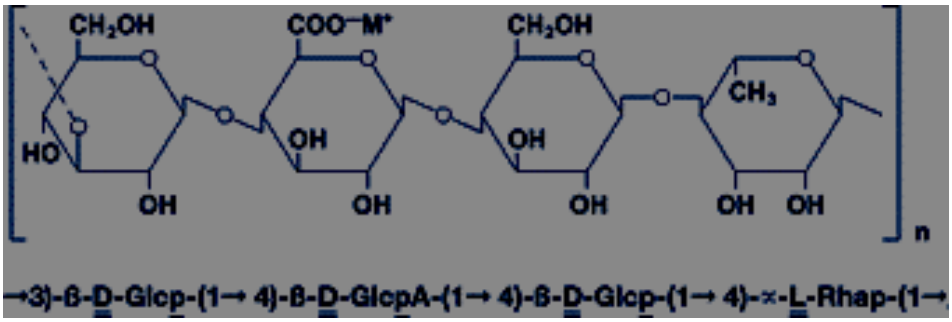



**Fig 5.1A**




β D-Glucose-β D Glucuronic acid-β D Glucose-α L Rhamnose

**Fig 5.1B**

**Fig 5.1**: Repeating unit of chemical structure of

(A) native (high acyl) gellan gum

(B) deacetylated (low acyl) gellan gum

## 5 A.3: MATERIALS AND METHODS:

### 5 A.3.1: Materials:

Gellan gum (Kelcogel F – low acyl and Kelcogel LT100 – high acyl) was obtained from CP Kelco U.S. Inc., USA. Tetra methyl ammonium chloride (TMACl) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai. All other chemicals were of analytical grade from local suppliers. Yeast cells *Kluyveromyces marxianus* NCYC 2675 were maintained routinely on MSYP medium containing 2 g % sucrose.

### 5A.3.2: Methods:

#### 5A.3.2.1: Preparation of gellan gum beads:

Gellan gums (Kelcogel F and Kelcogel LT100) were used in this study. Polymer was dispersed in double distilled preheated water (80$^{o}$C) by gentle stirring. The temperature was raised to 90$^{o}$C to achieve complete hydration of the polymer. Gellan beads were prepared using gellan solutions of different strength and combinations (1% w/v, 1.5% w/v, 2% w/v) by dropping this solution into $CaCl_2$ solution (2%w/v) under gentle stirring. The beads were filtered and dried at room temperature.

#### 5A.3.2.2: Cell Loading:

Whole cells of *Kluyveromyces marxianus* were immobilized using gellan gum by gel entrapment method. With the aim of lowering the gel setting temperature during the cell entrapment process while maintaining high mechanical properties, gellan beads of low acyl gellan gum (2%w/v) containing EDTA (0.8%w/v) was used to entrap yeast cells *Kluyveromyces marxianus*. Concentrations of cells ranging from 5%w/v to 20%w/v were evaluated.

#### 5A.3.2.3: Enzyme activity assay:

The enzyme activity of cells in immobilized form towards sucrose (2% w/v) was estimated by 3, 5-dinitro salicylic acid (DNSA) method as described in CHAPTER 2 of the THESIS.

#### 5 A.3.2.4: Swelling Studies:

Swelling study for different gellan concentrations were performed to determine the water holding capacity of the polymer. Water sorption capacity of gellan beads was determined by swelling the dried gel beads (0.1 gm) in deionized water (50 ml). Swelling study of the optimized batch (M-6) was carried out to check the water holding capacity of the polymer before and after immobilization.

**5A.3.2.5: Factorial design:**

Factorial design approach was adopted which showed interactions between factors that a "one factor at a time" model couldn't reveal. The levels of factors were independently varied, each at three levels (Table-5.3). The responses Yi were measured for each trial. A polynomial equation was constructed where the coefficients in the equation were related to the effects and interactions of the factor. Interactive statistical first order complete model was first generated to evaluate the selected responses.

$$Y = \beta_O + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 \quad \text{(Equation 1)}$$

The main effects ($X_1$ and $X_2$) represent the average result of changing one factor at a time from its low to high value. The interaction ($X_1X_2$) shows how the dependant variable changes when two or more factors are simultaneously changed. UNISTAT® version 3 for Windows™ was used to obtain the equation. Multiple regression analysis and f statistics were used to identify statistically significant terms [Bolton 1997]. Only those terms, which were statistically significant, were retained in the equation. The wet weight of yeast cells *Kluyveromyces marxianus* was kept constant (15% w/v) for all the batches from M-1 to M-9 (Table 5.3). Different parameters like gelling temperature, mean particle size, enzyme activities with and without glutaraldehyde were determined to characterize the loaded gellan beads.

**5 A.3.2.6: Operational stability:**

Invertase activity of immobilized cell system was assayed in 10ml reaction mixture at 40$^{o}$C in a jacketed vessel under stirring. Aliquots of 0.1ml were withdrawn after every one hour to estimate the glucose formed. Cross-linking with glutaraldehyde prevented leaching of the cells from the matrix into the assay medium. The parameters for cross-linking were optimized by varying the concentration of glutaraldehyde. 0.01% w/v, 0.05% w/v and 0.1% w/v were used. After every cycle the beads were washed thoroughly with double distilled water followed by the addition of fresh substrate [Bahulekar *et al* 1993].

**5A.3.2.7: Rheology study of gellan immobilized cells and gellan gum:**

To evaluate the gellan gum as an entrapment matrix, temperature dependent rheological study was performed. Rheology study was done on ARES – Rheometric scientific strain controlled parallel plate rheometer. Elastic modulus (G') and complex viscosity (Eta) were determined. To promote molecule hydration and to lower the gel setting temperature of gels, monovalent cation TMACl was added.

**5A.4: RESULTS:**

**Table 5.1**

**Preparation of Gellan beads**

| Batch No. | Concentration of Gellan Gum (%w/v) | |
|---|---|---|
| | Low Acyl | High Acyl |
| 1. | 1.0 | - |
| 2. | 1.5 | - |
| 3. | 2 | - |
| 4. | 2 | 0.5 |

**Table 5.2**: **Effect of cell loading on enzyme activity**

| Concentration of cells | Enzyme activity(U) |
|---|---|
| 5.0% | 2.32 |
| 10.0% | 9.7 |
| 15.0% | 17.43 |
| 20.0% | 23.55 |

Maximum enzyme activity was obtained at 20% cell loading (Table 5.2).Considering the feasibility for immobilization and easy scale up, 2% w/v low acyl gellan gum and 15%w/v concentration of cells were selected for cell immobilization and factorial design study.

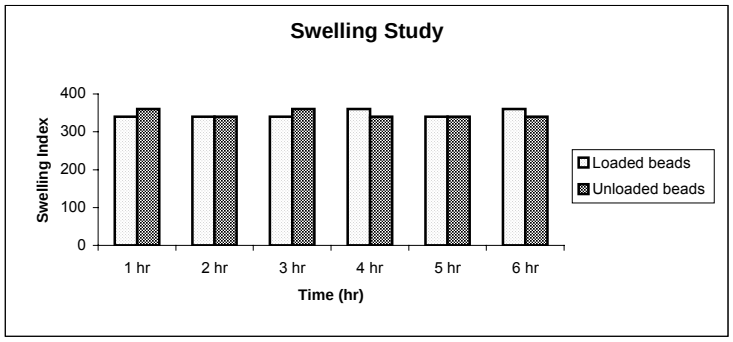



**Fig 5.2:Swelling study of beads**

As shown in **Fig 5.2** overall water uptake for loaded and unloaded beads were similar, which suggested that immobilization did not affect the swelling characteristic of the polymer [Mao *et al* 2001].

**Table-5.3: Evaluation data of different batches**

| Batches | Variable level in coded form* | | Gelling Temperature (ºC) | Mean Particle Size (μm) | Enzyme Activity (U) | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | | | With Glutaraldehyde | Without Glutaraldehyde |
| **M-1** | -1 | -1 | 37.5 | 170.87 | 12.83 | 6.63 |
| **M-2** | 0 | -1 | 40 | 175.15 | 18.14 | 16.17 |
| **M-3** | +1 | -1 | 44 | 285.98 | 20.21 | 24.96 |
| **M-4** | -1 | 0 | 33 | 121.42 | 13.49 | 14.65 |
| **M-5** | 0 | 0 | 39 | 147.01 | 15.16 | 22.49 |
| **M-6** | +1 | 0 | 43 | 135.10 | 22.69 | 26.78 |
| **M-7** | -1 | +1 | 40 | 130.94 | 12.68 | 14.45 |
| **M-8** | 0 | +1 | 41 | 140.01 | 20.11 | 23.30 |
| **M-9** | +1 | +1 | 47 | 158.64 | 30.42 | 24.96 |

| *Coded values | Actual values | |
|---|---|---|
| | $X_1$- Amount of Polymer | $X_2$ - Amount of TMACl |
| -1 | 1%w/v | 0.025 M |
| 0 | 1.5% w/v | 0.05 M |
| +1 | 2% w/v | 0.075 M |

Equation $Y = \beta_O + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2$

As shown in Table 5.3 (M-6 batch) both the yield (> 95%) and enzyme activity (> 15 U) were satisfactory.

The following equation was obtained for the gelling temperature.

$$Y = 38 + 4.416\ X_1 + 4.25\ X_2\ X_2 \quad \text{(Equation 2)}$$

The average gelling temperature obtained was 38ºC. Value of both the coefficient indicated that both the variables affected the gelling temperature positively. As gellan gel formation occurred through ion-induced association of double helices, the gelling temperature was increased with increasing ionic concentration of gellan gum. TMA – gellan molecules in low ionic strengths of TMACl behaved as highly elongated, non – aggregating chains. The TMA salt of the gellan gum was readily soluble in salt free water, but addition of higher concentrations of TMACl resulted in precipitation or gel formation, depending on the concentration of salt and gum [Gunning and Morris 1990]. This was clearly seen in the curvilinear relationship obtained in the graph (Fig5.3a).

The following equation was obtained for the particle size,

$$Y = 133.208 + 25.06\ X_1 - 34.210\ X_2 + 48.158\ X_2X_2 - 21.88X_1X_2 \quad \text{(Equation 3)}$$

The average particle size obtained was 133.20 μm. It can be seen that the mean particle size was significantly affected by both polymer and TMACl concentrations. As the gellan gum solution concentration increased the viscosity was higher and hence larger droplet size was obtained, which did not undergo size reduction at the given shear stress. The rpm was kept constant for all the batches and was not effective to break the particles into smaller size at higher polymer concentration. This resulted in greater particle size with increasing polymer concentration and hence a positive coefficient for $X_1$. Predominantly affecting factor was TMACl concentration with a negative sign of the coefficient indicative of inverse relationship. TMA – gellan molecules in low ionic strengths of TMACl behaved as highly elongated, non-aggregating chains. TMA salt of the gum was readily soluble in salt free water, but addition of TMACl to high concentrations resulted in precipitation or gel formation, depending on the concentration of the salt and gum [Gunning and Morris 1990]. As the amount of polymer was increased, the viscosity of the dispersion went on increasing but with the zero level concentration of TMACl viscosity of the solution was optimum (Fig 5.3b). The rpm was kept constant for all the batches and was not effective to break the particles into smaller size at higher polymer concentration. The negative value of coefficient $\beta_{12}$ indicated a decrease in particle size when the two factors were simultaneously increased.

Following equation was obtained for enzyme activity with glutaraldehyde

$$Y = 17.37 + 5.648\ X_1 + 2.5341\ X_2 + 2.605\ X_1X_2 + 1.705\ X_2X_2 \quad \text{(Equation 4)}$$

For gellan beads cross-linked with glutaraldehyde, mean enzyme activity was 17.37 U. Higher amount of polymer (2% w/v) was efficient for better ionotropic gelation leading to higher enzyme activity. Above discussion can be illustrated in surface graphs (**Fig 5.3B and 5.3C**).

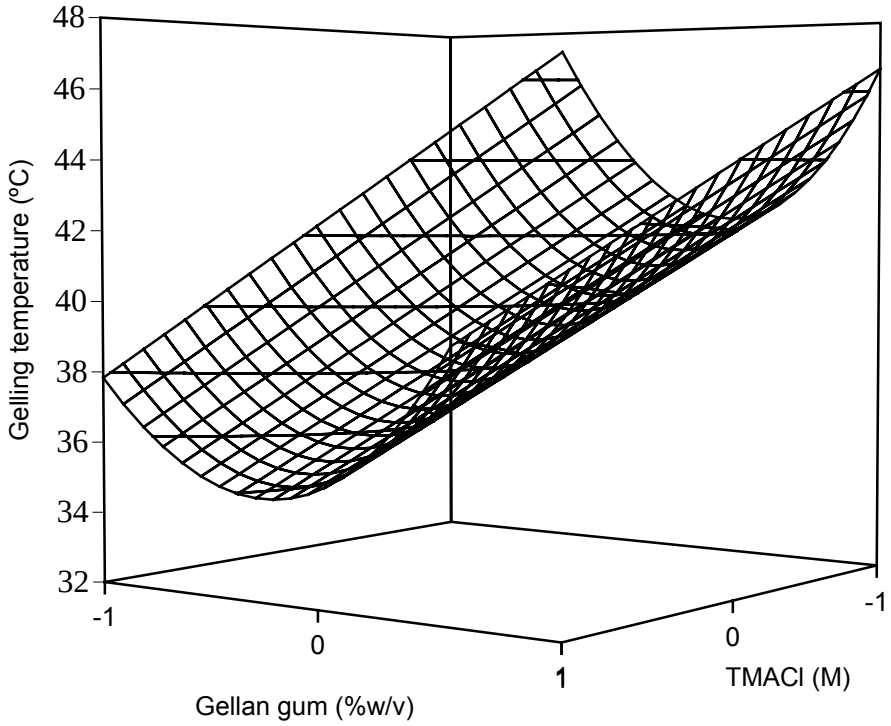



**Fig 5.3A Surface graph showing effects of variables on gelling temperature**

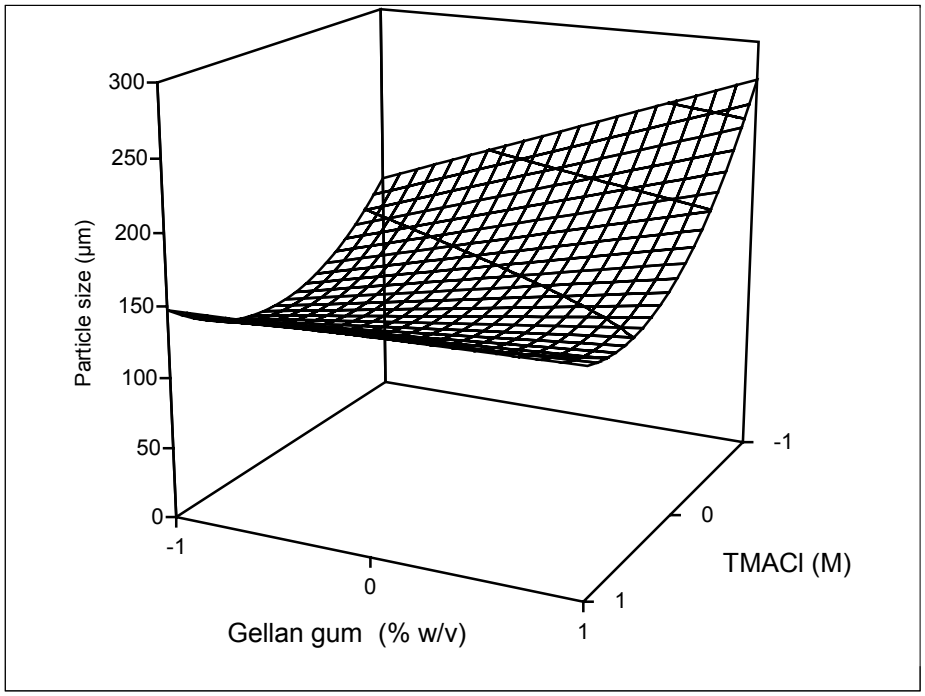



**Fig 5.3B**: **Surface graph showing effects of variables on mean particle size.**

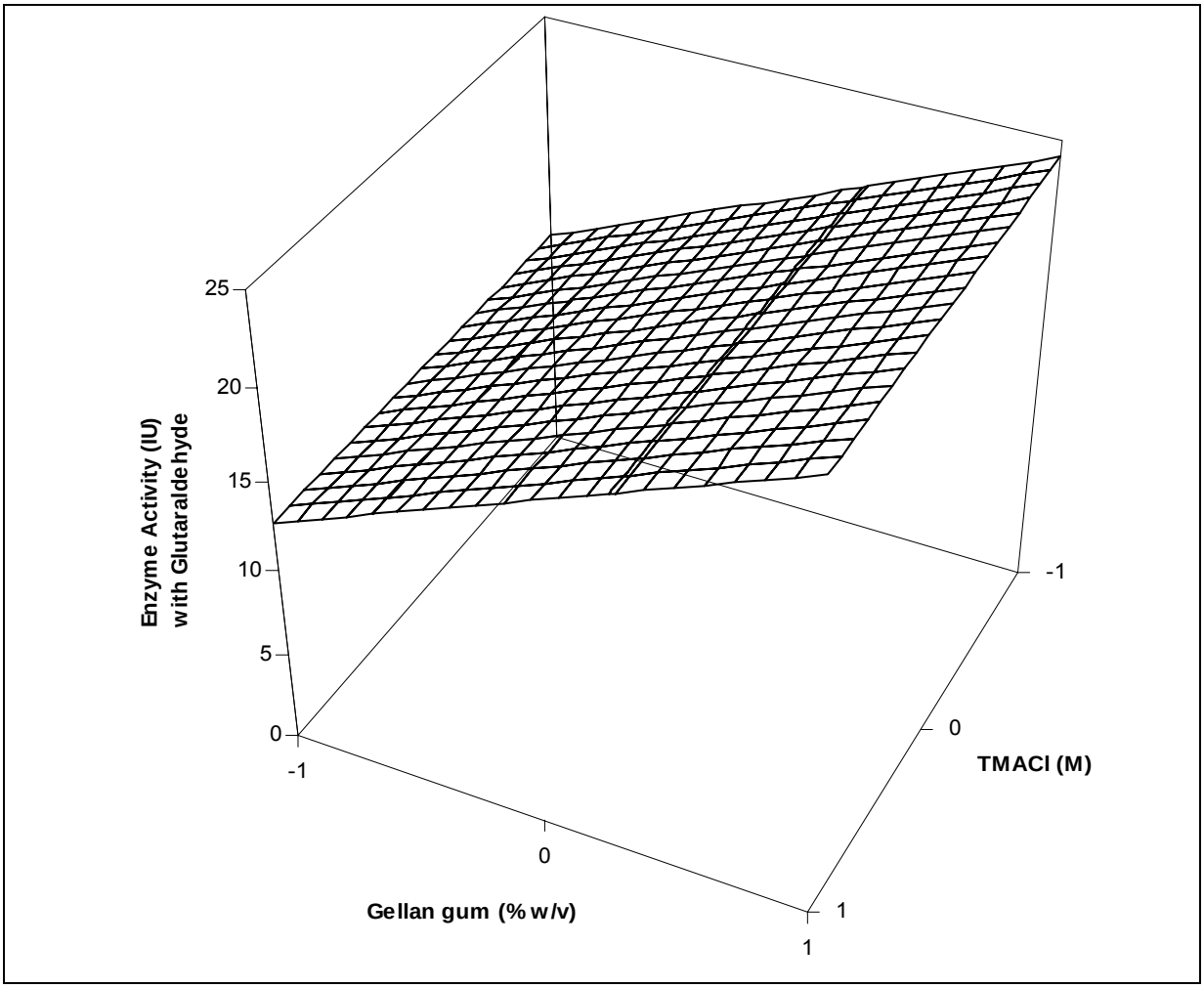



**Fig 5.3C**: **Surface graph showing effects of variables on enzyme activity with glutaraldehyde.**

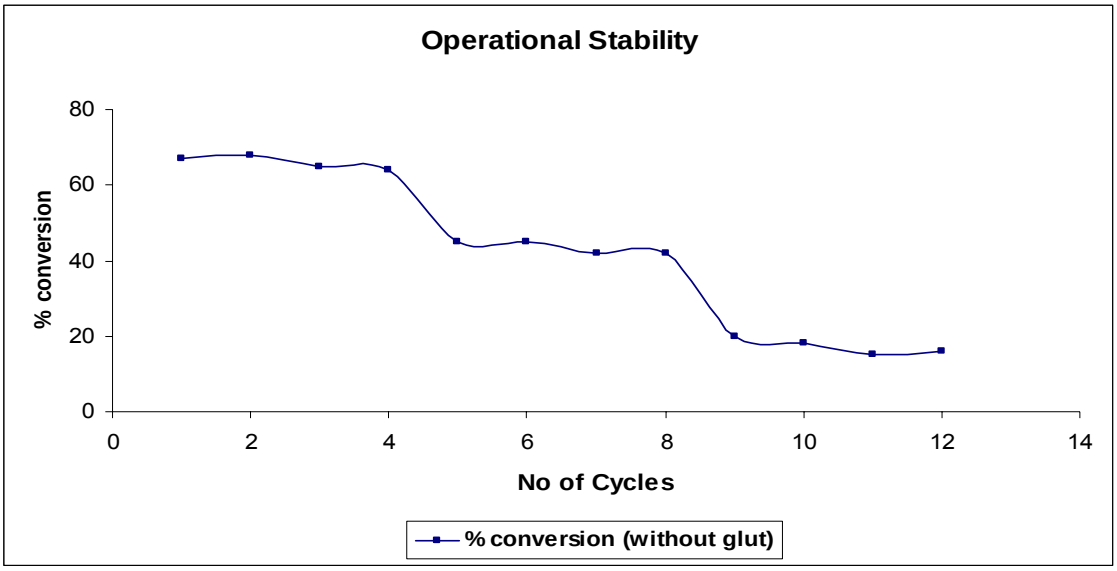



**Fig 5.4 A**

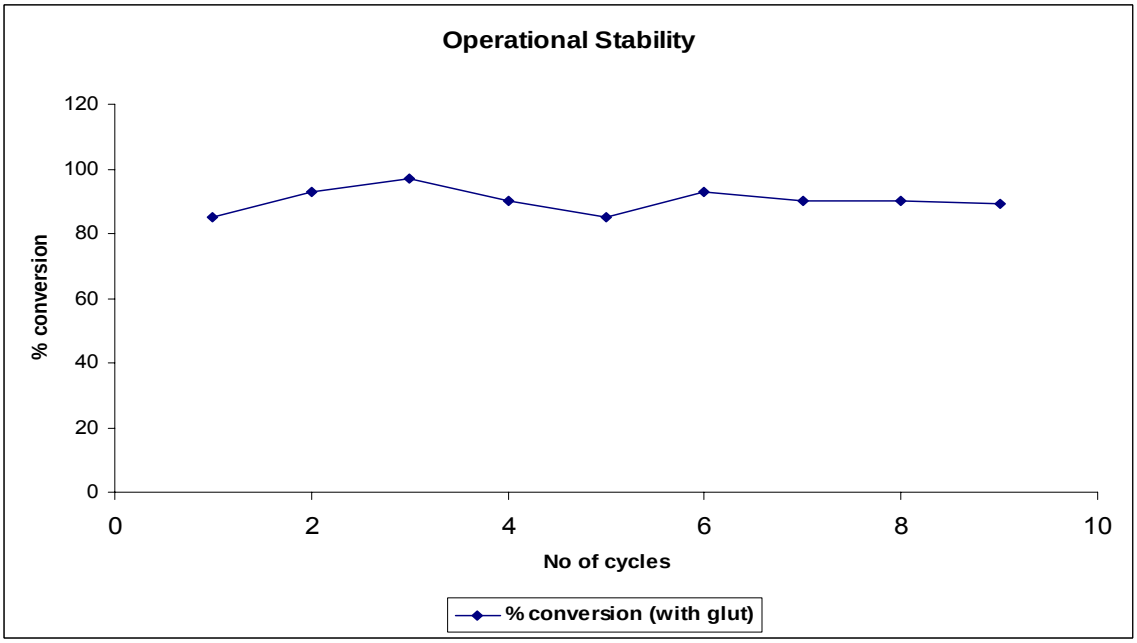



**Fig 5.4 B**

**Fig 5.4(A) and (B): Operational stability of *Kluyveromyces marxianus* immobilized on gellan gum (A) without glutaraldehyde and (B) with glutaraldehyde**

Operational stability was evaluated in batch mode as stated in MATERIALS AND METHODS. Reaction was terminated when half-life of the system was attained. It was found that entrapped cells were leached after every cycle and resulted in decreasing percentage of conversion with maximum conversion of 70%. To retain the cells cross-linking with bifunctional reagent glutaraldehyde was used, as it is relatively non-toxic

having mild reaction conditions. Use of glutaraldehyde (0.05% w/v) maintained maximum conversion level for nine cycles (>90%) preventing loss of the cells

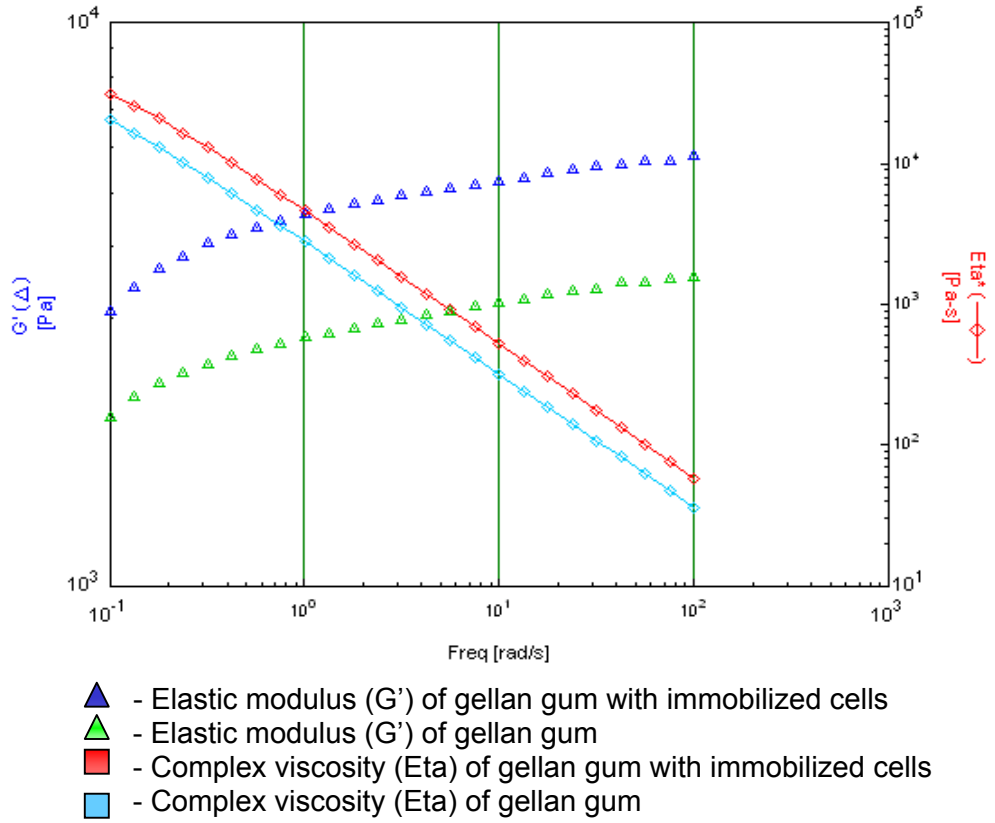
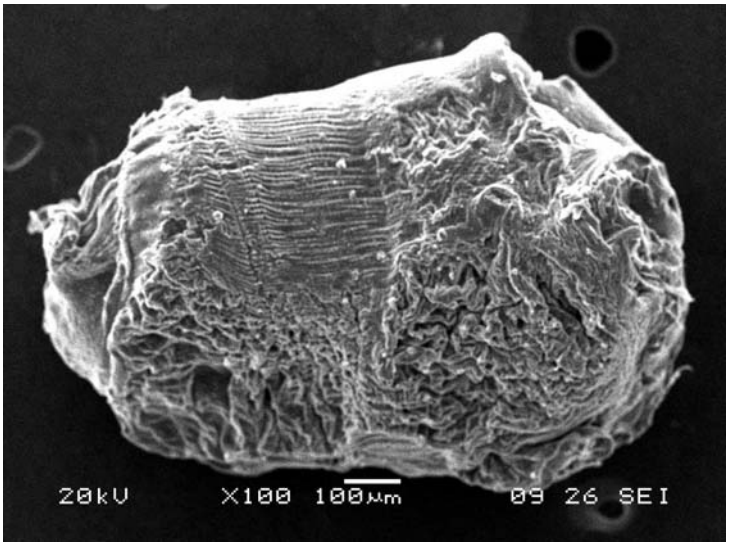


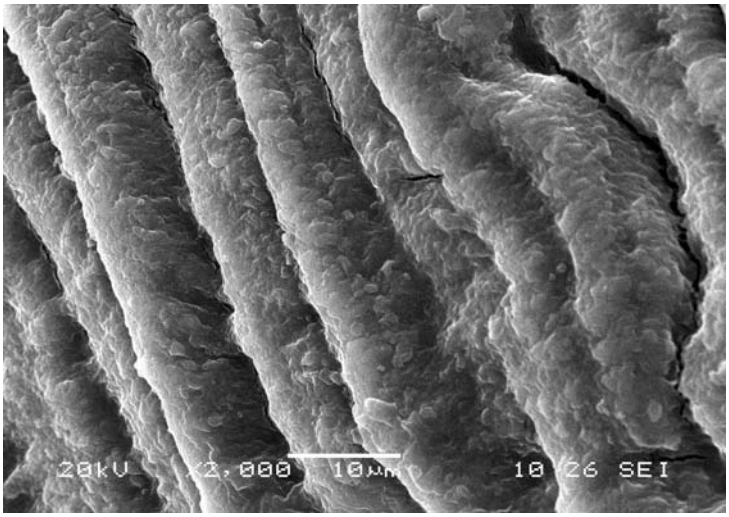

**Fig 5.5**: **Rheological study of gellan immobilized cells and gellan gum.**

The complex viscosity (Eta) was measured in the linear regime by applying 0.004 % strain (Fig5.5). In our study, at low frequency, complex viscosity values of gellan gum immobilized cells and gellan gum were 31,319 PaS and 20,259 PaS respectively. Rheology study of elastic modulus (G') and complex viscosity (Eta) of gellan immobilized cells and gellan gum indicated better elasticity and rigidity of polymer-immobilized cells as compared to gellan gum.

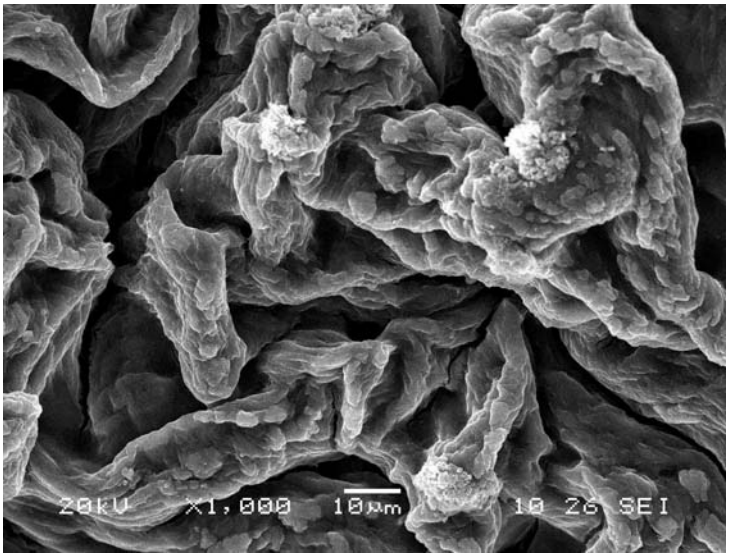



**Photograph 5.1 A**

**SEM of Gellan Bead**




**Photograph 5.1 B**
**SEM of Gellan Bead & Yeast**




**Photograph 5.1 C**
**SEM of Gellan Bead & Yeast**

**5A.5: DISCUSSION:**

Gellan forms double helices via carboxylate groups and subsequently aggregates to form a three-dimensional network in an appropriate aqueous environment. Both monovalent and divalent cations stabilized the network through cross-linking. Monovalent cations ($M^+$) cross link double helices indirectly (double helix – $M^+$ – water – $M^+$ – double helix), while divalent cations ($M^{++}$) cross link double helices directly (double helix – $M^{++}$ – double helix) [Chandrasekaran and Radha 1995,Tang *et al* 1997]. It is believed that gel formation occurs through ion-induced associations of double helices, which explains why the hydration of gellan gum was not possible above certain ionic concentration.

Rheological properties of aqueous gellan gum solutions were expected to change with the conformational transformation of gellan gum molecules. The dynamic rheology and confocal laser scanning microscopy (CLSM) of gellan at low concentrations (0.005-0.05w/v) in the presence of 10mM $CaCl_2$ have been investigated by Rodriguez et al in 2003.

Preliminary studies on the rheology and swelling properties of gellan gum suggested its potential for industrial and therapeutic applications. In this work *Kluyveromyces marxianus* NCYC 2675 cells were used as a source of β-D- fructofuranosidase enzyme source. Addition of monovalent cation like TMACl (0.05M) was effective for improving applicability of gellan gum (2% w/v) as an immobilization matrix. Enzyme activity of yeast cells (15% w/v) was optimum and amenable to easy scale-up. The factorial design study indicated that the parameters, polymer and TMACl concentrations selected had effects on particle size of the beads, percentage yield and enzyme activity. This system showed excellent storage capability when stored at 10˚C for more than three months with no loss of activity. The effect of cross-linking agent like glutaraldehyde was successfully evaluated to minimize leaching of the cells. Continuous product formation with maximum percentage conversion of >90% was achieved using this system in batch mode.

Polysaccharide gel matrices, more particularly Ca-alginate hydrogels [Gerbsch and Buchholz 1995, Kailapathy 2002] are by far the most frequently used materials for harmless cell entrapment. Further, a novel matrix such as gellan gum can also be used for controlled delivery of therapeutic molecules and for the formulation of sustained release beads [Rajnikanth *et al* 2006].

## 5 B: IMMOBILIZATION OF INVERTASE ON MESOPOROUS SILICA -SBA 15:

### 5 B.1: SUMMARY:

Invertase, which catalyses the hydrolysis of sucrose, is one of the simplest commercial carbohydrases and is used for the production of invert syrup (equimolar mixture of glucose and fructose). Fructose is the sweetest natural sugar having beneficial medicinal effects and is emerging as an alternative sweetener to sucrose in the pharmaceutical industry. β fructofuranosidase (E C 3.2.1.26) purified from *Kluyveromyces marxianus* NCYC 2675, a thermotolerant yeast, was immobilized on functionalized mesoporous silica SBA- 15. Attempts were made to immobilize invertase on functionalized silicalite and cross linked with glutaraldehyde so as to obtain a highly active and stable immobilized enzyme preparation suitable for commercial exploitation. Cross linked enzyme silica complex as well as enzyme silica complex showed very good temperature and pH stability. Extrudates of the mesoporous silica also showed similar results. Bound enzyme showed excellent stability to repeated use and retained 90% of its initial activity after ten cycles of reuse in batch mode.

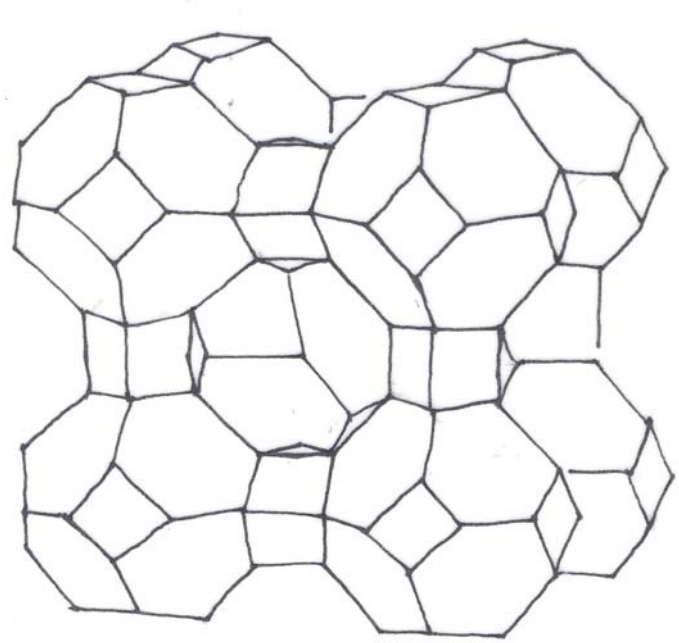
### 5 B.2: INTRODUCTION:

There are various reports of invertase immobilized on organic/ inorganic supports such as rice husk, corn grits [Monsan and Combes 1884], clay, lectin, polyacrylamide, chitosan [Cheng et al 2005] etc. Invertase has been immobilized via physical adsorption on hydrogels [Arslan et al 2000], covalent bonding [Bora *et al* 2005 ionic bonding [Tomotani and Vitolo 2004] and entrapment in alginate beads / capsules [Meena and Raja 2004], sol-gel beads [O'Neil *et al* 2002] and by bioaffinity layering [Yavuz *et al* 2004]. There are many reports of immobilization on polymers/ copolymers [Yildiz *et al* 2005] and microspheres [Arica 2001].

Silica supports have number of advantages, including chemical and mechanical stability, non hydrophobicity, biological inertness and resistance to microbial attack, and are stable at elevated temperatures. Silica sol-gel particles have been used for entrapment of enzymes [Pierre 2004]. Invertase has been immobilized on aminopropyl silica activated with humic substances [Rosa *et al* 2000]. Mesoporous silica materials have 1) a uniform pore size distribution 2) a high surface area and pore volume and are used to immobilize biocatalysts. The immobilization on SBA-15 of different proteins and enzymes such as cytochrome C, trypsin, amylase, horseradish peroxidase, lipase, and lysozyme has been recently investigated [Salis *et al*, Pandya *et al* 2005]. However there are no reports of immobilization of invertase on mesoporous silica SBA- 15.

We have developed method using functionalized mesoporous silica SBA 15 for immobilization of invertase. Cross-linking the enzyme with glutaraldehyde could enhance stabilization of the immobilized system. Extrudate-enzyme complexes showed retention of enzyme activity to a greater extent. An advantage of using extrudates is their easy separation and ease of handling, with no loss of matrix and rigid material. They are useful in the continuous production of invert syrup.

**Photograph 5.2 Zeolite 4A°**



**5 B.3: MATERIALS AND METHODS:**

**5 B.3.1: Materials:**

3-5 Dinitrosalicylic acid was obtained from Koch Light Laboratories Ltd. USA. All other chemicals used were of analytical grade. All buffers were essentially prepared in glass-distilled water. Mesoporous silica was obtained from Division of Catalysis; NCL, Pune.

**5 B.3.2: Methods:**

**5 B.3.2.1: Synthesis of SBA-15:**

SBA-15 was prepared by using the templating effect of non-ionic triblock copolymer surfactants according to a previous report by Zhao *et al*. Briefly 4g of Pluronic P123 copolymer was dispersed in 30g of distilled water and stirred for 6 hours. To the

resultant solution 120g of 2M HCl was added under stirring. After one hour of stirring at 40˚C, 8.5g of tetraethylorthosilicate (TEOS) was added drop wise and the mixture was maintained at 40°C for 24 hours under stirring conditions. It was then aged for 48 hours at 100˚C under static conditions in a Teflon lined autoclave. The crystallized product was filtered, washed with warm distilled water and dried at 100°C for 24 hours and then calcined at 550°C in nitrogen. Calcination was done at the rate of 1˚C/min and maintaining the furnace at 550°C for 4 hours. The structure was confirmed by XRD and surface area.

**5 B.3.2.2: Chemical modification of SBA-15:**

In a typical surface modification process (as described by Salis *et al* 2005), 3g of Silica SBA-15 was freshly activated at 150°C under vacuum, refluxed in 50ml toluene, dried and distilled with 1g of 3- aminopropyl triethoxysilane for 3 hours under an inert nitrogen atmosphere. The solid was filtered, washed with diethyl ether and soxhlet extracted with 250ml dichloromethane, obtaining covalently anchored 3 aminopropyl triethoxysilane moiety. It was dried at 80°C under vacuum.

**5 B.3.2.3: Enzyme Purification and assay:**

Enzyme purification was carried out as described in CHAPTER 3 of the THESIS after growing the cells on medium having the following composition in g/L.$Na_2HPO_4.7H_2O$ 12.8, KH2PO4 3.1, NaCl 0.5, NH4Cl 1, MgSO4.7H2O 0.5, Glucose 4.0, Yeast extract 2.0, Sucrose 50.0. Enzyme activity was performed as described in CHAPTER 2 of the THESIS.

**5 B.3.2.4: Immobilization of enzyme on functionalized SBA15:**

Formation of (M- E) complex:

1 g of functionalized SBA –15 powder was suspended in approximately 10 ml of acetate buffer, pH 4.5 (50 mM). 1 ml of purified enzyme (6000 IU / ml, protein 2.2 mgs/ml) was added and kept at 10°C overnight (24 hours) with slow stirring. M-E complex was separated by centrifugation. Several washings with the same buffer removed unbound enzyme. The complex was further distributed into aliquots for pH, temperature and substrate stability studies.

Preparation of cross-linked enzyme:

Mesoporous silica enzyme and glutaraldehyde complex (M-E-G) was essentially prepared as mentioned above followed by cross-linking with glutaraldehyde (1% v/v) for one hour at 10°C. (M-E-G) complex was separated by centrifugation. Several

washings were given with 50mM acetate buffer to remove unbound enzyme and glutaraldehyde. The M-E-G complex was further distributed into aliquots.

**5 B.3.2.5: Preparation of Extrudates:**

These complexes were made using two different combinations such as functionalization before addition of Boehemite and after addition of Boehemite.

Extrudate X**:** The binder used for making the extrudate is Boehemite. 1g of pre functionalized mesoporous silica was mixed with 0.3g Boehemite and water to make extrudates of size 3-4 mm length and 1-2 mm diameter. They were dried in an oven at 100°C. Functionalization was done using 3 amino propyl triethoxy silane.

Extrudate Y**:** The method of preparation of these extrudates was same as described earlier. However the mixing of the Boehemite with mesoporous silica was done before functionalization of silica.

**5 B.3.2.6: Immobilization of enzyme on mesoporous silica extrudates:**

To ensure easy handling and recovery, zeolite extrudates X and Y were used for enzyme immobilization and following complexes prepared.

*Complex 1***:** The mixture contained functionalized mesoporous silica extrudates X (0.15gms), 1ml acetate buffer (50mM), 25μl glutaraldehyde (50%v/v). This was allowed to stand for one hour at 10°C (with intermittent stirring). The supernatant was decanted to remove excess glutaraldehyde. The complex was resuspended in 1ml acetate buffer containing 100μl of invertase and kept for one hour at 10°C.The complex was washed with acetate buffer and invertase activity was determined.

*Complex 2*: Same procedure was repeated as in Complex 1 except that extrudate Y was used.

All the four complexes (M-E, M-E-G, 1, 2,) were given ten washings with 0.05M acetate buffer. Activity of the supernatant and complexes was checked after each washing. Activity of the complexes was observed to be constant after repeated washings and no enzyme activity was observed in the supernatant.

**5 B.3.2.7: Temperature Profile:**

(M-E), (M-E-G) complexes [aliquot] were mixed with 0.5 ml acetate buffer [50mM, pH 4.5] and incubated at different temperatures ranging from 30°C to 80°C for 10 minutes. Enzyme assay was performed for each aliquot as described in enzyme assay.

Temperature profile of free enzyme was performed in a similar manner and served as control.

**5 B.3.2.8: Temperature Stability:**

(M-E) complex, (M-E-G) complex (aliquots) equivalent to free enzyme were mixed with 1ml acetate buffer 50mM, pH 4.5. The complexes were incubated at temperatures ranging from 30ºC to 80ºC for one hour. Supernatant was removed by centrifugation. Complexes were checked for the invertase activity. Temperature stability of free enzyme served as control.

**5 B.3.2.9: pH Profile:**

(M-E), (M-E-G) complexes equivalent to free enzyme were mixed with 0.5ml of buffers ranging from pH 4.0 to pH 10.0 and enzyme assay performed for each aliquot of complex. pH profile of free enzyme was also performed in a similar manner. Buffers used were acetate buffer pH 4.0 pH 5.0, phosphate buffer pH 6.0, pH 7.0, Tris buffer pH 8.0, carbonate bicarbonate buffer pH 9.0, pH 10.0.

**5.3.2.10: pH Stability:**

(M-E) complex (aliquot), (M-E-G) complex equivalent to free enzyme were mixed with 1 ml of buffer ranging from pH 4.0 to pH 10.0 and incubated for one hour at room temperature after thorough mixing. Supernatant was removed. Invertase activity of each aliquot was checked under standard conditions. pH stability of free enzyme served as control.

Similar profiles of pH and temperature study were carried out with complex 1 and 2 as described above.

**5 B.4 RESULTS:**

**Table 5.4**

Percentage Efficiency of the Complexes (activities of complex as compared with free enzyme)

| Complex | M-E | M-E-G | 1 | 2 |
|---|---|---|---|---|
| % Efficiency | 62±3 | 85±2 | 80±4 | 83±2 |

Enzyme activities of supernatant and complex were checked by invertase assay, and compared with that of free enzyme. Glutaraldehyde containing complex (M-E-G) showed higher invertase activity than the complex without glutaraldehyde (M-E). Therefore two types of complexes were further prepared using SBA-15 extrudates and glutaraldehyde as the coupling agent as described in methods. Complex 1 and Complex 2 also showed good efficiency (Table 5.4). All the complexes retained their activity when stored at 4°C for six months. Non functionalized SBA-15- enzyme complex showed leaching out of enzyme activity on repeated use. This supports the previous report of amylase [Pandya *et al* 2005] that in purely siliceous mesoporous solids, adsorption of the enzyme is relatively week which results into significant leaching of the enzyme during use.

**Table 5.5**

Temperature Stability (increase in % efficiency in comparison with free enzyme) of enzyme immobilized on SBA-15

| Temperature | 30°C | 40°C | 50°C | 60°C | 70°C | 80°C |
|---|---|---|---|---|---|---|
| Immobilized enzyme | -- | 5 | 20 | 45 | 50 | 60 |
| Cross-linked enzyme | 2 | 10 | 30 | 50 | 70 | 70 |

Each value is the ± standard error of the mean from five different replicates. One unit of enzyme is defined as the amount of enzyme required to liberate 1µ mole of reducing sugar/min under the assay conditions.

As seen in **Table 5.5** cross-linked immobilized enzyme showed good temperature stability at all temperatures ranging from 30°C to 80°C. Immobilized enzyme also showed an increase in efficiency as compared to free enzyme.

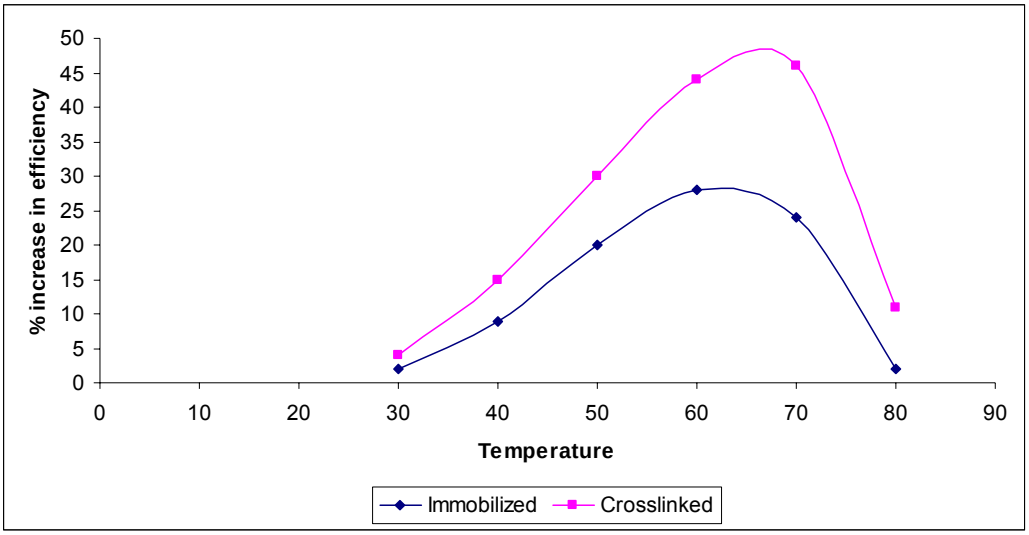



**Fig 5.7** Temperature profile of enzyme immobilized on SBA-15

As seen in **Fig 5.7**, enzyme immobilized on mesoporous silica and cross-linked enzyme showed slight increase in temperature profile in the range of 50 to 70°C as compared to free enzyme.

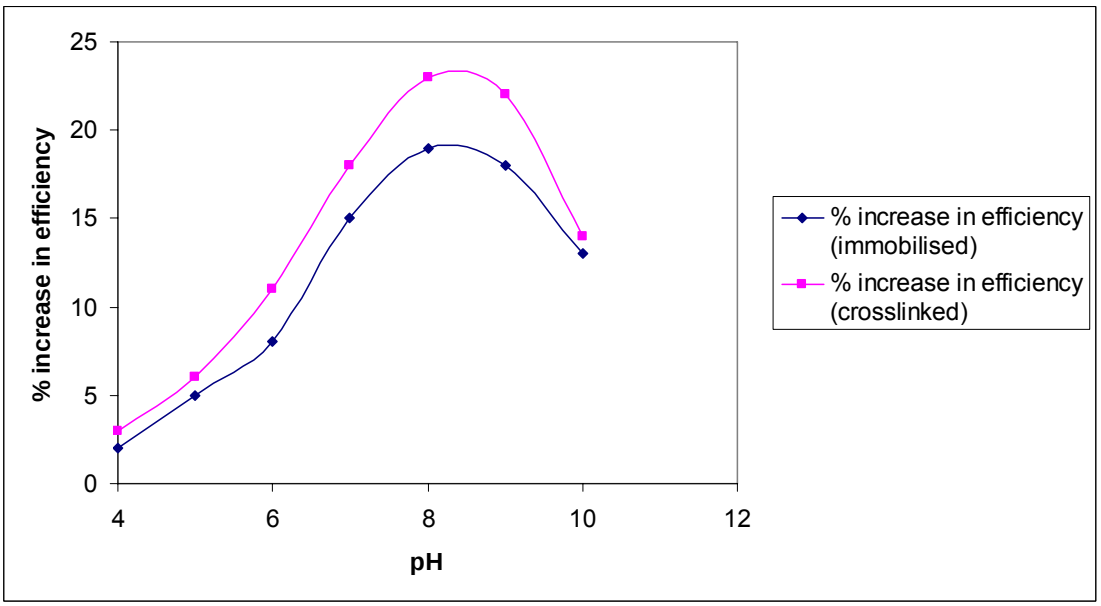



**Figure 5.7: pH profile of enzyme immobilized on SBA-15**

A change in pH profile was observed when compared with free enzyme as shown in **Fig 5.7**. Cross-linked enzyme showed a higher increase in efficiency than immobilized enzyme as compared to free enzyme.

**Table 5.6**

**pH stability (Increase in % efficiency in comparison with free enzyme) of enzyme immobilized on SBA-15**

| pH | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|
| Immobilized enzyme | no increase | no increase | 2 | 2 | 36 | 40 | 45 |
| Cross linked enzyme | 3 | 2 | 3 | 5 | 50 | 55 | 50 |

Data values given are means of three separate sets of experiments and the deviation from the mean is within 3%

**Table 5.6** shows pH stability of complexes, free enzyme loses 50% of it's original activity at alkaline pH above pH 8.0 whereas immobilized and immobilized cross linked enzyme shows apparently no loss of activity.

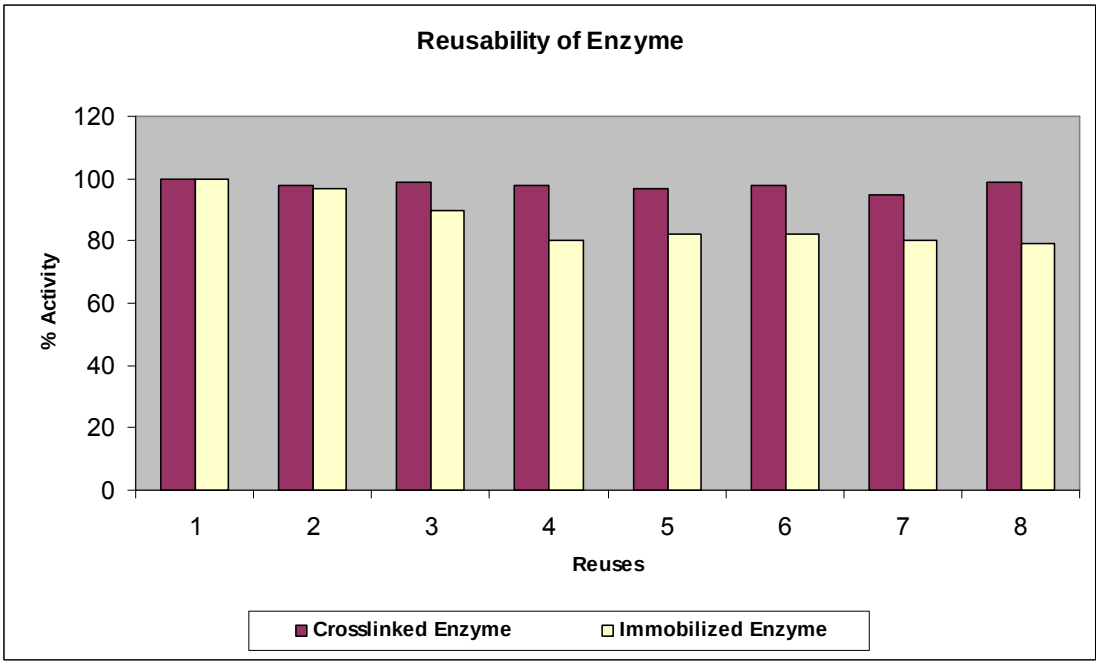



**Fig 5.8:Reusability of enzyme immobilized on SBA-15**

Enzyme silica complexes (with and without cross linking) were given 8 washes with acetate buffer and enzyme activity was checked after each wash. Reusability data of Fig 5.8 showed better stability of cross linked enzyme than that of the immobilized system without cross linking.

**5 B.5: DISCUSSION:**

Complexes 1 and 2 showed similar change in temperature and pH profile, and better stability was observed when compared with free enzyme. Functionalization before and after addition of Boehemite gave similar results. Pre-functionalized silica may provide free amino groups for attachment of glutaraldehyde molecules, which after addition of enzyme gives uniform distribution, and cross-linking of added enzyme.

The strong binding of the enzyme on the surface of the mesoporous silica is reflected in the absence of leaching of the biocatalyst from the support. Such improvement is believed to be due to the enhanced surface hydrophobicity and electrostatic interactions of the functional groups with the enzyme. Change in pH and temperature profile was observed which may be favorable for industrial use.

.

SBA-15 was functionalized as described in Methods. Small angle XRD, sorption studies and FTIR (IR) confirmed functionalization. Small angle XRD revealed that silylation has indeed occurred inside the mesoporous SBA-15. This was further confirmed by decrease shown in the specific surface area from 890 to 780$m^2$/g and decrease in pore size from 89 to 77$A^0$ was observed on modification with APTES. The FTIR spectra of $NH_2$-SBA-15 showed two bands at 3365 and 3298$cm^{-1}$ characteristic of the $NH_2$ groups (NH vibrations) and two bands at 2935 and 2872$cm^{-1}$ characteristic of asymmetric and symmetric vibrations of the $CH_2$ groups of the propyl chain of the silylating agent [Joseph *et al* 2003].In conclusion the immobilization of invertase with increased temperature and pH stability has been demonstrated. We investigated the thermal and pH stability of immobilized enzyme on functionalized SBA-15 and also of the enzyme immobilized on functionalized SBA-15 and cross-linked with glutaraldehyde. Glutaraldehyde is a widely used bifunctional cross-linking reagent that acts by formation of both intramolecular and intermolecular bond [Woodward 1977]. It was previously demonstrated that treatment of native *C. utilis* invertase with glutaraldehyde (0.01to.07%v/v) resulted in its inactivation (Zaborsky 1972). In solution, intermolecular bonds between protein molecules could result in inactive aggregates. However after immobilization, the formation of aggregates would be less likely because the enzyme

molecules are evenly dispersed within the zeolite. In this case, glutaraldehyde forms intermolecular bonds that result in a more stable enzyme preparation. Extrudates of the functionalized mesoporous silica were also prepared for ease of handling.

Glutaraldehyde acts as an efficient cross-linking agent, binding to amino groups created on functionalized mesoporous silica. Functionalized silica invertase complex showed a high pH, temperature and substrate stability. Glutaraldehyde enhances this effect. Longevity of the immobilized invertase was prolonged after treatment with glutaraldehyde. Extrudates of SBA 15 showed better operational stability even after repeated cycles of use. The high efficiency and reusability of the complexes using immobilized invertase indicates that this could be a convenient method for production of invert syrup. High temperature and substrate stability facilitates the use of this immobilized enzyme in confectionary. High temperature stability will be beneficial in the alcohol fermentation where addition of immobilized invertase will enhance the rate of fermentation with simple sugars such as glucose and fructose.

Shelf life of all the complexes were checked by storing these complexes at two different temperatures 30°C and 4°C.Activity was checked after every eight days. No significant loss of activity occurred after three months of storage at 30°C and at 4°C for immobilized and for cross linked enzyme whereas free enzyme looses 20 to 30% of its activity at 30ºC. No loss in free enzyme activity was observed at 4°C in presence of stabilizer such as glycerol.

These characteristics make the enzymatic sucrose hydrolysis process more attractive than the acid hydrolysis. Thus the immobilized system promises a wide variety of applications and merits in further investigation for large scale reactions and long term use.

## 5 C: IMMOBILIZATION OF *K MARXIANUS* ON SILICA AEROGEL:

### 5 C .1: SUMMARY:

*Kluyveromyces marxianus* NCYC 2675 a theromotolerant yeast was immobilized on glass wool reinforced silica aerogel. Silica aerogel, being nanoporous, has extremely low density with large open pores. High surface area and biocompatibility, makes it a promising material for immobilization of biologically active molecules notably enzymes. However it's brittle nature limits the performance as support material. Reinforcement of silica aerogel with glass wool increases its strength and flexibility making it useful for such applications. Electron microscopy and invertase activity measurements showed that aerogel provides a suitable platform for cell immobilization and can be reused without degradation.

### 5 C .2: INTRODUCTION:

Silica aerogel is one of the most fascinating artificially synthesized solid materials. Synthesis of aerogel was first reported by S. Kistler long back in 1932, but its potential has been understood only a few decades ago. Aerogel is a highly porous, ultra low density material having excellent insulating ability, high transparency and is environmentally benign having a plethora of applications (Hrubesh 1998, Burger *et al* 1997, Husing *et al* 1998, Pierre and Pajonk 2002). An added advantage of using aerogel for such applications is the flexibility in its preparation. It can be synthesized in various shapes and sizes with tunable pore sizes. Depending upon the application, aerogel can be treated with suitable molecules to make it hydrophobic or hydrophilic. In recent years, some novel applications of aerogel have emerged. It has been used as nanoglue for the immobilization of metal nanoparticles and magnetic materials [Morris *et al* 1999]. Embedding nanoparticles in silica xerogel stabilize them thermally [Hebalkar *et al* 2001]. The potential of silica aerogel has been realized in various biological applications such as biosensors for detection of bacteria [Power et al 2001), and host matrix for immobilization and regimentation of biologically active molecules [Bhatia *et al* 1998]. Silica aerogel has been shown to be an efficient encapsulation media for the enzyme lipase [Novak *et al* 2003, Maury *et al* 2005]. Yeast spores prepared from TMOS (tetramethoxysilane)-PEG (polyethylene glycol) solution with sol-gel process has been reported [Uo *et al* 1992]. Here we showed that silica aerogel provides an excellent platform for the immobilization of whole cells. Ultra low density silica aerogel is very brittle and fragile, which limits its performance in various applications. Strength and flexibility of silica aerogel can be enhanced by reinforcing it with glass wool.

*Kluyveromyces marxianus* NCYC 2675, a thermotolerant yeast was immobilized on silica aerogel reinforced with glass wool. Electron microscopy and invertase activity results of cells immobilized on aerogel sample show excellent performance.

**5 C.3: MATERIALS AND METHODS**:

**5 C.3.1: Preparation of silica aerogel reinforced with glass wool:**

Synthesis of silica aerogel was carried out by acid-base catalyzed sol-gel process. These gels were reinforced with glass wool to increase their strength and flexibility to make handling of aerogel easier while loading the cells. Silica gel was prepared by mixing 7.5 ml ethanol, 7 ml water, 0.03 ml ammonia, and 0.5 ml ammonium fluoride (0.1M) in a conical flask and stirred for 15 minutes at room temperature. 5 ml tetraethylorthosilicate (TEOS) was added to this solution and stirring was continued for 20-25 minutes, till the solution became transparent / colorless. At this stage, solution was transferred into plastic containers filled with glass wool. Commercially available glass wool was used for reinforcement without any further treatment. Gelation of sol takes place within 30 minutes and glass wool reinforced silica gels were obtained. These gels were allowed to age for 8 days to increase their strength and then soaked in ethanol bath. This allows complete exchange of the pore fluid (mixture of water and ethanol) with ethanol. These gels were then dried super critically at 280°C and pressure of 1250 psi to remove pore liquid. Monolithic silica aerogels of low density (0.1 g/cc) were obtained. These gels were used for the immobilization of yeast cells without any further treatment.

**5 C.3.2: Cell immobilization on aerogel:**

0.5 g of silica aerogel reinforced with glass wool was added to 100 ml of growth medium containing 5 g % sucrose and autoclaved at 10 lbs for 10mins. 10% inoculum of freshly grown *K marxianus* yeast cells was added to above 250 ml flask. The flask was kept on shaker (120 rpm) at 28˚C for 48 hrs. The flask was then kept stationary for one hour and the medium decanted. Cell count of supernatant was determined microscopically by Neubauer's chamber and found to be less than $10^4$ cells/ml.

**5 C.3.3: Invertase activity:**

The invertase activity was typically carried out in a reaction mixture containing 2g % sucrose as described by Gascon and Lampen in1978 and reducing sugar released by hydrolysis was measured (Miller 1959) as described in CHAPTER 2 of the THESIS.

**5 C.3.4: Reuses:**

A small portion of aerogel-whole cell complex was used for checking it's reusability in batch mode. After every use, the complex was washed thoroughly with acetate buffer 50 mM pH 4.5 for the next use. Five such uses were tried for conversion of sucrose to invert syrup. Enzyme activity was determined after every wash. The complex was stored at 4°C and cell count of supernatant and aerogel- complex was found to be constant even after six months.

**5 C.3.5: SEM**

Samples were studied using JEOL JSM – 6360 scanning electron microscope (SEM). The samples with yeast cells were fixed in 5% glutaraldehyde solution overnight. Samples were washed with water twice and finally suspended in water. Thin films of these samples were prepared on glass plates and platinum was sputtered on them to make them conducting.

**5 C.4: RESULTS:**

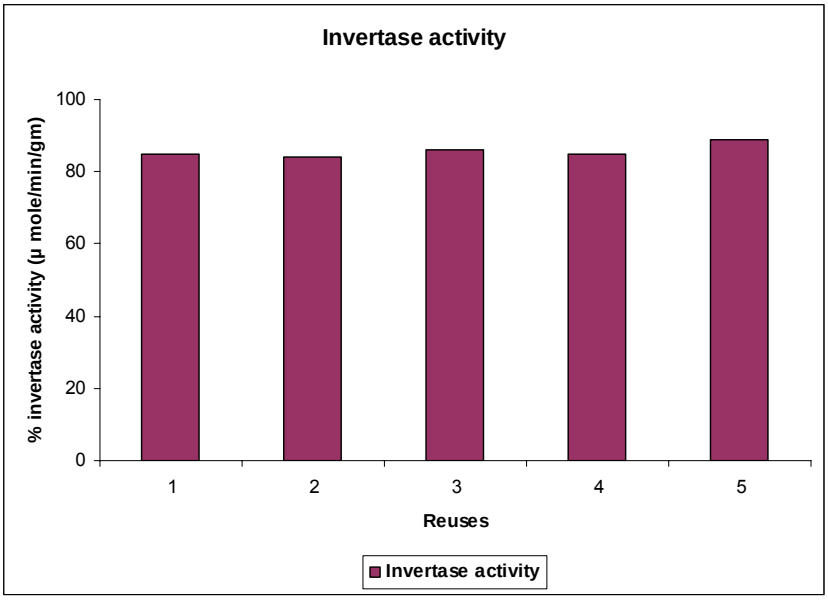



**FIG 5.9:Reuses in batch mode of enzyme immobilized on Aerogel**

As can be seen from **Fig 5.9**, cell based invertase activity was not lost even after 5 reuses in batch mode. Cell count in the supernatant was checked after every reuse and

was not more than 10 $^{4}$/ml. This showed the tight binding of the yeast cells to the matrix. Invertase activity remains constant even after five washes showing that cells remain bound to the aerogel.

Dividing and growing cells were observed within the silica aerogel (**Photo 5.3d**), since their open pores and interconnectivity allows free diffusion of liquid/air from pore to pore with limited restrictions and eventually through the entire material.

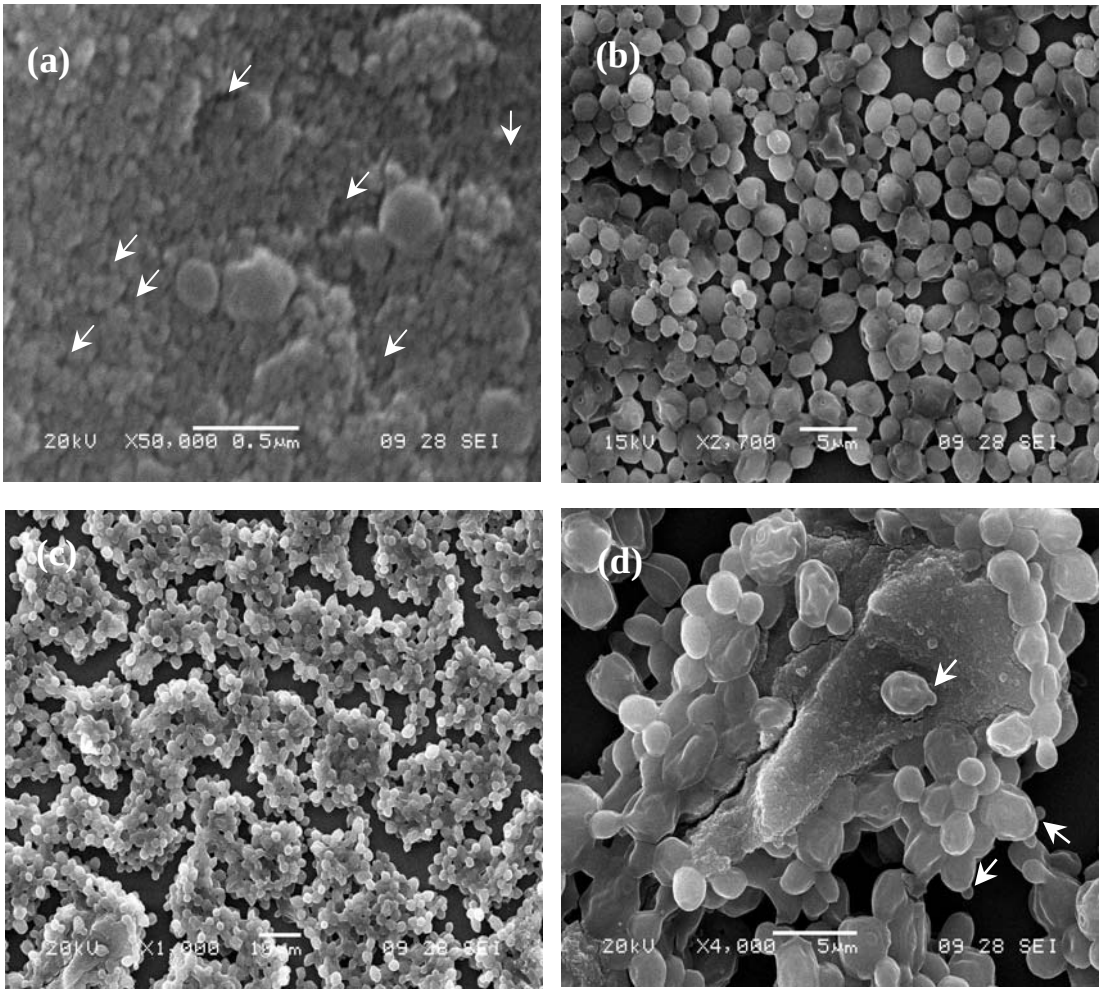



**Photo 5.3:** SEM images of (A) Glass wool reinforced silica (b) Whole cells of yeast Kluyveromyces marxianus(c) Aerogel-whole cell complex (d) magnified view of the aerogel-whole cell complex

The aerogel network can be seen with open pore structure having pore size~ 60-100 nm (arrows show open pores) as can be seen in **Photo. 5.3(a).**

**5 C.5: DISCUSSION:**

Currently there is a considerable interest in cell immobilization for the production of commercially important enzymes. The cost and production of these enzymes is

dependent on growth temperature and cell yield. Therefore selecting a suitable host matrix, which promotes the cell growth and the use of thermotolerant yeast, can be a better strategy. In this report we demonstrate that nanoporous aerogel can be used as a suitable host matrix for the immobilization of whole cells of thermotolerant yeast. Silica aerogel was synthesized by sol-gel method discussed above and is hydrophilic in nature. Yeast cells of size approximately 2 μm can be adsorbed in the pores of the aerogel. These immobilized cells on aerogel samples were reused in five successive reaction cycles without any loss of biocatalytic activity.

It can be seen from the images that aerogel is acting as a favorable support for the immobilization. The yeast cells have grown uniformly over the aerogel. Growing and budding cells can be seen in them. Although pore size of aerogel is smaller than the size of yeast cells, it could be noticed that cells can be adsorbed on it. However cells are not trapped inside the pores but have formed a bunch or colonies around the aerogel and have grown enormously. The porosity of aerogel seems to be a key feature in the growth of these cells, as porous medium ensures consistent oxygen and nutrient supply to the cells. Thus aerogel can be used as a solid support for immobilization of whole cells. Porous gel allows efficient mass transfer once immobilized and used further for transformation. Besides the silica being non toxic and environmentally benign it promotes cell growth. Thus aerogel can be used as a solid support for immobilization of whole cells.

Another field of scientific interest is the development of porous bioceramic materials that are used as human implants (Heidenau *et al* 2001).

We have demonstrated for the first time the immobilization of yeast cells on glass wool reinforced silica. Aerogel- whole cell complex showed no apparent loss in invertase enzyme activity when used repeatedly over 5 cycles for invert syrup production from sucrose. SEM results confirmed growing cells when observed within the silica aerogel. The inert nature of the synthetic matrix gives additional advantage of high rigidity with resistance to degradation through abrasion and microbial attack. Thus silica aerogels hold promise as a biocompatible scaffold for immobilization of cells in a variety of applications like fermentations, biotransformation and bioremediation of toxic materials.

### 5.6 C: FUTURE PROSPECTS

Achieving technical success is seldom sufficient; a lot of other factors have to be fulfilled before industrial success is assured. The cost considerations must take into account such factors as cell production, matrix type and form, immobilization conditions, reactor design and product purification. Economic and advantageous processes can be devised but their general industrial adoption will need time and encouragement. It is more likely

that future developments will come from within the industry itself and the role of the academic researchers will probably remain to some extent as the laying of the theoretical foundations for this work and development of new approaches.

Our culture *Kluyveromyces marxianus* NCYC 2675 has been adapted to grow in molasses and can tolerate high concentrations of sucrose. The organism is GRAS clear and hence safe to handle and can be used as animal feed or food additive. It is a hyperproducer of invertase and hence can be directly used for industrial applications avoiding the efforts needed of strain improvement. The flocculent characteristic of the organism will help in the economic downstream processing by reducing the cost of centrifugation. Being thermotolerant this strain can be used in regions where the temperature rises to 42˚C and above especially in Northern parts of India. Since the organism is thermotolerant, it not only prevents microbial contamination but also permits optimum enzyme production in shorter time compared to mesophilic organism.

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## PUBLICATIONS

### Papers

**1.** Immobilization of thermotolerant *Kluyveromyces marxianus* on silica aerogel for continuous production of invert syrup.

**S Karandikar,** A Prabhune, S Kalele, S Gosavi and S Kulkarni

*Research Journal of BioTechnology* **2006**; 1(2): 16-19.

**2.** Immobilization of Invertase on Functionalized Mesoporous Silica and Properties of the immobilized Biocatalyst

**S Karandikar**, S Pardhy, A Pundle, S Deshpande, and A Prabhune

*Research Journal of BioTechnology* **2007**; 2(1):12-17

**3.** Gellan Gum: A Novel Polysaccharide Matrix for Immobilization of thermotolerant yeast

M Nakrani, **S Karandikar**, A Prabhune, V Pokharkar

(communicated to 'Polymer' **2007)**

**4**. Evidence for the involvement of Tryptophan residue at the catalytic site of β-D fructofuranosidase from thermotolerant *Kluyveromyces marxianus*

**S Karandikar**, A Prabhune (communicated **2007**)

### Posters

**1.** Effect of synthetic zeolites on production of invertase from thermotolerant yeast *K marxianus*

**S Karandikar**, P Karandikar, S Pradhan, R Khan, A Chandwadkar, A Prabhune

Poster presented at **International Conference** of 10 $^{th}$ Congress of the Federation of Asian and Oceanic Biochemists and Molecular Biologist-Bangalore 7th to 10$^{th}$ December **2003**

**2.** Gellan Gum- A Novel Matrix for Enzyme and Whole Cell Immobilization

M Nakrani, **S Karandikar**, A Prabhune, V Pokharkar

Poster presented at 74$^{th}$ Annual Meeting. Society of Biological Chemists, Central Drug Research Institute Lucknow (India) Nov 7-10, **2005**

**3.** Synthesis of Sucrose Analogues and their role as glycoside inhibitors.

**S Karandikar**, M Rale, M G Kulkarni , A Prabhune

Poster presented at 74$^{th}$ Annual Meeting. Society of Biological Chemists Central Drug Research Institute Lucknow (India) Nov 7-10, **2005**

**4.** Immobilization of Invertase from thermotolerant yeast *K marxianus* on silica nanoshells

**S Karandikar**, S Kalele, S K Kulkarni, A Prabhune

Abstract accepted at 75th Society of Biological Chemists Jawaharlal Nehru University, 8-11th December **2006**

**5.** Biodegradation of Azo dyes by novel thermotolerant yeast

L Barhate, **S Karandikar**, P Desai and A Prabhune

Abstract accepted April **2006** NCEC Bits Pilani

**Patents Filed:**

**1.** An improved process for acceleration of hydrolyzing enzymes and production of alcohols.

NCL/9/2004, Filed on 31/3/2005, **1271/DEL/2005**

A Prabhune, A Chandwadkar, A Pundle, P Karandikar, **S Karandikar**.

**2.** An improved process for immobilization of hydrolyzing enzymes

NCL/58/2003, Filed on 29/9/2004, **1874/DEL/2004**

S Pardhy, A Prabhune, S Deshpande, A Pundle, **S Karandikar**

**3.** An improved process for the continuous manufacture of invert syrup and alcohol using heterogenized invertase on functionalized mesoporous silica

NCL/2/2004, Filed on 29/12/2004, **PCT/IN 2004/000438, 0490 NF 2004**

A Prabhune, S Pardhy, S Deshpande, A Pundle, **S Karandikar**.

**In House Project**:

Synthesis of Polymer Microspheres and their application for immobilization of industrially important enzymes.

**Project Code: MLP005226**

Date; 26th July 2004

P Shukla, A Prabhune, A Pundle, **S Karandikar**