STUDY OF TREHALOSE PRODUCTION AND METABOLISM IN *PROPIONIBACTERIUM SHERMANII* UNDER OSMOTIC STRESS

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDY OF TREHALOSE PRODUCTION AND METABOLISM IN PROPIONIBACTERIUM SHERMANII UNDER OSMOTIC STRESS**, in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, is an authentic record of my own work carried out during a period from Jan 2007 to Dec 2011 under the supervision of Dr. Bijan Choudhury, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Rohit

(ROHIT)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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The Ph.D. Viva-Voice Examination of **Mr. Rohit**, Research Scholar, has been held on 2/6/2012

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ABSTRACT

The current understanding of trehalose biosynthesis regulation under stress conditions is incomplete and needs further investigation. Furthermore, the knowledge of enhancement of trehalose accumulation under osmotic stress can be exploited for commercial production. Trehalose finds various pharmaceutical and industrial applications. With this view, we have investigated trehalose metabolism in the context of central carbon metabolism and glycogen and maltose accumulations under osmotic stress in a strain of dairy *Propionibacterium*. Similarly, suitability of crude glycerol obtained from biodiesel was studied for trehalose production. Interestingly, osmotic stress due to presence of KCl in crude glycerol was found to be reason for higher production of trehalose with crude glycerol. Therefore, two broad objectives of current study were to investigate trehalose metabolism under osmotic stress and to explore economical production of trehalose with cheap carbon source.

During our search for suitable strain for this study, four strains of dairy *Propionibacterium* available in culture collection centres of India were procured. These strains were screened for trehalose yield under osmotic and non stress conditions. Amongst the various strains procured from culture collection centers, *Propionibacterium shermanii* NCIM 5137 was considered for further studies. In screening study it was observed that all procured strains of *Propionibacterium* produced trehalose under osmotic stress. Since trehalose yield with respect to biomass was highest in *P. shermanii* NCIM 5137 in osmotic and non-stress conditions (60 mg/g of biomass and 15 mg/g of biomass) hence it was used for further studies. The selected strain has the ability to accumulate trehalose under non-stress condition so this can also be used for trehalose production using cheap carbon source in absence of osmotic stress agents.

Trehalose production was enhanced under osmotic stress as seen in static flask condition. So an effort was made to study the trehalose accumulation under osmotic stress in batch reactor where pH and dissolved oxygen were controlled at 6.8 ± 0.1 and 5% of saturation respectively.

The highest trehalose yield obtained in osmotic stress was 160 mg/g of biomass while in non-stress conditions it was 45 mg/g of biomass. In fact, the effect of osmotic stress was more prominent on final trehalose yield obtained in stationary phase and it was 44

mg/g of biomass in osmotic stress while 15 mg/g of biomass in non-stress condition. As Cardoso et al., 2004 also reported trehalose accumulation in various other carbon sources like lactose, lactic acid, hence a similar effort was made to study trehalose production with *P. shermanii* NCIM 5137. It was observed that use of lactic acid didn't result in improvement of trehalose production and growth was slow. In case of carbon source lactose, marginal improvement in yield was achieved in comparison to glucose media. So, these two carbon sources were not considered for further study.

In the present study, accumulation of trehalose with different carbon sources in *P. shermanii* NCIM 5137 was investigated. Since, use of lactic acid and lactose as carbon source didn't result in higher trehalose accumulation, hence we further used other carbon sources for trehalose production in static flask conditions and it was observed that trehalose accumulation was enhanced with disaccharides (maltose, 128.54 mg/g of biomass; sucrose, 170.47 mg/g of biomass) in comparison to monosaccharide (glucose 93.81 mg/g of biomass) while it was maximum in gluconeogenic substrate, glycerol (385 mg/g of biomass). Interestingly, maximum trehalose yield (with respect to biomass) achieved with these carbon sources were higher than the yield achieved with glucose media under osmotic stress conditions. Trehalose productions from various carbon sources like sucrose, maltose and starch are known in many microbes but accumulation of higher trehalose from glycerol is not reported in any microbe. Hence, to make trehalose production from glycerol commercially feasible, we studied the fermentative production of trehalose from crude glycerol using *P. freudenreichii subsp. shermanii* NCIM 5137 in batch reactor. Herein, the comparison of trehalose production in pure and crude glycerol media in batch reactor shows that maximum trehalose yield of approx 380 mg/g of biomass can be achieved and highest trehalose yield based on substrate consumed was improved three times with crude glycerol media as compared to that with pure glycerol media. Therefore, it was concluded that crude glycerol was superior as carbon source in comparison to pure glycerol. Thus an effort was made to elucidate the roles of various reported impurities of crude glycerol on enhanced trehalose accumulation in *P. freudenreichii*. In the present study, fatty acids like oleic acid, linoleic acid, stearic acid and palmitic acid and KCl were separately used in a chemically defined media (glycerol as carbon source) for investigating their effects on trehalose accumulation. It was observed that fatty acids have no beneficial effects on trehalose production but KCl at a concentration of 1% has significantly improved trehalose yields.

In summary, remarkable improvement in trehalose synthesis under osmotic stress was observed in the present study. Thus an effort was made to understand the variation in trehalose yield with respect to activities of various relevant enzymes and metabolites associated with trehalose biosynthesis. The probable role of TreS for synthesis of trehalose using glycogen as substrate under osmotic stress condition is presumed. However, this needs to be further elucidated with different sets of experiments. Another major observation was higher expression of ADP-glucose pyrophosphorylase over other nucleotide sugar synthesizing enzymes, indicating prominent role of ADP-glucose in osmotic stress condition. From this study, it became apparent that glycogen, maltose and trehalose synthesis are inter related during osmotic stress conditions. Over expressions of ADP-glucose pyrophosphorylase and TreS was the strategy probably adopted by *P. shermanii* for minimizing the effect of osmotic stress. Further effort was made to analyze the biosynthesis pathway variables (enzyme activities, metabolite concentration) using multivariate approach.

Although trehalose biosynthesis requires few metabolites and enzyme reactions but it seems to have a more complex metabolic regulation. The bacterial cell adapts to changing environments by changing the level of various biochemical metabolites and enzymes activities which leads to enhancement of trehalose biosynthesis. Understanding of this complex phenomenon is not very easy without any multivariate approaches. The advantage of multivariate approach includes reduction of number of variables and simultaneous assessment of effects by various variables on trehalose biosynthesis. Hence in the present study we had shown application of one such approach known as principle component analysis. Two principle components were extracted from the study of trehalose biosynthesis pathway under osmotic and non-stress conditions. From the score plots of principle components it was possible to discriminate osmotically stress cells from non-stress cells. Further loading plot was able to identify pathway variables which were responsible for discrimination of osmotically stressed cells from non-stressed cells. Beside data of trehalose biosynthesis pathway obtained from cells grown in glucose, and glycerol was projected on the score plot using the developed principle components.

As it was observed that osmotic stress results in higher trehalose accumulation so an effort was made to develop an osmotically sensitive mutant using EMS (Ethyl Methanesulphonate) as mutagen. From the mutation study, one mutant was selected

which was osmotically sensitive (non-viable in 3% NaCl media) and was able to accumulate higher trehalose even in non-stress condition. Specifically, decrease in trehalose content of cell in the stationary phase was reduced significantly as compared to parent strain. This osmotic sensitive mutant was also found to be resistant to nisin. The efficiency of this mutant for trehalose production was evaluated in glucose (Y_{tx} = 105 mg/g of biomass), sucrose (Y_{tx} = 148 mg/g of biomass) and pure glycerol (Y_{tx} = 233 mg/g of biomass) which was higher in comparison to parent strain (Y_{tx} = 15, 85 and 89 mg/g of biomass in glucose, sucrose and glycerol) respectively. During static flask studies with crude glycerol as carbon source, highest trehalose yield achieved with respect to biomass was 685 mg/g of biomass and final yield was 412 mg/g of biomass, this is probably the highest yield ever reported in *Propionibacterium*. In a recent study, with mutant strain of *Saccharomycopsis,* trehalose yield of 28% with respect to biomass (in the present study 41%) was reported using cassava starch as carbon source (Wang D-S et al., 2011). Similarly, recombinant *Corynebacterium* strain was able to produce trehalose with a yield of 31% of biomass with glucose as carbon source (Carpinelli et al 2006). In mutant, final trehalose yield achieved with respect to biomass was 3 times improved as compared to parent strain with crude glycerol. Likewise, trehalose yield achieved with respect to substrate consumed was increased from 21 mg/g of substrate consumed (parent strain) to 82 mg/g of substrate consumed (mutant strain). Along with this 0.42 g/g of propionic acid and 0.31 g/g of lactic acid were also obtained from crude glycerol. Importance of ADP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase over UDP-glucose pyrophosphorylase for trehalose biosynthesis was also clearly observed in the mutant as activities of these enzymes were much higher along with achievement of higher trehalose yield. In summary, it can be concluded that preference of ADP-glucose pyrophosphorylase over UDP-glucose pyrophosphorylase along with higher OtsA activity is the adopted strategy for enhanced trehalose biosynthesis in both parent and mutant strain. Since complete substrate consumption was not achieved in mutant hence an effort was made to increase biomass concentration by aerobic fermentation in flask cultures at 200 rpm using glycerol and crude glycerol (from biodiesel waste) as carbon sources. In aerobic condition, final biomass was increased ten times while trehalose titre reached 1.4 g/l with crude glycerol as carbon source with osmotic sensitive mutant. Interestingly, substrate consumption of 94% was achieved. Although, trehalose yield with respect to biomass decreased with aeration but trehalose titre increased due to increase in biomass

concentration under aerated conditions. The trehalose titre of 1.7 g/l was reported from recombinant *E. coli* with pure glycerol while in present study, final titer of 1.4 g/l and propionic and lactic acid yields of 0.53 and 0.21 g/g of substrate were obtained with crude glycerol media.

In conclusion, with respect to production, trehalose yields were increased from 1.5% of biomass (from glucose) to 39% of biomass (from crude glycerol) and 0.15 mg/g of substrate to 90 mg/g of substrate. However, trehalose titer of 1.5 g/l was also attained under aerobic condition. Similarly, new insights on trehalose metabolism under osmotic stress were obtained and major conclusions were difference in the role of different NDP-G pyrophosphorylase and probable role of TreS in trehalose synthesis from glycogen.

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Rohit

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ABBREVIATIONS

Fig	Figure
GC	Gas Chromatography
g	Gram
Hr	Hour
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
M	Molar
mM	Milli Molar
mg	Milli gram
min	Minutes
ml	Milliliter
µl	Microlitre
µM	Micromolar
UV	Ultra Violet
OD	Optical density
Pgm	Phosphoglucomutase
Iso	Isomerase
G6PD	Glucose-6-Phosphate dehydrogenase
UDPGD	UDP-glucose dehydrogenase
TreS	Trehalose synthase
Tase	Trehalase
GS	Glycogen synthase
PCA	Principal component analysis
UDP-G pyr	UDP-glucose pyrophosphorylase
GDP-G pyr	GDP-glucose pyrophosphorylase
ADP-G pyr	ADP-glucose pyrophosphorylase
G6P	Glucose-6-phosphate
Mal	Maltose
Glyc	Glycogen

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ABBREVIATIONS

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mM	Milli Molar
mg	Milli gram
min	Minutes
ml	Milliliter
µl	Microlitre
µM	Micromolar
UV	Ultra Violet
OD	Optical density
Pgm	Phosphoglucomutase
Iso	Isomerase
G6PD	Glucose-6-Phosphate dehydrogenase
UDPGD	UDP-glucose dehydrogenase
TreS	Trehalose synthase
Tase	Trehalase
GS	Glycogen synthase
PCA	Principal component analysis
UDP-G pyr	UDP-glucose pyrophosphorylase
GDP-G pyr	GDP-glucose pyrophosphorylase
ADP-G pyr	ADP-glucose pyrophosphorylase
G6P	Glucose-6-phosphate
Mal	Maltose
Glyc	Glycogen

Chapter 1

Introduction

Chapter 1

Introduction

Trehalose is a stable non reducing disaccharide which is widely distributed in nature (Schiraldi et al., 2002.). The properties of trehalose have made it a valuable biotechnological product with diverse applications, some of which have been developed for commercial use. Trehalose is used to store thermolabile enzymes like DNA polymerase and DNA ligase (Colaco et al., 1992). It can act as a stabilizer for complex molecules like antibodies which can be dehydrated at 37°C in the presence of trehalose without losing its activity (Roser, 1993). It is also used as a food additive in dried or processed food as it is safe for human consumption (Kidd et al., 1994). Use of trehalose as preserver of cells, tissues and organs was also reported (Eroglu et al., 2000; Guo et al., 2000). It is also reported for improvement of flower shelve-life (Iwaya-Inoue. et al., 2001). Trehalose use in cosmetic industries is also reported (Higashiyama, 2002). The medical use of trehalose in reducing the symptoms of illnesses such as Huntington's chorea and osteoporosis was reported previously (Higashiyama et al., 2002: Katsuno et al., 2004). Trehalose is found to be having nutraceutical value (Hugenholtz et al., 2002). It is half as sweet as sucrose, provides sustained energy and elicits a very low insulin response (Higashiyama et al., 2002; Elbein et al., 2003; Kroger et al., 2006). It is heat stable and in addition being a sugar it stabilizes protein or protein aggregation (Elbein et al., 2003). It had been reported that trehalose can protect cell from oxidative damage (Benaroudj et al., 2001). Trehalose is widely distributed in microbes (Arguelles, 2000). In nature, trehalose is formed as reserve compound and act as an agent which protects cells from various physical and chemical stresses (Argüelles, 2000; Schiraldi et al., 2002). Thus, understanding of trehalose metabolism will help in developing more stress resistant strains that are useful in commercial fermentation processes. On other hand, trehalose (due to its several applications) can be economically produced from a food microbe like Propionibacterium. Understanding how exactly trehalose interact with putative targets and activate metabolic and stress pathways is far from complete and more research must be conducted which in turn could impact industrial microbiology.

Various approaches for the industrial production of trehalose have been attempted which includes both enzymatic conversion and its accumulation during the fermentation of glucose using yeast cells (Chi et al., 2003). The trehalose produced by enzymatic conversion can be applied in food industries as final reaction product contain other sugars

in addition to trehalose, while the trehalose produced by fermentation can be applied in medical industries, as the extract produced by the yeast cells is relatively pure (Chi et al., 2003). Production of trehalose by fermentation is limited if glucose is used as a substrate hence alternative cheap carbon sources must also be explored for economical production of trehalose. Due to the potential applications of trehalose in diverse areas, development of economically feasible production systems has received much attention in recent years (Wang et al. 2011, Li et. al, 2011). The production of trehalose is known through many enzymatic and fermentation routes (Schiraldi et al., 2002). Similarly, the uses of maltose, sucrose and glucose as carbon source for trehalose productions are well known (Chi et al., 2009). Trehalose production from yeast is not successful due to its low yield (Maruta et al., 1996). Although enzymatic methods are efficient for trehalose production but microbial methods are preferable when use of wastes are desirable for commercial production. There are rarely any reports on use of cheap carbon source like waste for trehalose production.

In recent times, studies on metabolic responses against various abiotic stresses have resulted in gathering useful information regarding microbial phenotypes and metabolic regulation. Osmotic stress is one such stress phenomena which had been studied in microbial systems. It has been reported that under osmotic stress, cells accumulate compatible solutes like trehalose and glutamate and other osmoprotectants (Truper & Galinski, 1990). In particular, effects of osmotic stress on *E. coli, Corneybacterium sp. S. cerevisiae, Propionibacterium freudenreichii* had already been reported (Strom et al., 1993, Carpinelli et al., 2006, Voit et al., 2003, Cardoso et al., 2007). In all of these microorganisms, prominent role of trehalose biosynthesis pathway has been clearly demonstrated. Trehalose has been implicated as potential stress protectant that accumulates in yeasts during various stress conditions (Li et al., 2009). During the fermentation process, yeasts are subjected to a succession of stress conditions, such as high temperature, high sugar and accumulation of ethanol, which affect their viability and fermentation efficiency. In yeast, a strong correlation between trehalose content and stress resistance has been demonstrated for a variety of stresses such as heat, osmotic stress, and ethanol (Hottige et al., 1994; Zancan et al., 2005 Conlin et al., 2007). Furthermore, the stress response is mediated at the level of transcription, and a number of stress-induced transduction pathways concerning trehalose has already been reported (Ruis et al., 1995; Estruch et al., 2000). Thus, when the cells encounter such stresses, dynamic changes occur in the complex biological networks that comprise genes, proteins, metabolites, etc. and that

underlie cellular function (Belloch et al., 2008). The investigation of such molecular responses will help us to understand the molecular mechanisms by which cells adapt to fermentation conditions. Several studies have reported that trehalose is better as a protein stabilizer than any of a number of compatible solutes, because of its unusual ability to alter the water environment surrounding a protein, stabilizing the protein in its native conformation (Kaushik et al., 2003; Magazù et al., 2005).

In the present study, trehalose accumulation is studied in *Propionibacterium shermanii.* Dairy *Propionibacterium* has several application especially it is responsible for eye formation in Swiss cheese (Langsrud and Reinbold, 1973). It is used in the production of Vitamin B_{12} and propionic acid (Cardoso et al., 2004). It is having properties of probiotic (Jan et al., 2002). *Propionibacterium* during fermentation produces various organic acids like lactic acid, propionic acid, acetic acid, succinic acid. (Hettinga and Reinbold, 1972). Trehalose is widely distributed in *Propionibacterium* (Deborde et al., 1996). Hence, it is not surprising that the potential of this microorganism as a cell factory for the production of flavours, texturizers, and nutraceuticals has been explored to a great extent (Thiery et al., 2011). The use of *Propionibacterium* strains in fermentation processes depends on functional properties (flavour and texture development) as well as growth performance and robustness. Furthermore, during culture handling, storage and product processing, *Propionibacterium* have to cope with dehydration (freeze-drying), elevated temperatures ($\geq$ 41°C, *e.g.*, in cheese processing), cold stress (2-6°C), among other stresses. In this context, it is clear that a good performance in industrial applications largely depends on the ability of *Propionibacterium* to withstand various stresses, and in particular osmotic stress. Realizing the importance of *Propionibacterium* as producer of various food additives and its long history of safe use it was planned to explore it for the trehalose production capability.

Considering the importance of trehalose it had been studied in *E .coli, Corynebacterium, Mycobacterium and Saccharomyces.* In microbes, intracellular trehalose biosynthesis is known to occur through three pathways - OtsAB, TreYZ and TreS pathways (De Smet et al., 2000). OtsAB is the best characterized pathway which involves condensation of UDP-glucose and glucose-6-Phosphate to form trehalose-6-phosphate by enzyme trehalose-6-phosphate synthetase (OtsA) followed by de-phosphorylation to trehalose by trehalose-6-phosphate phosphatase (OtsB) (Cardoso et al., 2007). However, it is worth mentioning that OtsA can also utilize ADP-glucose and GDP-glucose as a substrate instead of UDP-

glucose. The specificity of OtsA towards ADP-glucose and GDP-glucose was not clearly established in all these organisms. Importance of TreS pathway has also recently been reported in *Mycobacterium* sps. (Pan et al., 2008) and its role as a catalyst for inter-conversion of trehalose and maltose were also reported in *Propionibacterium* (Cardoso et al., 2007) and *Corneybacterium* sp. (Wolf et al 2003). The role of TreYZ pathway in trehalose biosynthesis has been reported in *Corneybacterium sp.* (Wolf et al., 2003) but its absence in *Propionibacterium freudenreichii* was also reported recently (Falentin et al., 2010, Cardoso et al 2007). Among these three metabolic pathways for trehalose biosynthesis, it was reported that under osmotic stress, over-expressions of OtsAB were observed in *E. coli, S. cerevisiae* and *Propionibacterium sp.* (Strom et al., 1993, Voit et al., 2003, Cardoso et al., 2007). Under osmotic stress, over-expressions of enzyme responsible for synthesis of UDP-glucose (UDP-glucose pyrophosphorylase) was also observed in yeast (Voit et al., 2003) but no efforts were made to study the stress effects on other nucleotide sugar synthesizing enzymes and enzymes associated with trehalose biosynthesis pathway. As *Propionibacterium* followed OtsAB pathway for trehalose biosynthesis hence importance of UDP-glucose as a substrate is important. This nucleotide sugar UDP-glucose is synthesized by enzyme UDP-glucose pyrophosphorylase. UDP-glucose synthesis occurs from UTP and glucose-1-phosphate which comes from glucose-6-phosphate by the enzyme phosphoglucomutase (Qian et al., 1994; Degeest et al., 2000). These enzymes have been studied in *Lactococcus lactis* and *Streptococcus thermophilus* for the exo-polysaccharide accumulation (Boels et al., 2001, Levander et al., 2004.) and have been found to play important role in exo-polysaccharide synthesis (Degeest et al., 2000) but their influence on trehalose synthesis is not studied in *Propionibacterium.* In the present study, an effort was made to assess the importance of trehalose biosynthesis pathway under osmotic stress by simultaneous monitoring of various enzymes associated with trehalose biosynthesis pathway. In all previous studies, role of individual enzyme on trehalose biosynthesis was evaluated but no effort was made to evaluate global effects of various enzymes or metabolites on trehalose production.

A large amount of biodiesel waste produced from biodiesel manufacturing industries poses major environmental risks (Yazdani et al., 2007, Pyle et al., 2008). Thus many investigations reported utilization of this crude glycerol for various alternative uses like combustion, composting and biological conversions. Similarly, microbial conversion of crude glycerol into value added products is an alternative way for utilisation of this

waste and it seems to be economically attractive. Several microbial products were produced from crude glycerol like 1, 3 propionediol using *Clostridium* and *Klebsiella* (Himmi et al., 1999, Hiremath et al., 2011), hydrogen from *Enterobacter aerogens* (Ito et al., 2005), succinic acid using *Anaerobiospirillum succiniciproducens* (Lee et al., 2001) omega-3 polyunsaturated fatty acids from algal fermentation (Pyle et al., 2008), docosahexaenoic acid using *Schizochytrium* (Ethier et al., 2011), surfactin (Fonseca de Faria et al., 2011) and ethanol (Oh et al., 2011) respectively. In the present study, crude glycerol from biodiesel waste was used as carbon source for parent and osmotic sensitive mutant of *Propionibacterium freudenreichii subsp shermanii* for the production of trehalose together with organic acids like propionic acid and lactic acid.

An attractive advantage of microbial-based processes is the ability to efficiently utilize agricultural or industrial wastes as substrates for the production of valuable metabolites. Therefore, microbial production of trehalose in high yield could offer an economically advantageous and environmentally-friendly alternative to currently employed enzymatic processes. Understanding the metabolic pathways involving trehalose in microorganisms is a prerequisite for developing genetically engineered strain. In the present study, considering the importance of trehalose, an investigation regarding economical production of trehalose was conducted along with the study of metabolism of trehalose biosynthesis.

Based on these observations we devised our strategy to get detailed insight into trehalose biosynthesis pathway (especially under osmotic stress) and finally proposed the suitability of crude glycerol for economic production of trehalose (presence of osmotic stress agent like KCl in crude glycerol was responsible for higher trehalose accumulation) (Ruhal et al., 2011). Thus objectives of current investigation were

- Screening of suitable strain of *Propionibacterium* obtained from Indian culture collection centers for higher trehalose production under osmotic stress.
- Study of trehalose accumulation in selected strain under osmotic stress in batch reactor and comparison of yield obtained with other carbon source (lactose, lactic acid and glucose)
- Effect of different cheap carbon source on trehalose production and suitability of crude glycerol obtained from biodiesel waste for simultaneous productions of trehalose and propionic acid.

- Study of trehalose metabolic pathway under osmotic and non-stress conditions
- Analysis of trehalose biosynthesis pathway using principle component analysis
- Trehalose production with an osmotically sensitive and nisin resistant mutant with crude glycerol.

Chapter 2

Literature review

Chapter 2

Literature Review

2.1 Accumulation of compatible solutes (including trehalose) under osmotic stress in microbes

The cytoplasmic membrane is permeable and acts as an effective barrier for solutes present in the environment of microbes or the cytoplasm. In particular, the total concentration of intracellular osmotically active solutes is higher than in the outside, which leads water to flow down its chemical potential into the cell. Therefore, a hydrostatic pressure, the so-called turgor, is exerted by the cytoplasmic membrane toward the cell wall. Consequently, turgor balances the difference in osmotic pressure between the cell interior and its surroundings. Turgor is maintained throughout the growth cycle as the cell elongates, and it is important for enlargement of the cell envelope and hence for growth and division (Koch, 1983). Although, quantifying turgor is very difficult in bacteria (Wood, 1999), but the values of 3 to 5 bar for Gram-negative bacteria and approximately 20 bar for Gram-positive bacteria have been estimated (Whatmore, 1990). Due to large cytoplasmic solvent pool required for expansion of the multilayer peptidoglycan the turgor value of gram positive bacterium is considered high. Bacteria are forced to adapt to the environment with frequent fluctuations in osmolality (Morbach & Kramer, 2005). It can be through decrease or increase in the external osmolality, or osmotic hypo or hyper shift. The two main adaptation strategies found in microbes are the release of solutes out of the cytoplasm after an osmotic downshift and the accumulation of so-called compatible solutes after an osmotic upshift (Kramer, 2002). Betaine, proline, glutamine, ectoine, and trehalose were found to be effective as osmoprotectant (fig 2.1 A). In the cellular context, they cause rehydration of the cytoplasm by elevating the internal osmolality because they can be accumulated up to molar concentrations without disturbing cellular functions (Csonka, 1999). At the molecular level, compatible solutes are thought to stabilize and protect enzymes by preventing dehydration of the protein (Fig 2.1 B) (Arakawa et al, 1985; Youxing, 1998). Similarly, various adaptation are motivated by microbes under temperature stress, alkaline/acidic stress (Singh et al., 2011; Goitis et al., 2010). As the bacteria faces the challenge of osmotic stress, instant water efflux occurs and cell dehydrates. Consequently, it may lead to slow growth or can stop growth. In general,

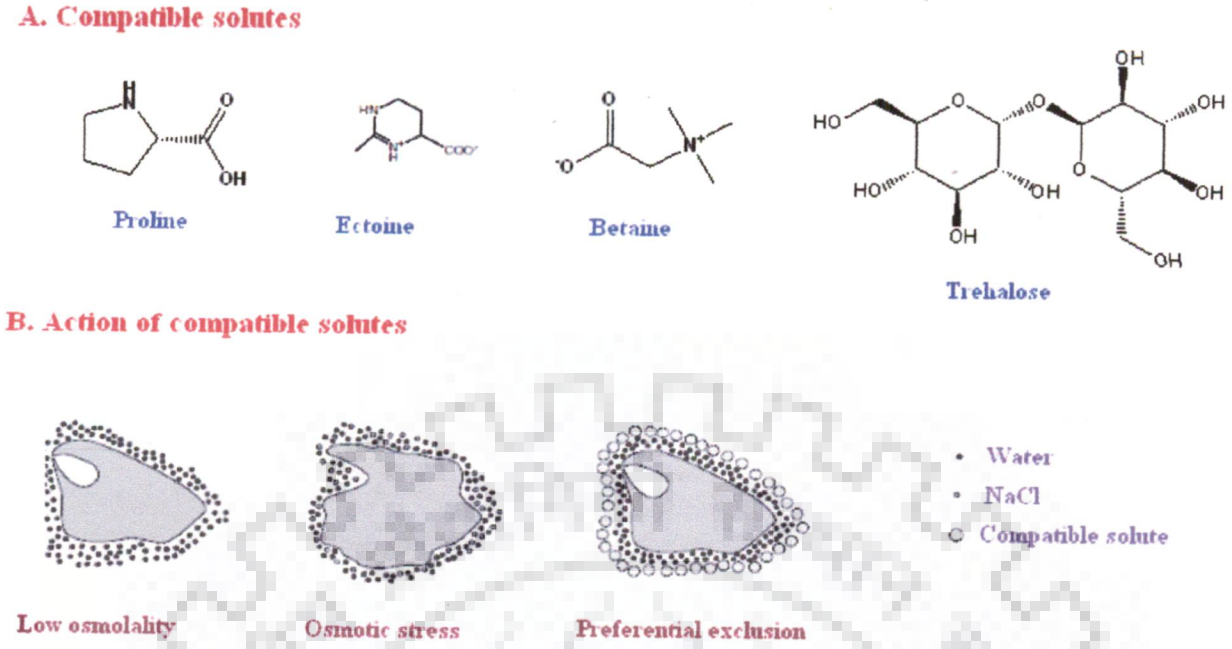

Fig 2.1 (A) Various compatible solutes accumulated in microbes under osmotic stress (B) Action of compatible solutes when present extracellular during low osmolality, osmotic stress. (Morbach & Kramer, 2005)

response of bacteria to hyper osmotic stress is considered in three overlapping phases (Wood, 1999) as illustrated in fig 2.2.

In the first phase cytoplasm undergoes dehydration (Fig 2.2). During second phase, the cytoplasm rehydrates by adjustment of the cytoplasmic solvent composition (mainly by accumulation of ions or compatible solutes). Subsequently, in the third phase, the remodelling of the cell occurs, which is characterized by changes in gene expression profiles and by exchange of ionic osmolytes against compatible solutes (Fig 2.2). As a result, growth is resumed. Various microbe including budding yeast *Saccharomyces cerevisiae*, *Propionibacterium freudenreichii* and *Clostridium* are widely used in the fermentation and brewing industries. During the fermentation process, these are subjected to a succession of stress conditions, such as high temperature, high sugar and accumulation of ethanol, which affect their viability and fermentation efficiency. When the cells encounter such stresses, dynamic changes occur in the complex biological networks that comprise genes, proteins, metabolites which are responsible for cellular function. The investigation of such molecular responses will help us to understand the molecular mechanisms by which cells adapt to fermentation conditions. Several studies have reported

that trehalose is better as a protein stabilizer than other reported compatible solutes, because of its unusual ability to alter the water environment surrounding a protein, thus stabilizing the protein in its native conformation. A strong correlation between trehalose content and stress resistance has been demonstrated for a variety of stresses, such as heat, osmotic stress, and oxidative (Li et al., 2009). Furthermore, the stress response is mediated at the level of transcription, and a number of stress-induced transduction pathways concerning trehalose was also reported (Kramer et al., 2000).

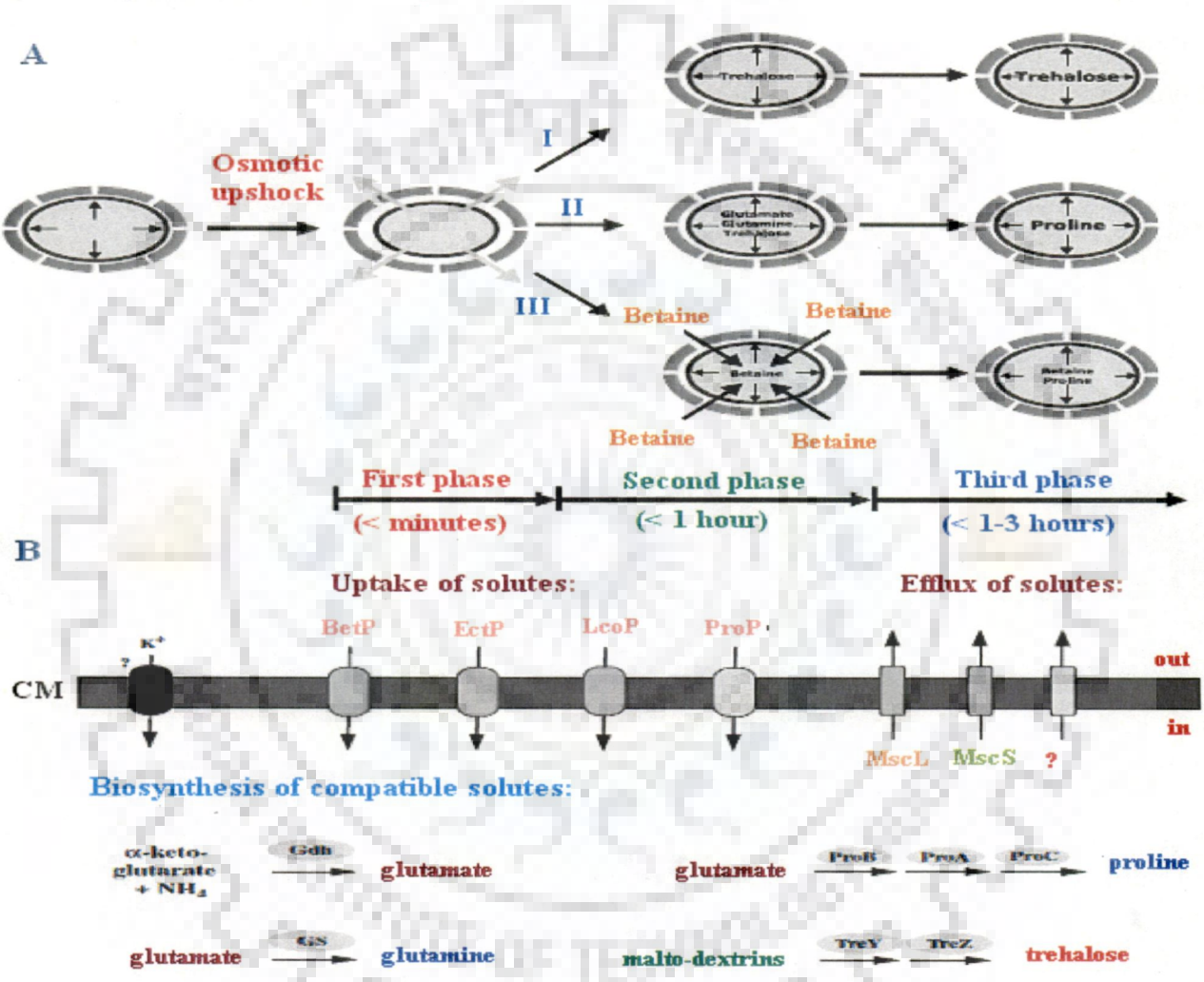

Fig 2.2 Response of osmotic stress in *Corynebacterium glutamicum* [A] in first phase after sudden osmotic shock leads to loss of water subsequently synthesis of compatible solutes trehalose (I), while in nitrogen surplus betaine and glutamine are synthesised (II & III) (Morbach & Kramer, 2005)

Thus trehalose is one of important compatible solute accumulated in microbes under osmotic stress (Arguelles, 2000). Therefore, it is important to understand trehalose

metabolism with respect to stress tolerance in microbes, which can be further exploited for its production. Trehalose metabolism under hypertonic stress can be viewed in fig 2.3. Fig 2.3 shows trehalose metabolic situation in *E. coli* under hypertonic and hypotonic conditions (Li et al., 2011). Glucose is transported into the cells by glucose phosphotransferase system (EIIAGlc). Under hypertonic conditions, trehalose is synthesized by trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB), using glucose-6-phophate and UDP-glucose as substrates. However, trehalose is degraded by both periplasmic (TreA) and cytoplasmic (TreF) trehalase enzymes. Excretion of trehalose is facilitated by stretch activated proteins (SAP) in the plasma membrane. Under isotonic conditions, trehalose is transported into the cells by enzyme EIICBTre (TreB) of the phosphotransferase system. Trehalose-6-phosphate is hydrolyzed into glucose and glucose-6-phospate by trehalose-6-phosphate hydrolase (TreC). Free glucose is phosphorylated by glucokinase (GlK).

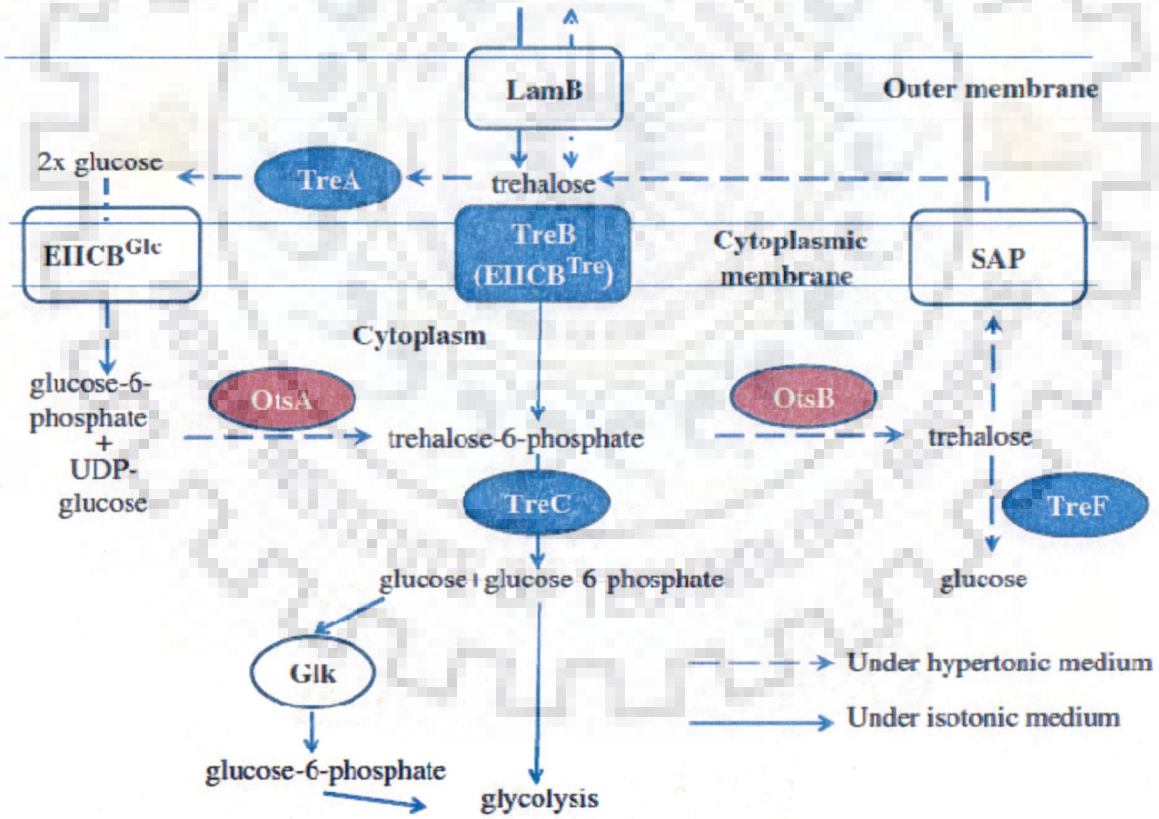

Fig 2.3 Trehalose metabolism in *E. coli* during hypertonic and isotonic situation (Li et al., 2011)

2.2 Trehalose

Trehalose is a naturally-occurring sugar having similar functionality as sucrose but with greater stability and less sweetness. It can be used by product developers either to improve existing products or to create innovative new products. Trehalose is a multi-functional sugar. Its mild sweetness, low cariogenicity, low hygroscopicity, high freezing-point depression, high glass transition temperature and protein protection properties are all of immense benefit to food technologists. Trehalose has no laxative effects and after ingestion is broken down in the body to glucose. It has a moderate glycaemic index with low insulinemic response. Trehalose accumulation is reported under osmotic stress in microbes including yeast (Argüelles, 1997 & 2000; Zaragoza et al., 2000; Pedreño et al., 2002; González-Párraga et al., 2007 & 2011; Sengupta et al., 2011)

Trehalose (α, α -trehalose) is a disaccharide formed by α-1, 1-glycosidic linkage of two d-glucose molecules (Fig 2.4) (Cardoso et al., 2007). It is a non-reducing sugar that is not easily hydrolyzed by acid, and the glycosidic bond is not cleaved by α-glucosidase. The molecular formula and weight are $C_{12}H_{22}O_{11}$ and 342.31, respectively. When purified it is usually found in the dihydrate form, which is the typical commercial product. Although there are three possible anomers of trehalose, that is, α, β-1,1-, β, β -1,1-, and α, α 1,1-, only the α, α -trehalose (Fig 2.4) has been isolated from and biosynthesized in living organisms. This naturally occurring disaccharide is widespread throughout the biological world. It is known to be one of the sources of energy in most living organisms and can be found in many organisms, including bacteria, fungi, insects, plants, and invertebrates.

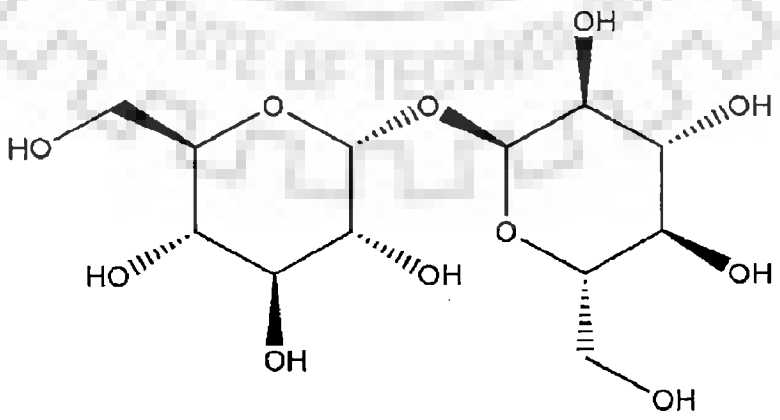

Fig. 2.4 Structure of trehalose

The properties of trehalose are shown in Table 2.1. Its relative sweetness is 45% of sucrose. Trehalose has high thermo stability and wide pH range stability. Therefore, it is one of the most stable saccharides. When 4% trehalose solutions having 3.5 to 10 pH were heated at 100°C for 24 hr, no degradation of trehalose was observed in any case. Because of non-reducing sugar, this saccharide does not show Millard reaction with amino compounds such as amino acids or proteins. Its particular physical features make it an extremely attractive substance for industrial applications. Furthermore, this saccharide shows good sweetness like sucrose, and in the food industry, this saccharide is used as a sweetener (Iturriaga et al., 2009).

Table 2.1 Physical properties of trehalose

Appearance	White orthorhombic crystals
Relative sweetness	45 % of sucrose
Melting point	
dehydrate	97.0 °C
anhydride	210.5 °C
Solubility	68.9 g/100 g H_2O at 20 °C
Heat of fusion	
dehydrate	57.8 kJ/mol
anhydride	53.4 kJ/mol
pH stability of solution	>99 % (pH 3.5–10, at 100 °C for 24 h)
Optical rotation	[α]D +178°
Heat stability of solution	>99 % (at 120 °C for 90 min)
Density	1.58 g/cm^3 at 24 °C

2.2.1 Trehalose applications

The trehalose properties have made it a valuable biotechnological product with various applications and this has made it important for commercial use (Iturriaga et al., 2009). So far, there are most common and promising uses for this disaccharide.

Trehalose can be used to store thermolabile enzymes such as DNA polymerase, restriction enzymes and DNA ligase at ambient temperature (Colaco et al., 1992). It can be used as stabilizer and protector for complex molecules: Unstable molecules such as antibodies dehydrated at room temperature or 37 °C in the presence of trehalose, maintaining their activity after various months in storage (Roser, 1993). It can also be used as foods additive as trehalose can be used in dried or processed foods such as fruits and vegetables, in order to preserve aromas and their organoleptic properties, this disaccharide is not toxic and it is already consumed as part of the human diet, as it is present in bread, honey, mushrooms, wine, beer, etc (Kidd et al., 1994). Application of trehalose as preserver of cells, tissues and organs was also reported. Cells, tissues and even organs can be preserved for months in the presence of trehalose, either dried or frozen, improving shelf-life in comparison with other substances (Eroglu et al., 2000; Guo et al., 2000). Improvement of flower shelve-life: Addition of 50 to 100 mM trehalose to tulips and gladioli increases their shelf-life in a vase after cutting, as it apparently avoids transpiration (Iwaya-Inoue. et al., 2001). Cosmetic industries have shown several beneficial effects of trehalose as it traps and reduces bad odours emitting from human skin by up to 70%, making it a useful additive for facial or body creams and for deodorants (Higashiyama, 2002). Various possible medical uses of trehalose has been reported which includes the role of trehalose in reducing the symptoms in illnesses such as Huntington's chorea and in osteoporosis has been explored. In the former, trehalose prevented the formation of polyglutamine protein in the brain and in the second study, the consumption of trehalose was found to reduce the degeneration of bones in female rats whose ovaries had been removed. However, the mechanisms involved in these uses are not fully understood (Higashiyama et al., 2002: Katsuna et al., 2004).

Trehalose is a unique disaccharide sugar capable of protecting biomolecules against environmental stress (Arguelles, 2000). It is a stable, colourless, odour-free and is widespread in nature. Trehalose has a key role in the survival of some plants and insects which are termed as anhydrobionts, in harsh environments, even when most of their water body is removed. The unusual phenomenon of these plants and insects drove attention towards the study of trehalose. Since then, it proved to be an active stabilizer of enzymes, proteins, biomasses, pharmaceutical preparations (especially in vaccines) and even organs

used in transplantation. Recently, trehalose has been accepted as a safe food ingredient by the European regulation system following approval by the US Food and Drug Administration. The wide range of applications of this sugar has increased the interest of many research groups into the development of novel and economically feasible production systems.

2.2.2 Trehalose production

In the early 1990, the cost of 1 kg of commercialized trehalose could reach US$ 700 (Paiva et al., 1996). Since then many other processes have been proposed to reduce the production costs. A group of Brazilian researchers developed a method to overproduce trehalose in *Saccharomyces cerevisiae*, by applying stress conditions and this resulted in accumulation of trehalose upto a 20% of cell dry weight (Schilardi, 2000). Other groups studied the physiology of yeast when immobilized on a solid matrix and found an increase in final trehalose content (Schilardi, 2000). These achievements already contributed in the price reduction of trehalose by 50%. Although the cost was now affordable for very specific and value added applications, for example in the pharmaceutical industry, it was not compatible with emerging uses in the food industry. Because of the promising applications of trehalose in the developments of novel foods and novel preservation strategy, research continued on novel process technologies. The most interesting one have been filed for patents and successfully exploited in production processes leading to a major reduction in the commercial price of trehalose to 5–6 US$ kg^{-1} (Schiraldi et al., 2000)

Producrion of trehalose in yeast by following the pathway is shown in Fig. 2.5 (type I) has been reported. In 1994, two patents were issued by Hayashibara Biochemical Laboratories Inc. (Okyama, Japan) disclosing a novel non-reducing saccharide forming enzyme (maltooligosyl trehalose synthase, MTSase; EC 5.4.99.15) (Maruta et al., 1994) and a trehalose releasing enzyme (maltooligosyl trehalose trehalohydrolase, MTHase; EC 3.2.1.141) (Maruta et al., 1994) obtained from the culture of microorganisms such as *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q 36 (Fig. 2.5, type II). The first enzyme acts on reducing maltodextrins having a polymerisation degree (DP) higher than three and catalyses the conversion of the α-1,4 linkage at the reducing end to an α-1,1. The second enzyme specifically acts on the α-1,4 linkage adjacent to the α-1,1, liberating trehalose and a lower molecular weight maltooligosaccharide. Maruta *et al.* reported that this novel enzymatic system, was produced in a fermenter with a medium containing complex components. The biocatalytic activity was present (complete reaction) in the culture broth

(0.9 U ml^{-1}) as well as in the microbial cells (0.6 U ml^{-1}). The crude enzyme solutions have been purified, following conventional procedure, and fully characterized. The enzymatic synthesis route was proposed to follow type II pathway, when two reactions are uninvolved (Fig. 2.5, type II). The conversion and the product formation kinetic were studied using maltodextrins containing up to seven glucose residues and found to have a very poor activity with maltotriose (Schiraldi et al., 2000). Three main enzymatic routes for trehalose synthesis were discovered: a phosphorilase system found in fungi and yeast; a glycosyltransferase–hydrolase system that occurs in mesophilic bacteria such as *Arthrobacter* sp. but also, more interestingly, in extremophilic microorganisms; and a trehalose synthase catalyzing an intra-molecular transglycosilation from maltose, Type III (*Pimelobacter* sp., *Thermus* ssp., etc.). These routes have been intensely discussed in the literature and few biotechnological processes were developed exploiting these enzymatic systems, which are the subjects of several patents (Schiraldi et al., 2000). Comparison of various enzymatic methods is listed in table 2.2.

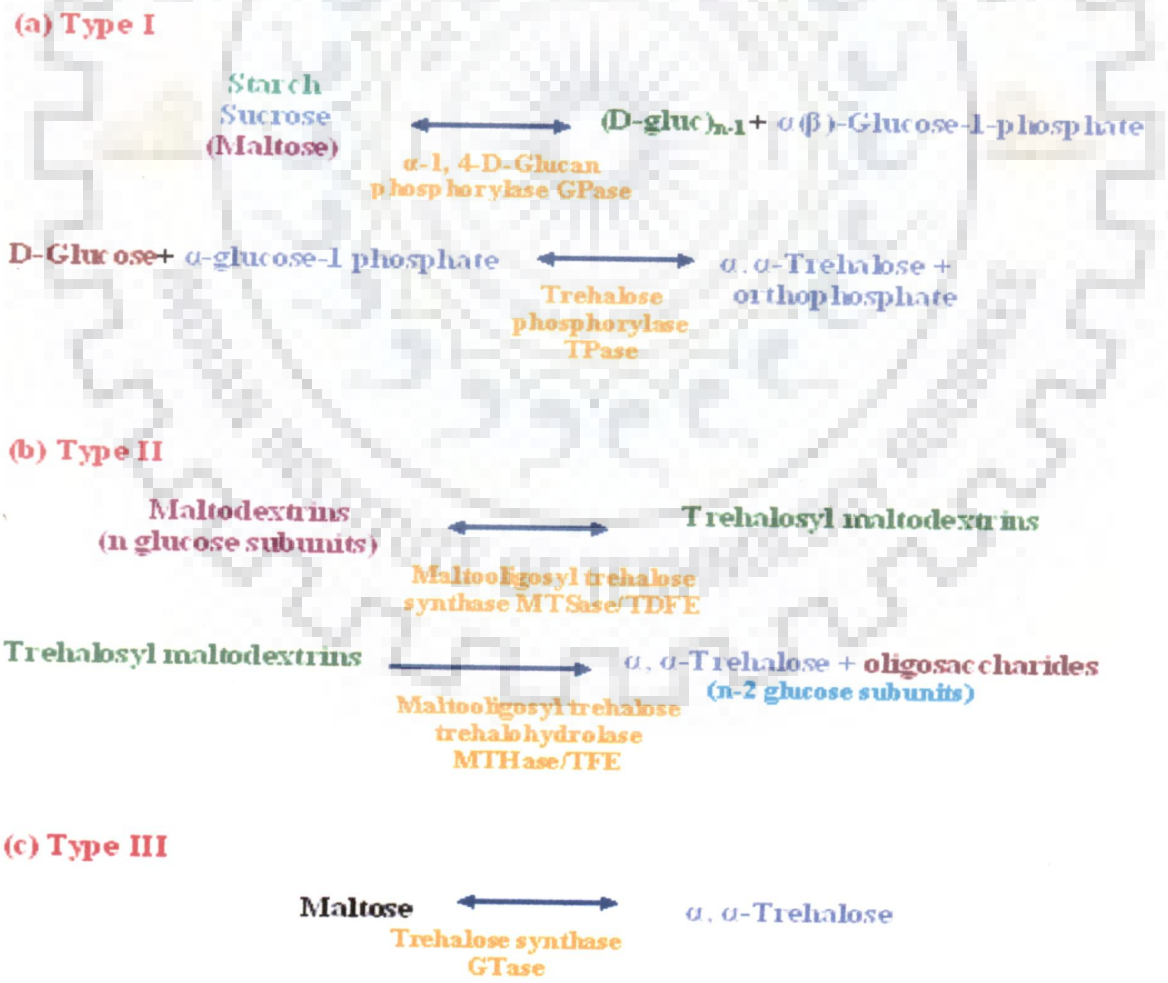

Fig 2.5: Bioconversion of different substrates into non-reducing sugar trehalose (Schilraldi et al., 2002)

Table 2.2: Comparison of various enzymatic methods for trehalose production (Schiraldi et al, 2002)

Microorganisms	Enzymes	T_{OPT} (°C)	pH_{OPT}	Km(mM)	Production	Tehalose yield (%)
Fungi and yeasts						
	TPS-ase (1^{st} step)	50	6.8	2.8(MD)	1-1.2U $g_{biomass}^{-1}$	n.r.
	TPPase (2^{nd} step) GPase TPsae	25	6.9	-	-	40
Bacteria						
Pimelobacter R48	GTase	45	7.0	n.r.		
Pseudomonas putida		37	10			
Thermus aquaticus	GTase	65	6.5	n.r.	31.4U $g_{biomass}^{-1}$	81(40°C) 71(60°C)
Arthrobacter sp.Q36	MTS	40	7.0	1.4 (M6)	16.3U $ml_{culture}^{-1}$	92
	MTS	40	7.0	7.0(MT4)	25.1U $ml_{culture}^{-1}$	
Brevibacterium helvolum	MTS	35	6.5	-	8U $ml_{culture}^{-1}$	90
	MTH			-	12 U $ml_{culture}^{-1}$	
Rhizobium sp.M-11	MTS	40	7.0	-	1.5 U $ml_{culture}^{-1}$	60
	MTH	45	7.0	-	2.0 U $ml_{culture}^{-1}$	
Archaea						
Sulfolobus acidocaldarius	MTS	75	5.5-6	5.7(M6)	1.2 U $g_{biomass}^{-1}$	82
	MTH	75	5-5.5	3.7(MT4)	3.4 U $g_{biomass}^{-1}$	
Sulfolobus solfataricus KM1	MTS	70-80	5.0-6.0			80-83
	α-amylase	70-85	4.5-5.5	-		
Escherichia coli JM109 expressing	MTS			n.r.	10 U $g_{biomass}^{-1}$	
Sulfolobus solfataricus KM1enzymes	α-amylase			n.r.	2822U $g_{biomass}^{-1}$	
Sulfolobus shibatae	TDFE	70	4.5-5.5	2.0(M6)	1.2 U $g_{biomass}^{-1}$	75-80
	TFE	85	4.5-5.5	1.0(MT4)	3.5 U $g_{biomass}^{-1}$	
E.coli Rb-791expressing	TDFE	75	5	-	36 U $g_{biomass}^{-1}$	80-85
Sulfolobus solfataricus MT4 enzymes	TFE	85	5	-	726 U $g_{biomass}^{-1}$	

Various methods for large-scale production of trehalose have already been reported. They include: (1) Production of trehalose from starch or maltose by thermostable enzymes of thermophilic bacteria (Mukai et al., 1997; Yoshida et al., 1997; Kobayashi et al., 1997). (2) Accumulation of trehalose from glucose by fermentation using *Saccharomyces cerevisiae* and Basidiomycotinous yeast (Schick et al., 1995; Miyazaki et al., 1996). (3) Synthesis of trehalose from glucose and glucose-1-P by trehalose phosphorylase of *Pichia fermentans* (Schick et al., 1995). (4) Sucrose was converted into glucose1-phosphate and fructose by sucrose phosphorylase, fructose was further converted into glucose by glucose isomerase. Then, trehalose phosphorylase catalyzes the condensation of glucose-1-phosphate and glucose to form trehalose (Satto et al., 1998). It is reported that trehalose produced by the enzymatic conversion can be used in food industries as the final reaction product contains other sugars in addition to trehalose (Mukai et al., 1997), while trehalose produced by the fermentation process can be applied in medical industries as the trehalose in the extract of the yeast cells is very pure (Miyazaki et al., 1996). However, at present a larger application of trehalose is limited by its relatively high price, since trehalose is extracted from yeasts and the fermentation substrate is glucose. Although the enzymatic conversion has many advantages, but it needs at least three enzymes (Mukai et al., 1997).

The conventional process for producing trehalose using *Saccharomyces cerevisiae* has a relatively low production yield and has been replaced by an enzymatic conversion process (Maruta et al., 1995; Li et al., 2011). Nowadays, however, overproduction of microbial metabolites is possible by the rational design of microorganisms due to advances in genetic and metabolic engineering tools. An attractive advantage of microbial-based processes is the ability to efficiently utilize agricultural or industrial wastes as substrates for the production of valuable metabolites (Li et al., 2011). Therefore, microbial production of trehalose in high yield could offer an economically advantageous and environmentally-friendly alternative to currently employed enzymatic processes. Understanding the metabolic pathways involving trehalose biosynthesis in microorganisms is a prerequisite for developing an appropriate strain.

Recently, trehalose titre upto 1.7 g/L in controlled bioreactor cultures was achieved using synthetic M9 medium supplemented with 40 g/L glycerol, 0.1mM validamycin A, and 300 mM NaCl in *E. coli* (Li et al., 2011). But the limitation of the process is use of validamycin (very costly) and NaCl (not preferable). In one of previous studies, it was found that *S. fibuligera* sdu cells could accumulate trehalose upto18.0% (w/w) of biomass from soluble starch in SSY medium (Chi et al., 2001). However, it seems that corn starch

as a substrate for trehalose production is better than soluble starch because corn starch is more easily obtained and much cheaper than soluble starch. However, enhanced conversion of corn starch to trhalose was obtained upto 22% of biomass (Chi et al., 2010). A thermo sensitive mutant of *S. wibuligera* was reported to accumulate trhalose upto 28% of biomass of trehalose using cassava starch (Wang D-S et al., 2011). In *Corynebacterium* recombinant strain over-expressions of OtsAB and TreYZ leads to trehalose yield (g/g biomass) of 31% (Carpinelli et al., 2006).

In the present study an effort was made to improve the trehalose production by fermentation route using cheap carbon sources like crude glycerol.

2.3 *Propionibacterium freundereichii*

Propionibacteria were first described by E. Von Freudenreich and S. Orla-Jensen, during their study on propionic acid fermentation in cheese, leading to propose the genus *Propionibacterium* (Jensen et al., 1909). *Propionibacteria* are firmicutes with a high G + C content, gram-positive, non-sporing, non-motile pleomorphic rods. They are anaerobic to aerotolerant and generally catalase positive. They grow optimally at 30°C and neutral pH. Cells are hetero-fermentative and can metabolise a range of substrates such as carbohydrates (including glucose, galactose, fructose and lactose), alcohols (glycerol and erythritol) and organic acids (lactate and pyruvate). *Propionibacteria* present a particular central carbon metabolic pathway, propionic fermentation. This fermentation involves the Wood-Werkmann cycle (Wood et al., 1991) which produces propionate, acetate, succinate and carbon dioxide. *Propionibacterium freudenreichii* has been used for a long time as a ripening culture in Swiss-type cheese manufacture, and is more and more considered for its potent probiotic effects. It accumulates inorganic polyphosphate (Poly P) as energy reserve, carbon storage compounds (glycogen), and compatible solutes such as trehalose. *P. freudenreichii* can exert health-promoting activities, such as bifidogenic effect in the human gut and promising immunomodulatory effects (Thiery et al., 2011). Many *P. freudenreichii* strains are involved in adaptation, cheese ripening, bio-preservation and probiotic effects and are highly strain-dependent. The elucidation of the molecular mechanisms is now facilitated by the availability of genome sequence and molecular tools. Propionibacterium is found in cheese together with lactic acid bacteria (Marty et al., 2011; Ben et al., 2003). It is also known for producing antiyeast metabolites and antifungal compounds (Miescher et al., 2008 & 2011; Lind et al., 2010). It has also been reported to have improved bile salt tolerence (Reimann et al., 2011).

2.3.1 Taxonomy, diversity and probiotic properties

The genus *Propionibacterium* is divided in two groups based on habitat of origin: classical or dairy *Propionibacteria* (mainly isolated from dairy products such as cheese) and cutaneous *Propionibacteria* (typically found on skin). *Propionibacteria* belong to the Actinobacteria class, comprising high G+C content Gram-positive bacteria (Stackebrandt et al., 1997). Dairy propionibacteria have been traditionally isolated from milk and dairy products. Four typical dairy species were early described: *P. freudenreichii, P. acidipropionici, P. jensenii and P. thoenii* (Thierry et al., 2011). Cutaneous propionibacteria are commensal of mammals including humans, the most studied species within this group being *P. acnes*, involved in acne and in post surgery infections (Thierry et al., 2011).

The genome size of *P. freudenreichii* ranges around 2.6 Mb and its G+C content is 67%. The first genome of a *P. freudenreichii* strain (CIRM-BIA1T) has recently been sequenced (Falentin et al., 2010). Recently, fingerprinting methods such as Pulsed-Field Gel Electrophoresis and Randomly Amplified Polymorphic DNA-PCR were reported to characterise *P. freudenreichii* at the strain level (Meile et al., 2008). Similarly, multi-locus sequence typing which is based on the sequence analysis of internal fragments of seven genes has recently been developed for *P. freudenreichii*. It was applied to 113 strains of different phenotypes and origins (Dalmasso et al., 2011).

Some strains of dairy *Propionibacteria* are also used in probiotic preparations, alone or in combination with lactic acid bacteria and/or bifidobacteria. A probiotic is defined as "a live microorganism which, when administered in adequate amounts, confers a health benefit on the host" (FAO report, 2006). An increasing number of reports on potential probiotic properties of *Propionibacteria* have been published (Thierry et al., 2011).

Finally, the interest in *P. freudenreichii* for its various probiotic activity which were reported, are production of bifidogenic compound it produces, 1, 4-dihydroxy-2-naphthoic acid (DHNA) (Isawa et al., 2002; Kaneko et al., 1999). This compound stimulates growth of bifidobacteria, which are beneficial for human health (Bougle et al., 1999; Hojo et al., 2001). *P. freudenreichii* adapts very well to gastric and bile salt stresses (Jan et al., 2002; Leverrier et al., 2003) and is able to survive and maintain active metabolism *in vivo* in the rat or human gut. Live freeze-dried strains of *Propionibacteria* are already commercially available as tablets to improve intestinal transit (Jan et al., 2002). In vitro, *P. freudenreichii* produces beneficial metabolites; including short chain fatty acids, and conjugated linoleic

acid and some strains like *P. freudenreichii* JS also exhibit immunomodulatory activity (Kekkonen et al., 2008). *P. freudenreichii* has lots of evidence for safe use in human diet and animal feed. *P. freudenreichii* has been granted the Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration (Mogensen et al., 2002). *P. freudenreichii* also belongs to the list of agents recommended for Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA, 2009). Similarly, *Propionibacterium* can be exploited for in-situ production of trehalose in cheese, since various developments in developing strategies for probiotic potential of cheese has been reported (Grattepanche et al., 2008).

2.3.2 Important Metabolic pathways in *Propionibacterium*

Propionibacteria exhibit some unusual metabolic pathways that have been investigated in detail. The metabolism of *Propionibacterium* is very complex, because several interconnected pathways are used simultaneously. *Propionibacteria* are anaerobic but are also aerotolerant. They have low nutritional requirements and are able to fit, survive and remain active in various environments. *P. freudenreichii* is able to grow, under anaerobic conditions, in a minimal medium containing a carbon source, ammonium as the sole nitrogen source, minerals, and two to four vitamins (Falentin et al., 2010). *P. freudenreichii* strains, is able to use a variety of carbon substrates, including sugars (lactose, galactose, D-glucose, D-mannose), alcohols (erythritol, glycerol, adonitol), and acids (lactic acid, gluconic acid) (Falentin et al., 2010). Genome annotation clearly confirmed that this strain was able to import these carbon sources and to catabolise them by different pathways (glycolysis, pentose phosphate, and Entner-Doudoroff pathways) (Falentin et al., 2010). The use of other carbon substrates, such as D-fructose, L-arabinose, ribose, D-raffinose, saccharose, xylitol, and gluconic acid, is reported to be strain-dependent in *P. freudenreichii* (Falentin et al., 2010).

Propionic acid fermentation was reported via the Wood Werkman cycle as shown in fig 2.5. Propionic acid is the major end product of fermentation in *Propionibacteria* and confers their typical flavour to Swiss-type cheeses. The well known pathway for propionic acid production is known as Wood-Werkman cycle, involves succinyl-CoA and methylmalonyl-CoA as intermediates. It was first described in *P. freudenreichii* and *Pelobacter propionicus* (Schink et al., 1987), and is present in other bacterial species such as *Bacteroides fragilis* (Macy et al., 1978). The Wood-Werkman cycle was extensively investigated in *P. freudenreichii* at biochemical (Falentin et al.,

2010) and genetic levels. It includes a methylmalonyl-CoA carboxytransferase, a methylmalonyl-CoA epimerase, and a methylmalonyl-CoA mutase (Fig 2.6). The key feature of the Wood-Werkman cycle in *P. freudenreichii* is a transcarboxylation reaction without the involvement of free CO_2. The enzyme catalyzing this reaction is a methylmalonyl-CoA carboxytransferase, transferring a carboxyl group from methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA (Fig. 2.6). The enzyme involved has been fully characterized and its structure resolved. It is a biotindependent carboxytransferase (EC 2.1.3.1) composed of three subunits. The complete Wood-Werkman cycle is presented in Fig. 2.6.

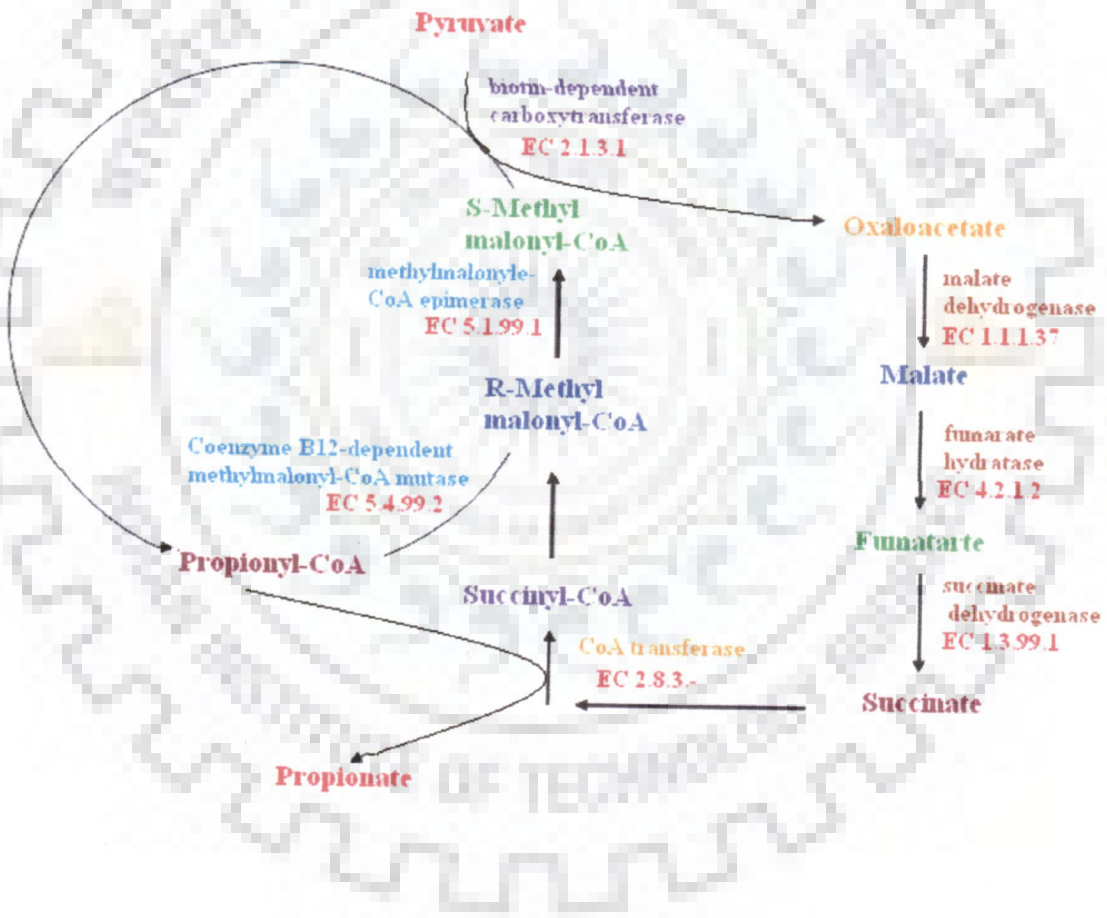

Fig 2.6 Wood workman cycle for Propionic acid fermentation (adapted from Thiery et al., 2011).

P. freudenreichii is usually grown under anaerobic or microaerophilic conditions and described as an anaerobe. However, early studies also reported oxidative activity with free oxygen on a variety of substrates in *Propionibacteria* (Vorobjeva, 1999). Accordingly, all

the genes required for aerobic respiration were also identified in *P. freudenreichii* genome: genes encoding NADH dehydrogenase, succinate dehydrogenase, cytochrome bd complex, ATPase and the complete pathway for heme synthesis (Falentin et al., 2010). A switch from anaerobic to aerobic culture induces the consumption of the propionate produced in anaerobic conditions. Such a shift has been applied to improve the yield of vitamin B_{12} production (Ye et al., 1999) and 1,4-dihydroxy-2- naphthoic acid production (Furuichi et al., 2007). Under anaerobic conditions, the electron acceptor in *P. freudenreichii* can be sulphate, fumarate, nitrate, menaquinone (vitamine K2), or a pool of ferrous iron and humic acid in soil (Benz et al., 1998).

2.3.2.1 Vitamin B_{12} biosynthesis pathway

Fig 2.7 shows Vitamin B_{12} biosynthesis in *Propionibacterium*. At present, production of vitamin B_{12} by chemical means is not feasible. Vitamin B_{12} is produced by fermentation in an industrial scale using *Propionibacterium freudenreichii*. It has demonstrated that *Propionibacteria* species has the highest potential to accumulate vitamin B_{12} intracellularly. It has been exploited for Vitamin B_{12} production with co-culture lactic acid bacteria (Hugenschmidt et al., 2010 & 2011).

At present, production of vitamin B12 by chemical means is not feasible. Vitamin B12 is produced by fermentation in an industrial scale using *Pseudomonas* sp. (Florent and Ninet, 1979), *Propionibacterium freudenreichii* (Crespo et al., 1991; Nakano et al., 1996), *Eubacterium limosum* (Lebloas et al., 1984) and *Methanosarcina barkeri* (Mazumder et al., 1986). It has been demonstrated that *Propionibacteria* species has the highest potential to accumulate vitamin B_{12} intracellularly.

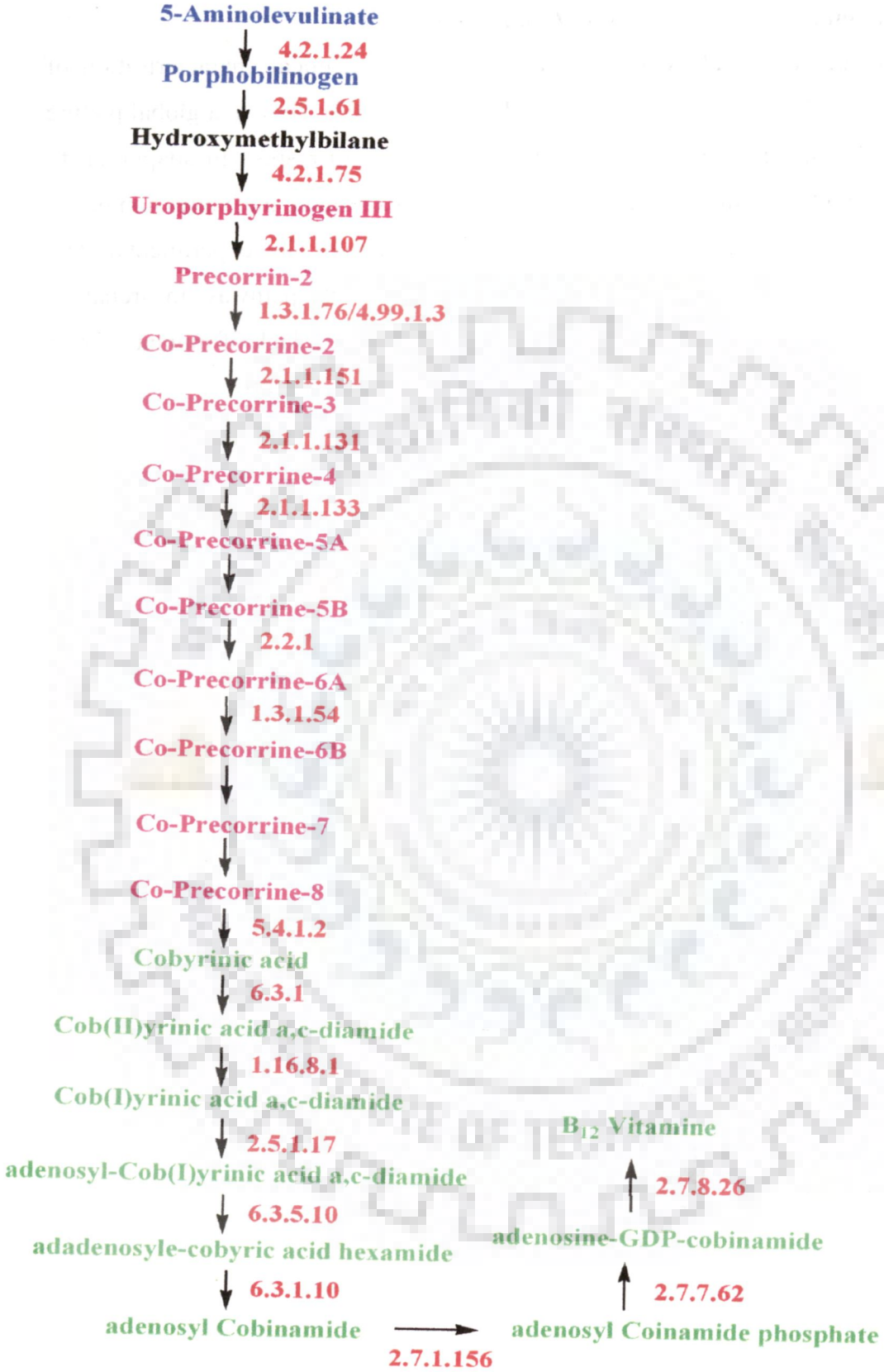

Fig 2.7: Vitamin B_{12} synthesis in *Propionibacterium* (Falentin et al., 2010)

2.3.2.2 Trehalose metabolic pathway in *P. freudenreichii*

In one of previous study with *P. freudenreichii* biochemical characterisation of trehalose synthesis pathway was carried out (Cardoso et al., 2007). Herein, a global picture of trehalose metabolism in *P. freudenreichii* was obtained with respect to adaptation to osmotic, oxidative and acid stress. Under all types of stress examined, the expression of the OtsAB pathway for trehalose synthesis was clearly enhanced. The experimental data presented here strongly suggest the involvement of the TreS pathway in trehalose catabolism; however, conclusive evidence was not obtained due to lack of mutants. Their proposed pathway is presented in fig 2.8 adapted from Cardoso et al., 2007.

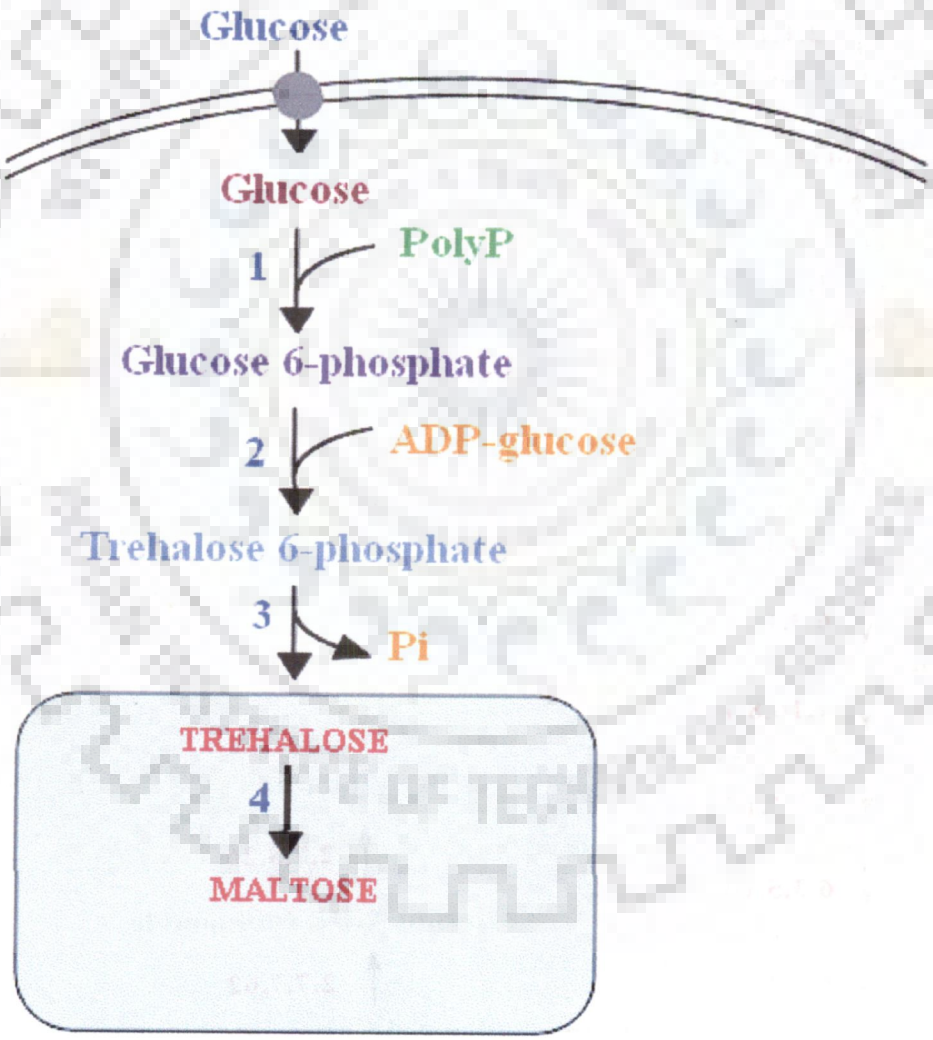

Fig 2.8 Proposed trehalose metabolic pathway in *P. freudenreichii* (Cardoso et al., 2007). 1, 2, 3 and 4 are phosphoglucomutase, OtsA, OtsB and TreS.

2.3.3 Intracellular storage for survival

P. freudenreichii displays numerous features which allow its long term survival, including the accumulation of energy and carbon storage compounds, the accumulation of compatible solutes, and the induction of a multi-tolerance response under carbon starvation. Interestingly, *P. freudenreichii* is able to accumulate inorganic polyphosphate (polyP) as an energy reserve whereas most bacteria utilize ATP. Only bacteria particularly adapted to extreme environments are able to use polyP. The key enzyme involved in the synthesis of polyP in bacteria is polyphosphate kinase (PPK). PPK synthesizes polyP by transferring the terminal phosphate of ATP to polyp (Falentin et al., 2010).

P. freudenreichii is able to synthesize glycogen, as reported for the first time using in vivo ^{13}C NMR analysis of cells grown in the presence of ^{13}C glucose (Meurice et al., 2004). The genes potentially involved in glycogen metabolism had not been previously described in *P. freudenreichii*. Six genes related to glycogen metabolism were identified in the genome and they are encoding ADP-glucose pyrophosphorylase, a glycogen synthase, a glycogen branching enzyme, an glucan phosphorylase, and two glycogen de-branching enzymes, respectively (Falentin et al., 2010). Four of these six genes were also found in *P. acnes*. Since phenotypic data indicate that neither *P. freudenreichii* nor *P. acnes* is able to ferment extracellular glycogen, these enzymes must be involved in intracellular glycogen accumulation and/or hydrolysis.

P. freudenreichii strains are able to synthesize and accumulate trehalose from glucose and pyruvate (Cardoso et al., 2004). The synthesis of trehalose is enhanced at the beginning of the stationary phase and under oxidative, osmotic, and acid stress conditions (Cardoso et al., 2004, 2007) and this ability is strain-dependent. Trehalose is most commonly synthesised in bacteria via the trehalose-6- phosphate synthase/phosphatase (OtsA and OtsB) pathway and catabolised by trehalose synthase (TreS). The genes OtsA, OtsB, and TreS were previously identified in strain NIZO B365. These three genes (PFREUD_12170, PFREUD_12160, PFREUD_10650) were similarly organized in CIRM-BIA1T and the corresponding proteins showed 99%, 99% and 100% similarity, respectively, to the previously reported sequences. *P. freudenreichii* is also known to accumulate glycine betaine. In addition to osmotic stress adaptation (Falentin et al., 2010) glycine betaine participates in long-term survival, as does trehalose, by acting as a chemical chaperone. Genes supporting glycine betaine transport and biosynthesis reflect this ability. Glycine betaine is synthesized by oxidation of choline (dehydrogenase, PFREUD_19130), leading to betaine aldehyde, which is then oxidized to glycine betaine

(Betainealdehyde dehydrogenase, dha1, PFREUD_01860). Similarly, accumulation of exopolysaccharides is reported in *Propionibacterium* (Thiery et al., 2011). In *Lactobacillus* production of exopolysaccharide is reported hence it can be further exploited in *Propionibacterium* (Champagne et al., 2007 ; Patel et al., 2010 & 2011).

2.4 Metabolic pathways of Trehalose biosynthesis

The OtsAB pathway, the most common route known to be involved in the stress response of *E. coli* and yeast, proceeds from UDP-glucose and glucose-6-phosphate to form trehalose-6-phosphate, which is subsequently dephosphorylated to yield free trehalose (Fig 2.8, 2.9). The reactions are catalyzed by trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively (De Smet et al., 2000). Less-prominent routes for trehalose synthesis are the TreYZ (De Smet et al., 2000, Kobayashi et al., 1996, Maruta et al., 1996) and the TreS pathways (De Smet et al., 2000; Cardoso et al., 2007) (Fig 2.9 & 2.10). The substrates of the TreYZ route are oligomaltodextrins or glycogen (Maruta et al., 1996). In the first reaction step, TreY (maltooligosyl trehalose synthase) transglycosylates a terminal maltosyl residue into a trehalosyl residue before trehalose is liberated through the activity of TreZ (maltooligosyl trehalose hydrolase). Finally, it was described that TreS (trehalose synthase) transforms maltose in a single transglycosylation reaction into trehalose (De Smet et al., 2000; Cardoso et al., 2007).

Trehalose phosphorylase (TreP) has been reported in *Agaricus bisporus*, *Catellatospora ferruginea, Euglena gracilis* and *Flammulina velutipes*. TreP catalyzes a reversible reaction *in vitro*, which hydrolyzes trehalose and transfers a glucose molecule to the inorganic phosphate, to form glucose-1-phosphate and release free glucose (Wannet et al., 2005; Ren et al., 2005) (Fig 2.9 & 2.10)

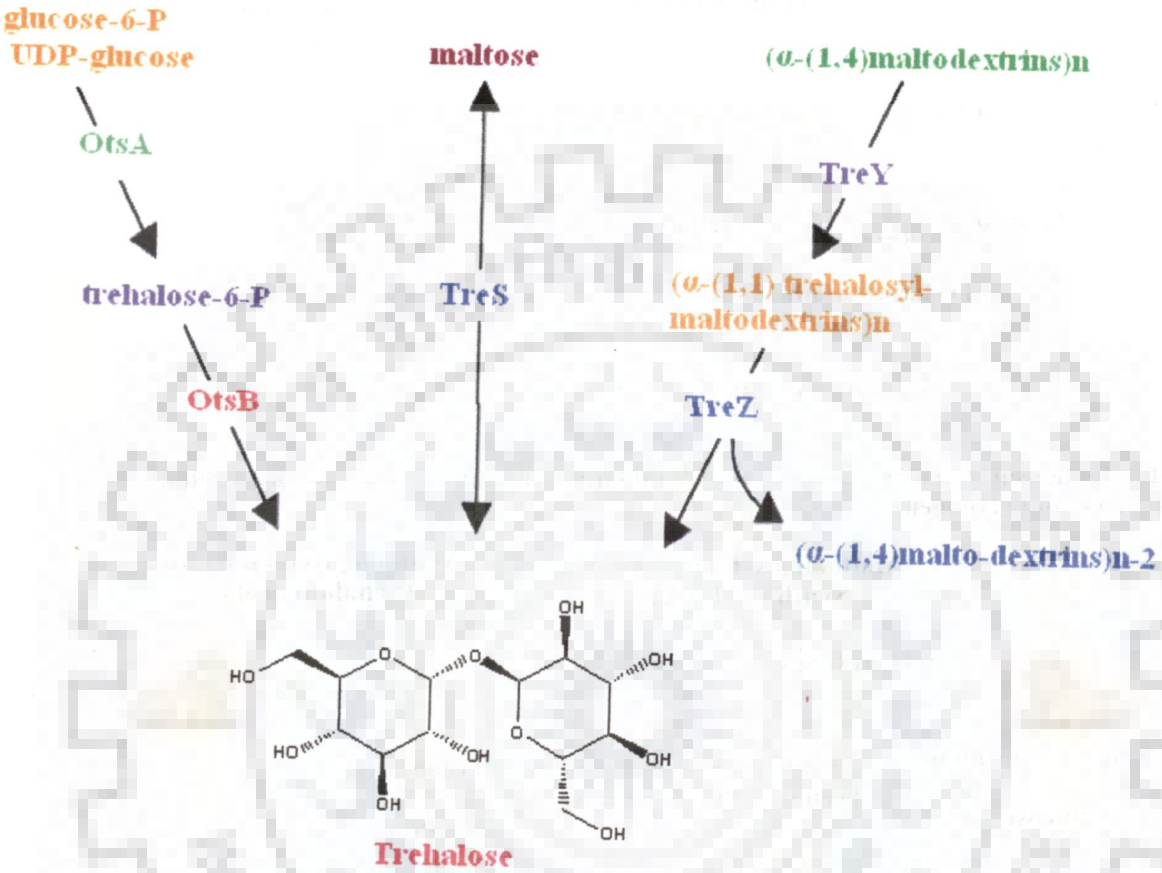

Fig 2.9: Three pathways of trehalose biosynthesis generally found in nature (Morbach & Kramer, 2005)

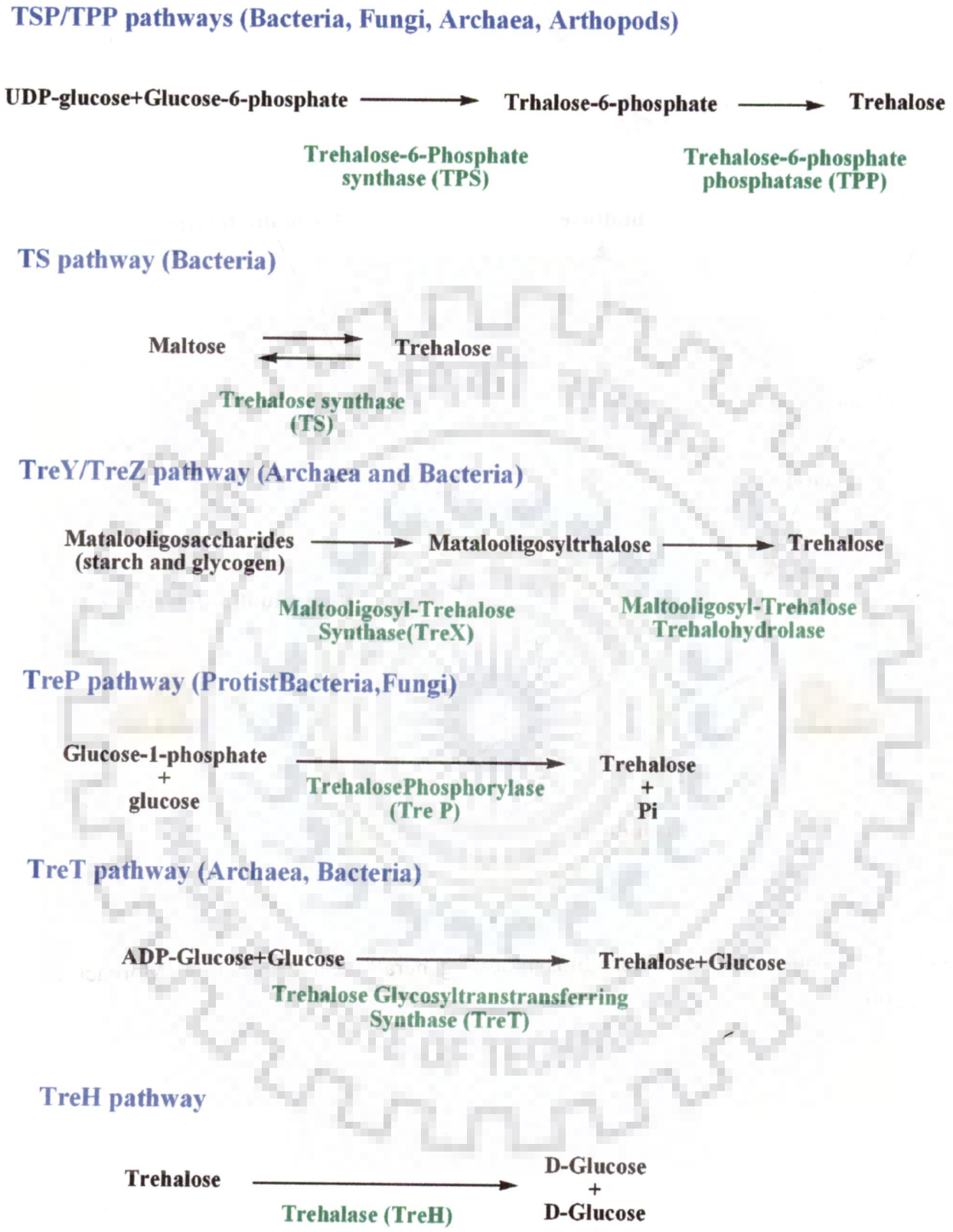

Fig 2.10: Different pathways of trehalose synthesis (Iturriaga et al., 2009)

2.4.1 Role of different enzymes of central metabolic pathway at glucose-6-P, glucose-1-P and UDP-glucose nodes for trehalose biosynthesis

Three important nodes of central metabolic pathway are glucose-6-phoshate (pathway towards glycolysis and pentose phosphate pathway), glucose-1-phosphate (UDP-glucose/ADP-glucose/GDP-glucose) and UDP-glucose node as shown in fig 2.11. Several reports on influences of these various enzymes have been reported previously in *Corynebacterium* and yeast.

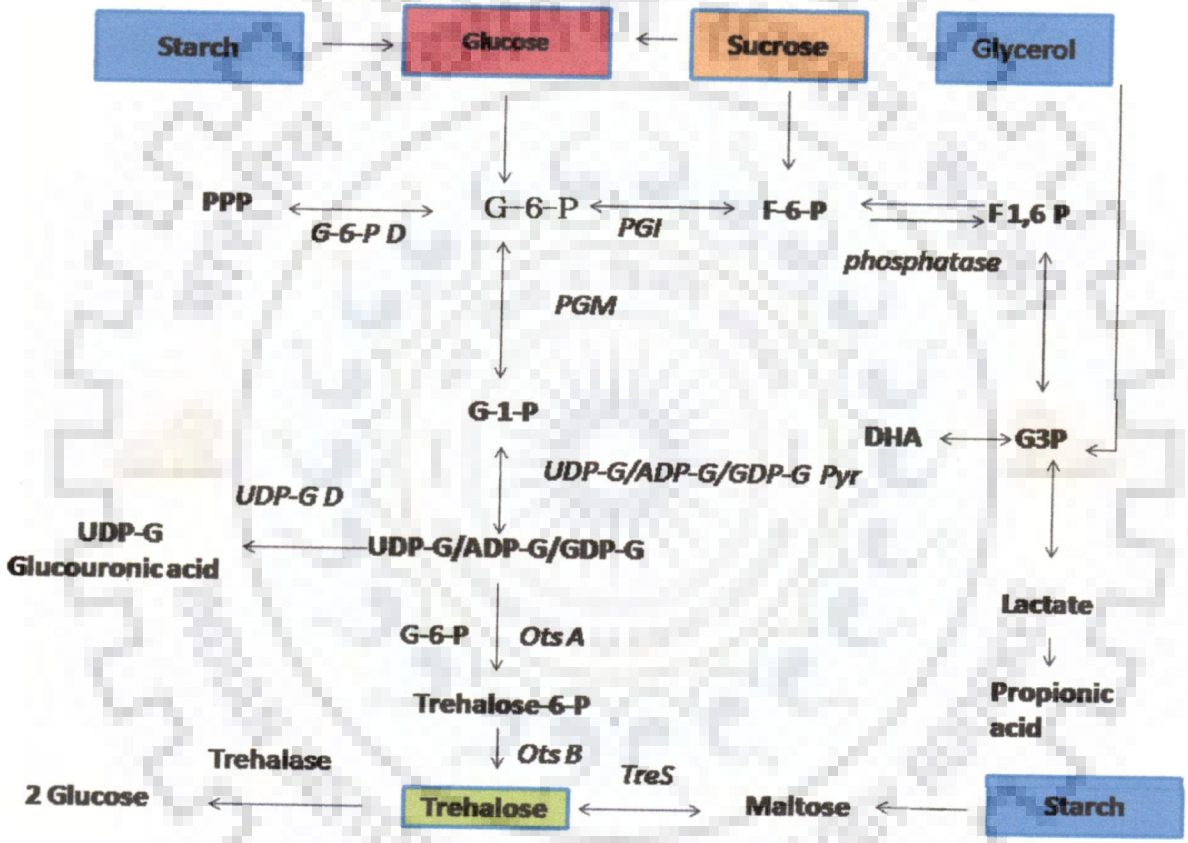

Fig 2.11: Important enzymes which may influence trehalose synthesis at three nodes of central metabolic pathway

2.4.1.1 ADP-glucose/UDP-glucose/GDP-glucose pyrophosphorylase

These three enzymes are important for the synthesis of important substrate for OtsA known as nucleotide sugars ADP-glucose, UDP-glucose and GDP-glucose. In previous reports influence of UDP-glucose pyrophosphorylase (Gal U) has been studied in *Corynebacterium* and yeast. In a study on heterologous expression of *E. coli* genes of gal

U in *Corynebacterium* it was reported, that gal U over-expression along with OtsAB had increased trehalose yield but no improvement was observed on individual over-expression of gal U or OtsAB (Padilla et al., 2005). In yeast during heat shock, 12 fold increase in UDP-glucose pyrophosphorylase resulted in modest increase in trehalose yield (Voit et al., 2003). NDP-glucose is involved in various other cellular metabolic activities like synthesis of exopolysaccharides or cell wall formation, thus to regulate the trehalose biosynthesis individually, cells may adopt NDP-glucose pyrophosphorylase as a controlling element (Fig 2.11). This fact was further supported in *P. freudenreichii* where it was found that OtsA was more specific to ADP-glucose in crude extract while pure enzyme was more specific to UDP-glucose followed by GDP-glucose and ADP-glucose (Cardoso et al., 2007).

2.4.1.2 Phosphoglucomutase

In yeast, role of phosphoglucomutase depends on various other factors like availability of glucose-6-phosphate (Voit et al., 2003). No direct study of influence of phosphoglucomutase on trehalose bio-synthesis in *Corynebacterium* or other microbe was ever reported.

2.4.1.3 Concentration of metabolites

The trehalose is synthesised from substrates, UDP-glucose and glucose-6-phosphate, hence their concentrations may also have an influence. Thus availability of UDP-glucose can be regulatory in nature. The effect of glucose-6-phosphate on trehalose biosynthesis was predicted to be insignificant in yeast (Voit et al., 2003). Similarly, glycogen, maltose formation may have influence as they are linked to trehalose biosynthesis through TreS and TreYZ pathway (Cardoso et al., 2007).

2.4.1.4 Glycolysis and pentose phosphate pathway

In yeast, it was proposed that the effect of glucose-6-phosphate dehydrogenase was insignificant while in *Corynebacterium* over-expression of glucose-6-phosphate dehydrogenase reduced the trehalose yield by 33% (Voit et al., 2003; Becker et al., 2007).

2.4.1.5 Trehalase and TreS

Another important enzyme, trehalose synthase (TreS) have important role in trehalose metabolism. This pathway was reported in the complete genome sequence of *P.*

freudenreichii (Falentin et al., 2010). TreS was a part of catabolic pathway of trehalose in *P. freudenreichii* (Cardoso et al., 2007). But it should be noted that catabolic nature of TreS was reported under osmotic stress. TreS is reversible and can interconvert trehalose and maltose; probably anabolic nature of this enzyme may be dependent on carbon sources In bacteria *Pimelobacter* and *Thermus aquaticus* TreS synthesize trehalose by converting α 1-4 linkage of maltose into α 1-1 linkage (to form trehalose) (De Smet et al., 2000). Hence effects of carbon source and osmotic stress can be different for TreS pathway. Role of trehalase has been reported extensively in yeast and it was shown too involved in catabolic pathway of trehalose (Arguelles, 2000).

2.4.1.6 UDP-glucose dehydrogenase

The role of enzyme, UDP-glucose dehydrogenase was difficult to correlate it with trehalose biosynthesis. UDP-glucose dehydrogenase converts its substrate UDP-glucose to UDP-glucouronic acid (Chong et al., 2005, Granja et al., 2007), thus this enzyme competes with OtsA for substrate UDP-glucose. This enzyme needs co-factor like NAD^+/$NADP^+$ along with UDP-glucose thus its effects may be indirectly correlated with other part of biosynthesis pathway. There were no reports on the role of UDP-glucose dehydrogenase on trehalose production.

2.4.1.7 Over-expression of Fructose 1, 6 biphosphatase

In *Corynebacterium* over-expression of fructose1,6 biphosphotase led to higher yield of lysine but there was decrease in trehalose content in the recombinant strain (Becker et al., 2005).

It can be concluded that no particular enzyme has total control on trehalose biosynthesis and similar conclusion was also predicted in yeast where optimum ratios of particular enzymes found to have influence on trehalose biosynthesis (Jung et al., 2003). In the present study molecular level understanding of trehalose biosynthesis was carried out by monitoring enzyme activity and metabolites concentrations associate with glucose-6-phosphate, glucose-1-1 phosphate and UDP-glucose node.

2.5 Use of crude glycerol from biodiesel waste for microbial conversion

Biodiesel manufacturing industries are rapidly expanding and their wastes are a major concern for environmentalists. Biodiesel production is one of the possible solutions to energy crisis and it provides a sustainable and economically feasible source of

alternative energy (Yusuf et al., 2011). Biodiesel is produced, primarily, by the trans-esterification of triglycerides with methanol or ethanol. After separation, two phases are obtained: an oil phase, consisting of alkyl esters (methyl or ethyl esters), and a phase rich in glycerin, consisting of glycerol, soap, alcohol and hydroxides. Glycerin is the main coproduct from biodiesel production by trans-esterification and it is obtained in the raw form, which has a low commercial value due to the presence of several impurities. However, this coproduct may be used as a source of raw material for the production of high value added product through chemical or biochemical conversion, such as polymers and additives for fuels, esters and ethers of glycerin. The production of biodiesel increased from almost zero in 1991 to 16000 million litres in 2010 (Gui et al., 2008; Yusuf et al., 2011). In general, crude glycerol is a major by-product of biodiesel industry. Crude glycerol contains methanol, soaps and salts as impurities. For industrial biodiesel production, most preferred method is alkaline trans-esterification (Fjerbaek et al., 2009). This method uses fat, lard, tallow or vegetable oil and alkaline catalyst (dissolved in methanol) which results in separate layers of biodiesel and its waste. The waste is a rich source of crude glycerol but due to the presence of impurities, its purification is not economical (Mu et al 2009). Un-reacted methanol and soaps formed during trans-esterification reaction were considered as two major impurities of crude glycerol (Alhanash et al., 2008). Further, during pre-treatment of biodiesel waste with HCl (adjustment of pH from 12 to 1), soaps are converted into free fatty acids and salts like KCl or NaCl. Many investigations reported utilization of crude glycerol for various alternative uses like combustion, composting and biological conversions. In fact, the conversion of this waste by a thermo-chemical method to propylene glycol and acetol was reported previously (Chiu et al., 2006). Uses of crude glycerol for the productions of food additives, pharmaceuticals, and synthesis of polyethers, polyols and production of detergents have also been reported (Katrynoik et al., 2011). Oxidation of glycerol results in many values added products like formic acid, lactic acid, acetic acid, oxalic acid, glyceric acid, tartronic acid and dihydroxyacetone (DHA) (Katrynoik et al., 2011). Similarly, microbial conversions of crude glycerol into value added products are an alternative way for the utilisation of this waste and apparently it seems to be economical and environmentally attractive. In biological conversion approaches, crude glycerol is used as a carbon source by microbes to form microbial biomass, along with other desirable microbial products. Several microbial products were produced from crude glycerol like 1, 3 propanediol (Himmi et al., 1999), hydrogen (Ito et al., 2010), succinic acid (Lee et al., 2007) and

omega-3 polyunsaturated fatty acids (Pyle et al., 2008) using *Clostridium butyricum*, *Enterobacter aerogens*, *Anaerobiospirillum succiniciproducens* and algal fermentation respectively. In the present study, an effort was made to explore the utilization of crude glycerol for fermentative productions of trehalose and propionic acid.

Trehalose is a non-reducing disaccharide accumulated in microbes under stress conditions (Arguelles, 2000). Trehalose has many advantages and production of this nutraceutical is of commercial importance (Higashiyama, 2002). Trehalose is currently produced by enzymatic conversion and it is preferably used in food industries as it contains mixture of other sugars along with trehalose, while trehalose produced by the fermentation route can be used for pharmaceutical industries as the trehalose in the extract is reported to be relatively pure (devoid of other sugars). At present a larger applications of trehalose are limited due to its higher price and non-availability of relatively pure trehalose. Amongst the microbes, the production of trehalose from yeast is well known and the conversion of cassava starch to trehalose by a mutant strain, *Saccharomycopsis fibuligera* A11 was also reported (Chi et al., 2003). Similarly, the uses of maltose, sucrose and glucose as carbon source for trehalose productions are well known (Zhang et al., 2010). However, there are no reports on the use of glycerol as a carbon source for trehalose production. According to the GRAS notification for Hayashibara trehalose submitted by Hayashibara International Inc, annual production of trehalose was 20000 tons with a market value of approximately 50 million US dollars (Oda et al., 2001).

2.5.1 Composition of Crude Glycerol

The crude glycerol produced during the biodiesel production process is impure and of little economic value. The impurities include methanol and soaps. Biodiesel producers use excess methanol to drive the chemical transesterification and do not recover the entire methanol. Therefore, it is present in the glycerol layer. Also, free fatty acids present in the initial feedstock can react with the base to form soaps that are soluble in the glycerol layer. In addition to methanol and soaps, crude glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, or sulphur (Thompson and He, 2006).

It has been reported that glycerol constitutes anywhere from 65% to 85% (w/w) of the crude glycerol streams (Gonzalez-Pajuelo et al., 2005; Mu et al., 2006). The remaining portion in the crude glycerol streams is mainly methanol and soaps (Thompson and He, 2006). The wide range of the purity values of crude glycerol can be attributed to different glycerol purification methods used by the biodiesel producers and the different feedstock

used in biodiesel production. For example, Thompson and He (2006) have characterized the glycerol produced from various biodiesel feed stocks. It was found that the crude glycerol from any feedstock is generally between 60 and 70 % (wt) glycerol. Mustard seed feedstock had a lower level (62%) of glycerol, while soy oil feedstock had 67.8 % glycerol and waste vegetable had the highest level (76.6 %) of glycerol. Thompson and He (2006) also investigated the elemental composition of crude glycerol in crude glycerol stream. The elements present in the glycerol produced from most feedstock (such as mustard seeds, canola, soybean, and waste vegetable oil) were similar. Calcium was in the range of 10-20 ppm, magnesium was 3-7 ppm, phosphorous was 10-60 ppm, and sulphur was 14-21 ppm. It should be noted that when crambe, an oilseed crop, was used as feedstock, the crude glycerol contained the same elements, but at vastly different concentrations. Schröder and Südekum (1999) have also reported the elemental composition of crude glycerol from rapeseed oil feedstock. Phosphorous was found to be between 1.05 % and 2.36 % (w/w) of the crude glycerol. Potassium was between 2.20 % and 2.33%, while sodium was between 0.09% and 0.11%. Cadmium, mercury, and arsenic were all below detectable limits.

2.5.2 Uses of Glycerol

Because there is a glut of this impure glycerol, there have been many investigations into alternative uses for it. Combustion, composting, animal-feeding, thermo-chemical conversions and biological conversion methods for glycerol usage and disposal have all been proposed (Pyle et al., 2008). Some simple methods for the usage of glycerol have been proposed. For example, Johnson and Taconi (2007) reported that combustion of crude glycerol is a method that has been used for disposal. However, this method is not economical for large producers of biodiesel. It has also been suggested that glycerol can be composted (Brown, 2007) or used to increase the biogas production of anaerobic digesters (Holm-Nielsen et al., 2008). DeFrain et al. (2004) attempted to feed biodiesel-derived glycerol to dairy cows in order to prevent ketosis, but found that it was not useful. Also, Lammers et al. (2008) studied supplementing the diet of growing pigs with crude glycerol. Their study found that the metabolizable to digestible energy ratio of glycerol is similar to corn or soybean oil when fed to pigs. Therefore, the study concludes that "crude glycerol can be used as an excellent source of energy for growing pigs", but also cautions that little is known about what the impacts of impurities in the crude glycerol. Furthermore, Cerrate et al. (2006) have had some success with feeding glycerol to broiler chickens. Birds fed 2.5 % to 5% glycerine diets had higher breast yield than the control group, but the authors

caution that there is still concern about methanol impurities within the glycerol. These simple methods may be useful in disposing of excess glycerol, but higher-value processes for glycerol utilization should also be investigated. For example, glycerol can be thermochemically converted into propylene glycol (Alhanash et al., 2008; Dasari et al., 2005), acetol (Chiu et al., 2006), or a variety of other products (Johnson and Taconi, 2007). Glycerol can also serve as a feedstock in fermentation processes. For example, Lee et al. (2000) have used glycerol in the fermentation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid. Glycerol has also been used as a carbon source in the fermentation of *E. coli*. This leads to a mixture of products such as ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi et al., 2006).

Recently, a process using crude glycerol as a substrate for the fermentation of the microalgae *Schizochytrium limacinum* has been developed (Chi et al., 2007). Rooney et al. 2009 previously isolated rhamnolipid-producing bacteria from soils at a biodiesel facility. On the basis of their ability to grow on glycerol as the sole carbon source, and Morita et al.2007, described the use of waste glycerol from biodiesel industry in the production of mannosylerythritol lipids by *Pseudozyma antarctica* JCM 10317. In contrast to the relatively expensive purified glycerol, which is an important ingredient in food, drug, cosmetic and chemical production, raw glycerol contains impurities, such as salts and other organic compounds, and is a potentially inexpensive carbon source for the microbial production of chemicals (deSilva., 2009;. Pauchauri, 2006).

Glycerol is non-fermentable by most microorganisms, with the exception of a group of bacteria including *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* species (Zheng et al., 2008). Pilot-scale fermentation of glycerol by *Klebsiella pneumoniae* M5al, at the 5000 l scale, under nitrogen gas delivered at 0.15 volumes/minute (vvm), yielded ethanol to a maximal level of 18 g/l and with productivity of 0.28 g/l/h (Cheng et al., 2007). Mu et al. (2006) reported production of ethanol from crude glycerol to a maximal level of 11.9 g/l, and reported a productivity level of 0.5 g/l/h. In the present work, trehalose production from glycerol (especially from crude glycerol derived from biodiesel waste) was reported. The advantage of this method is the production of intracellular trehalose together with high yield of extracellular propionic acid.

2.6 Propionic acid production

Propionic acid is considered an important chemical intermediate for the synthesis of cellulose fibres, herbicides, perfumes, pharmaceuticals and food preservatives (Zhang et

al 2009). Propionic acid is currently manufactured via petrochemical process which requires petroleum products (non-renewable) (Zhang et al 2009). However, rise in oil prices and demands for bio-based chemical products have generated significant commercial interests on producing propionic acid and other chemicals from bio renewable feedstock, including agricultural and industrial wastes. Hence development of a bio based method for the production of propionic acid is advantageous. At present, commercial production of propionic acid was around 200000 tonnes per year and is expected to grow at 1.8% per year (Coral, 2008). Large amount of crude glycerol obtained (99600 tonnes per annum in 2006) as waste in the biodiesel industry poses a significant environmental risk (Yazdani et al 2009). Hence, it is desirable to use crude glycerol as a renewable low-cost feedstock to produce industrial chemicals like propionic acid.

Propionic acid, an important mold inhibitor and chemical intermediate, is widely used in industry and in particular in the food industry (Zhang et al., 2009). Currently, almost all propionic acid is produced by petrochemical processes, but propionic acid biosynthesis is expected to be a promising option due to use of renewable raw materials and the overall increasing consumer demand for biological products. Although there has been great interest in producing propionic acid from biomass via fermentation using *Propionibacteria*, the relatively low propionic acid yield, and production rate of this approach have created major barriers for commercialisation (Suwannakham et al., 2005)

Propionic acid production by *Propionibacterium acidipropionici* ATCC 4965 was studied using a basal medium with sugarcane molasses (BMSM), glycerol or lactate (BML) in small batch fermentation at 30 and 36 °C. Results indicated that *P. acidipropionici* produced more biomass in BMSM than in other media at 30 °C (7.55 g l^{-1}) as well as at 36 °C (3.71 g l^{-1}). Propionic acid and biomass productions were higher at 30 °C than at 36 °C in all cases studied. The best productivity was obtained by using BML (0.113 g l^{-1} h^{-1}), although the yield of this metabolite was higher when glycerol was used as carbon source because there was no detection of acetic acid (Coral et al., 2008). Similarly, other process has been described for propionic acid production (Gupta et al., 2001; Goswami et al., 2000 & 2001; Balamurugan et al., 2000; Coral et al., 2008).

2.7 Study of metabolic regulation by monitoring enzyme activities and multivariate approaches

With regard to metabolism, the emerging field of metabolomics will generate systems variables in the form of the concentrations of large number of metabolites (Stephanopoulos et al., 2002). Analyzing these large volumes of data is becoming the main challenge in generating new knowledge from high throughput experiments. There is a clear need for computational methods that can integrate large sets of physiological data into a structured picture. The goal of these models will be to capture the complex relationships that are at the heart of the functioning of living cells and organisms with limited prior knowledge of the structure of these interactions. A fundamental premise of systems-based research is that the underlying mechanisms and interactions of a biological system can be probed by introducing a variety of perturbations and measuring the system response. Various approaches have been included for studying metabolic pathways like measurement of flux, gene deletion and over-expression methods (Stephanopoulos et al., 2002).

To study microbial physiology several methods were reported previously, one of them is through measurement of enzyme activities and metabolite concentration especially in exo-polysaccharide or pullulan or β-glucan synthesis (all polysaccharides). This kind of work has given new insight regarding influence of nucleotide sugars synthesizing enzymes on exo-polysaccharides production from different sugars, as reported by measuring enzyme activities in *Lactobacillus delbrueckii* (Grobben et al., 1996) and effect of galactose and glucose in *Lactobacillus casei* (Mozzi et al., 2001). Similarly, exopolysaccharides biosynthesis was correlated to enzyme activities of phosphoglucomutase, epimerase and UDP-glucose pyrophosphorylase in *Streptococcus thrmophilus* (Degeest & Vuyst, 2000). Metabolic pathway for β-glucan was proposed in *Pediococcus parvulus* with respect to carbohydrate by measuring enzyme activities (Velasco et al., 2007). This work reported about new insight with respect to activity of enzymes involved in sugar transport, sugar nucleotide biosynthesis and energy generation. They have also concluded on the basis of higher activity of phosphoglucomutase, that this enzyme plays an important role for EPS production. Similarly, metabolic pathway was proposed with the help of enzyme activities in *Lactobacillus helveticus* for exopolysaccharides synthesis (Torino et al., 2005). In fact, in a study for pullulan production in *Aureobasidium,* analysis of enzyme activities and measurement of UDP-glucose gave important conclusions, like it was observed that when more pullulan was synthesised less UDP-glucose was left in cell extract and higher activities of

phosphoglucomutase, UDP-glucose pyrophosphorylase and glycosyl transferase were observed when the bacteria was grown in different sugars (Duan et al., 2007). Since trehalose biosynthesis involves nucleotide sugar as important substrate hence regulation of these nucleotide sugars through their corresponding enzymes was expected.

Another method applied in present study is multivariate analysis. It is now possible to describe the whole genome of an organism. Similarly, the whole set of proteins that appear at a certain stage in a living cell can at least be considered, even if not quantitatively described, and the same should in principle hold for the total flow of metabolic products. The automated acquisition of large amounts of (omics genomics, proteomics or metabonomics) data results in exploratory and interpretative challenges. The abundance of data is not in itself a guarantee of obtaining useful information on major events taking place in an investigated system. On the contrary, data from the omics field need to be processed, analyzed, in order to highlight the useful information among the measurements. Since these data are highly multivariate in nature, one must use data analysis techniques which are able to cope with the challenges inherent in masses of data, notably noise, collinearities, and missing data. Only with careful data analysis it is possible to address central questions such as how to modify drug chemical structure in order to improve drug performance, or to understand why a certain test creature is a slow responder in a metabonomics assay. Multivariate data analysis (MVDA) involves observation and analysis of more than one statistical variable at a time. It is used extensively in the field of chemometrics, which is defined as the process of extracting useful and relevant information out of data with the aid of statistical tools and techniques. For complex datasets, such as the datasets were obtained from biotech processes, univariate or bivariate analysis is often inefficient and is likely to result in misinterpretation of data (Kourti et al., 2005). Use of projection methods can effectively deal with challenges such as multidimensionality of the dataset, multicollinearity, missing data, and variability from experimental error and noise (Martin etal., 2002). Principal component analysis (PCA), partial least squares (PLS) regression, principal component regression (PCR), discriminant partial least-squares (DPLS) regression, canonical variable analysis, and modified soft independent modelling of the class analogy (SIMCA) are some of the commonly used statistical approaches for data projection and/or regression.

Recently, several data mining algorithms based on projection methods have been successfully applied in the analysis of large amounts of microarray data. Gene clustering, identification of discriminatory genes, and determination of characteristic gene expression

patterns are examples of such applications (Misra et al., 2002; Stephanopoulos et al., 2002). The principal component analysis (PCA) projection method is of particular interest as an unsupervised method that can be applied to reveal the true dimensionality of data, identify redundancies and conveniently represent data in a reduced dimensional space. An introduction to PCA for the physiological oriented researcher has been provided by Benigni and Giuliani (Benigni and Giuliani, 1994). On the other hand, regression analysis is the major tool for obtaining models from measured data.

When measuring m independent variables, an m-dimensional description of the state of the system is obtained. However, some variables may be interrelated or in fact contain exactly the same information. The amount of redundancy is likely to be large in a sizable data set. Therefore, an equally satisfactory description of the data may be possible with fewer dimensions. One particular data reduction technique called principal component analysis (PCA) is used to reveal the true dimensionality of a data set. PCA defines a new lower dimensional space spanned by variables that are linear combinations of the original variables and account for as much of the original total variation as possible. The new variables are called latent variables or principal components. The PCA projection of matrix X is represented as follows:

$$X = TP^T + E$$

Here, matrix T (size n x d) is called the scores matrix and matrix P (size m x d) is called the loadings matrix, where d is the number of principal components. Matrix E is the residuals matrix. PCA is a stepwise optimization procedure where the successive principal components are extracted in such a way that they are uncorrelated with each other and account for successively smaller amounts of the total variation. It is possible to extract as many principal components as there are original variables, however, in most PCA applications the goal is to account for most of the total variation with as few principal components as possible.

The projection approach can be adapted to a variety of data-analytical objectives, (1) summarizing and visualizing a data set, (2) classification and discriminate analysis, and (3) finding quantitative relationships among the variables. This applies to any shape of a multivariate data-set, with many or few variables, many or few observations, and complete or incomplete data tables. In particular, projections handle matrices with more variables than observations very well, and the data can be noisy and highly collinear. Methods used

in this context are principal-component analysis (PCA) (Jackson, 1991; Martens, 1989; Wold et al., 1987) for projecting X down on to a few scores, also called latent variables, giving a summary of X, soft independent modelling of class analogy (SIMCA) (Wold et al., 1984), and partial least-squares discriminant analysis (PLS-DA) (Sjo¨stro¨m et al., 1985) for classification, and principal component regression (PCR) (Martens, 1989;Kalivas, 1999) and PLS for latent variable regression. Furthermore, hierarchical PLS and PCA are two recent modifications, which simplify interpretation in applications involving large number of variables (Eriksson et al 2001; Eriksson et al., 2002).

In the last few years, multivariate methods (''chemometry'') have been increasingly applied in several scientific fields, from analytical biochemistry to biomedical engineering, as well as for microbial monitoring and improvement (Eriksson et al., 2004; van der Werf et al., 2005 ; Robertson et al., 2007). The multivariate approach, however, has rarely been used for cellular physiology research (e.g., Chan et al., 2003; Hwang et al., 2004; Li and Chan, 2004; Persson et al., 1990; Schlosser et al., 1993; Teschendorff et al., 2007). These methods are appealing for our purposes because they allow a significant reduction in the complexity of the phenomena examined, while multivariate observations can be grouped according to their degree of similarity. All this provides a basis to identify the underlying causes in the analyzed phenomena (Brereton, 2003; Johnson and Wichner, 1998a).

The chemical industry has been an early adopter of chemometrics as a quick and economical method of extracting real-time information from the data and thus leading to improved process monitoring and control. PCA has been applied toward control of an activated sludge wastewater treatment plant and this has been shown to enable easier analysis, monitoring, and diagnosis of the system (Tomita et al., 2002). Combination of visible (vis) and near-infrared spectroscopy (NIR) and chemometrics has been used for discrimination between samples of Australian commercial white wines of different varietal origins (Cozzolino et al., 2003). Models developed using PCA, PCR, and DPLS gave an excellent discrimination between samples of the two varietal origins under consideration with an accuracy of up to 98%. These models could be used by the wine industry for identification of white wine varieties or their blends. Fourier transforms infrared spectroscopy (FTIR) and PCA has been used to study oxidation of lubricating base oils (Gracia et al., 2010). The principal components generated were able to explain 99.99% of the variance, with the second PC showing that iron favoured formation of alcohols and esters and thus influenced the oxidation process. Applications of chemometrics in pattern

recognition and multivariate calibration have been shown to result in greater profits for industry due to better process control, faster verification of raw material identification and quality, and faster analysis of wastewater (Seasholtz, 1999). Chemometrics has also been applied for authentication of meat products, on-line estimation of the carcinogenic potential of lubricant base oil and rapid analysis of a gaseous effluent from a heterogeneously catalyzed reaction (Al-Jowder et al., 1999; Lima et al., 2003; Wilkin et al., 2003). PCA of data from over 50 batches at two different production scales (700 L and 1,500 L) was performed (Thomassen et al., 2010).

A variety of applications of chemometrics in biopharmaceutical processes have been published in the last decade (Gabrielsson et al., 2002; Johnson et al., 2007). They include, analysis of NIR spectral information for an antibiotic production process, multivariate statistical process monitoring for processing of pharmaceutical granules, the assessment of seed inoculum quality from a manufacturing process and development of an integrated online multivariate statistical process monitoring, product attributes prediction, and fault diagnosis framework for a fed-batch penicillin fermentation (Vaidyanathan et al., 2002; Undey et al., 2002; Cunha et al., 2002; Undey et al., 2003) In a recent publication, a flexible process monitoring method was applied for analysis of pilot plant cell culture data for fault detection and diagnosis (Gunther et al., 2007). A PCA model was constructed from 19 batches, and the model was shown to successfully detect abnormal process conditions and diagnose root causes.

In the present study, PCA was used to analyse multivariate data related to trehalose biosynthesis pathway under osmotic and non stress conditions.

Chapter 3

Material and Methods

Chapter 3

Material and Methods

3.1 Strains and media compositions

3.1.1 Strains

In the present study, the objective was to study effect of osmotic stress on trehalose production and metabolism in food microbe *Propionibacterium*. Therefore, screening for suitable strain was carried out by procuring various strains of dairy *Propionibacterium* available in culture collection centres of India. The cultures were acquired from culture collection centres MTCC Chandigarh and NCIM Pune India.

The cultures used for screening were *P. shermanii* MTCC 1371, *P. freudenreichii* MTCC 1950, *P. shermanii* NCIM 5137 *and P. freudenreichii* NCIM 2111. Cultures were maintained in stab of MRS media (Cardoso et al., 2004).

3.1.2 Composition of production media for trehalose biosynthesis studies

The trehalose production was studied in media reported by Cardoso et al., 2004 with slight modification whose composition is listed in table 3.1, 3.2 and 3.3. Composition of vitamin solution is listed in table 3.2 while composition of chemically defined media is listed in table 3.3.

Table 3.1 Composition of production media

Component	Composition
Tryptone	20 g/l
Peptone	20 g/l
Yeast extract	1 g/l
K_2HPO_4	0.25 g/l
Vitamin solution	20 ml/l
Carbon source	20 g/l

Table 3.2 Composition of Vitamin solution used in production media (pH 6.8)

Vitamin	**Composition (mg/l)**
Biotin	1.1
Folic acid	1.1
PABA*	110
Riboflavin	110
Pyrodoxine	220
Thiamine	220
Niacinamide	220

*Para-amino Benzoic acid

3.1.3 Composition of chemical defined media (pH 6.8)

Composition of chemically defined media is listed in table 3.3. Composition of vitamin, metal ion and nucleic acid bases solution used in chemically defined media are listed in table 3.4, 3.5, 3.6.

Table 3.3 Composition of chemically defined media (pH 6.8)

Components	**Composition**
Carbon source	20 g/l
K_2HPO_4	2.5 g/l
KH_2PO_4	3 g/l
Ammonium citrate	0.6 g/l
Sodium acetate	1 g/l
Cysteine-HCl	0.25 g/l
Vitamin solution	10 ml/l
Metal ion solution	10 ml/l
Nucleic acid bases solution	10 ml/l

Table 3.4 Composition of Vitamin solution (pH 7.0)

Vitamin	**Composition**
Pyridoxine-HCl	200 mg/l
Nicotinic acid	100 mg/l
Thiamine-HCl	100 mg/l
Riboflavin	100 mg/l
Ca-pantothenate	100 mg/l
Na-PABA	1 g/l
Biotin	100 mg/l
Folic acid	100 mg/l
Vitamin B_{12}	100 mg/l
Orotic acid	500 mg/l
2-deoxythymidine	500 mg/l
Inosine	500 mg/l
Pyrodoxamine HCl	500 mg/l

Table 3.5 Composition of metal ion solution (pH 7.0)

Metal	**Composition**
$MgCl_2.6H_2O$	20 g/l
$CaCl_2.\ 2H_2O$	5 g/l
$FeCl_2.4H_2O$	0.5 g/l
$ZnSO_4.7H_2O$	0.5 g/l
$CoCl_2.6\ H_2O$	0.25 g/l

Table 3.6 Composition of nucleic acid base solution (per 10 ml of 0.1N NaOH), the pH was adjusted to 7.0 with 0.1 N NaOH. Sterilisation was done by membrane filtration.

Nucleic acid base	Amount (mg)
Adenine	10
Uracil	10
Xanthine	10
Guanine	10

3.2 Static and shake flask studies

The cultures were grown in 500 ml culture flasks containing 200 ml of liquid media (in triplicate). All flasks were inoculated with freshly cultured stab and incubated at 30°C in static flask conditions (in triplicate). For shake flask studies, flasks were incubated at 200 rpm at 30°C. Growth was monitored by measuring optical density spectrophotometrically. For optical density measurement, 3 ml of culture was centrifuged at 10,000 rpm for 10 min at 4°C and washed pellet was re-suspended in 3 ml of isotonic solution (0.85% NaCl) and optical density was determined at 600 nm. Optical density was converted into dry weight as described in section 3.7.6. Further 10 ml of cell culture samples were harvested at regular intervals and rapidly centrifuged at 12, 000 rpm for 15 min at 4°C, supernatants (cell free supernatant) were kept stored at -20°C until used for substrate concentration analysis while washed cell pellet (only pellet) was kept in refrigerator (-80°C) for further analysis of trehalose content, enzyme activities and other metabolites.

3.3 Batch reactor studies

A Three litre (working volume 3 liter) New Brunswick autoclavable fermenter (Fig 3.1) was used for all batch reactor studies. 100 ml of 24 h grown static flask culture was used as inoculum. After autoclaving the fermenter with media (only yeast extract, peptone, tryptone), sterilised carbon source solution was added separately and inoculum was finally added. The final working volume was 2 litres, pH was maintained at 6.8 by automatic

addition of NaOH, dissolved oxygen was maintained at 5% of air saturation by automatic passing of nitrogen and air gases and foaming was prevented by adding antifoam for 24 h. Samples were taken at regular intervals (4-6 h), which were used for substrate and product analysis. These were always stored at -80°C.

Fig 3.1: New Brunswick Batch fermenter used for the present study.

3.4 Crude glycerol or biodiesel waste preparation and pre-treatment

To evaluate the suitability of crude glycerol for trehalose and propionic acid productions, biodiesel waste was used which was prepared by base catalysed trans-esterification reaction of soya bean oil (Ruhal et al., 2011); briefly 1.8 g of KOH was dissolved in 33.5 ml of methanol and was added to soya bean oil (120 ml) (i.e. oil to methanol 3.6:1 (v/v)) at room temperature with constant stirring and was left in separating funnel for at least three to five hours. After separation, two layers were collected as shown in Fig 3.2; lower layer was biodiesel waste which consists of glycerol, methanol and soaps.

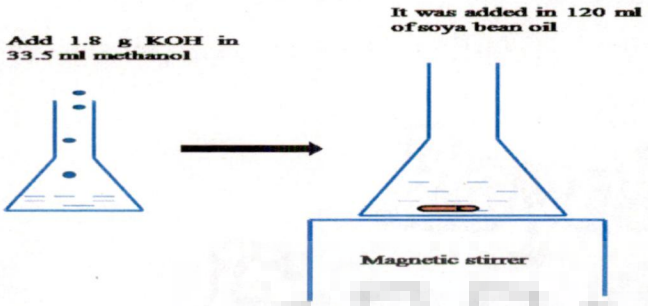

Fig 3.2 Crude glycerol from biodiesel waste (lower brownish red). Upper layer is biodiesel.

Crude glycerol was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, and then pH of the fluid was adjusted to 3 with hydrochloric acid to convert soap into free fatty acids. The formed precipitate was separated from the crude glycerol solution by centrifugation at 5000 rpm and subsequently pH of the supernatant was again adjusted to pH 12 with base (KOH), followed by separation of formed precipitate by centrifugation. Finally pH of the supernatant obtained after second centrifugation was adjusted to 6.8. Methanol was removed during autoclaving. The compositions of crude glycerol and treated glycerol are listed in table 3.7. Glycerol was measured by periodate method as described in section 3.7.1, methanol was detected by gas chromatography (Dani manufactures) using sol-gel wax capillary column (0.25 x 20 x 0.12) with FID detector and nitrogen as a carrier gas (section 3.7.5). KCl was estimated by Atomic absorption spectroscopy (section 3.7.7). The concentrations of glycerol and

methanol were determined using standard plots obtained with analytical grade chemicals. Soaps which included fatty acids were precipitated by pH adjustments and subsequently weight of dried precipitates were measured (Initially pH adjusted to 3 and then after centrifugation precipitate was collected. The pH of the supernatant was again changed from 3 to 12 and formed precipitate was collected after centrifugation. Both precipitates were combined and dried at 105^0C). Similarly, ash content was obtained after heating the crude glycerol at 550°C for overnight in a furnace. Likewise, moisture content was determined from weight differences obtained after keeping the crude glycerol at 110°C for overnight.

Table 3.7 Composition of crude glycerol before and after pre-treatment

Composition	**Concentration untreated**	**Concentration after pre-treatment and autoclaving**
Glycerol	657±.4 g/l (62% w/w)	132±0.2 g/l (13.2% w/w) (since 5 times diluted)
Methanol	171±6 g/l	0.0±0
Soap content	19±0.08 g/l	4±0.4 g/l
Moisture content	162±0.8 g/l	-
Ash content	24±0.3 g/l	14±0.4 g/l
Specific gravity	1.12±0.04	0.98±0.08
KCl content	-	7.2 g/l

For batch reactor and static flask studies, pre-treated crude and pure glycerol were used as carbon source. The sterilized nitrogen sources (tryptone 1%), peptone (1%), yeast extract

(0.1%)), and phosphate (0.025%) were added separately. Static flask studies were done in 500 ml Erlenmeyer flasks with 200 ml working media (initial pH 6.8) and incubated at 30^0C. Two litres, New Brunswick (USA) fermenter was used for all batch fermenter studies; all the media constituents were autoclaved in the fermenter except carbon source which was autoclaved separately. Sterilized carbon source, filter sterilized vitamin solution and 100 ml of inoculums were added aseptically. The pH was maintained at 6.8 by automatic addition of 2 N NaOH while dissolved oxygen was maintained at around 5-10% of air saturation by automatic passing of nitrogen gas or air for 24 h. The fermentation was carried out at 30°C and agitation speed was 200-600 rpm. Samples were collected at regular interval and rapidly centrifuged at low temperature (4°C). The cell pellet and supernatant were stored at -80°C till further use. Cell pellet was used for trehalose quantification while supernatant was used for lactic acid, propionic acid and glycerol quantifications.

3.5 Preparation of cell extracts

20 ml of broth was taken after every 4-6 h and was centrifuged at 12,000 rpm for 15 mins at 4°C. Supernatant was decanted and the cell pellet was washed with potassium phosphate buffer (5 mM, pH 7.0). The cell extract of *P. shermanii* NCIM 5137 was obtained using the method described by (Tobiassen et al., 1996) for *Propionibacterium* with brief modification; in brief cell pellet was incubated with 5 mg/ml of lysozyme for one h in 5 mM Tris buffer (pH 7) at 30°C and after that cell pellet were lysed using laboratory scale sonicator (Hielscher ultrasonic processor UP100H (100 W, 30 kHz) with 30 second sonication pulses and 30 second rest under chilled conditions at 40% amplitude together with 200 mg/ml of glass beads (100-200 μm) (Tobiassen et al., 1996). Cell debris were removed by centrifugation at 15,000 rpm for 20 mins and supernatant so obtained, is cell extract which was stored in -80°C. This cell extract was used for enzyme activity assay and determination of metabolites like UDP-glucose, glycogen and glucose-6-phosphate.

3.6 Extraction of trehalose, maltose and glycogen

Trehalose is intracellular in *Propionibacterium freudenreichii* as earlier reported by Cardoso et al 2004. With every 4-6 h interval, 10 ml of broth from the culture medium was taken and centrifuged at 12,000 rpm for 10 min. The bacterial cells were obtained as pellet while extracellular media as supernatant. The cell pellets were washed thrice with 0.85% saline solution. Trehalose was determined both intracellular (inside bacterial cell) and

extracellular (broth culture medium) by Trehalase enzyme (Sigma-Aldrich). No extracellular trehalose was found. For intracellular trehalose, the washed cell pellet was suspended in 2 ml of 80% of ethanol and boiled in water bath till the volume reduced to 0.2-0.3 ml. In the extract, sodium citrate buffer (pH 5.5, 0.1 M) was added to make the final volume to 1 ml and after centrifugation the clear supernatant was used for trehalose quantification. For maltose, 0.1 M potassium phosphate buffer (pH 7) was used instead of 0.1 M sodium citrate buffer. For glycogen cell extract obtained as described in previous section (3.5)

3.7 Analytical methods.

3.7.1 Substrate analysis

Glycerol was measured by periodate method as described previously (Bondioli et al., 2005). First calibration curve was prepared with different concentrations of glycerol (fig 3.3). For calibration curve, 1.2 ml of 10 mM sodium periodate solution was added in each standard concentration of glycerol and mixed for 30 second. After that, 1.2 ml of a 0.2 M acetylacetone solution was added and incubated at 70°C for one min. Absorbance at 410 nm were taken in spectrophotometer. For sample analysis, 1 ml of sample was dissolved in 4 ml of hexane and 4 ml of extraction solvent (95% ethanol) followed by vigorous shaking for 5 min. After centrifugation at 2000 rpm for 15 min, lower layer (0.5 ml) was separated into another tube and 1.5 ml of 95% ethanol was added, followed by addition of 1.2 ml of sodium periodate solution and mixed for 30 sec. Further, 1.2 ml of 0.2 M acetyl acetone solution was added and incubated at 70°C for one min. Spectrophotometric absorbance at 410 nm were noted.

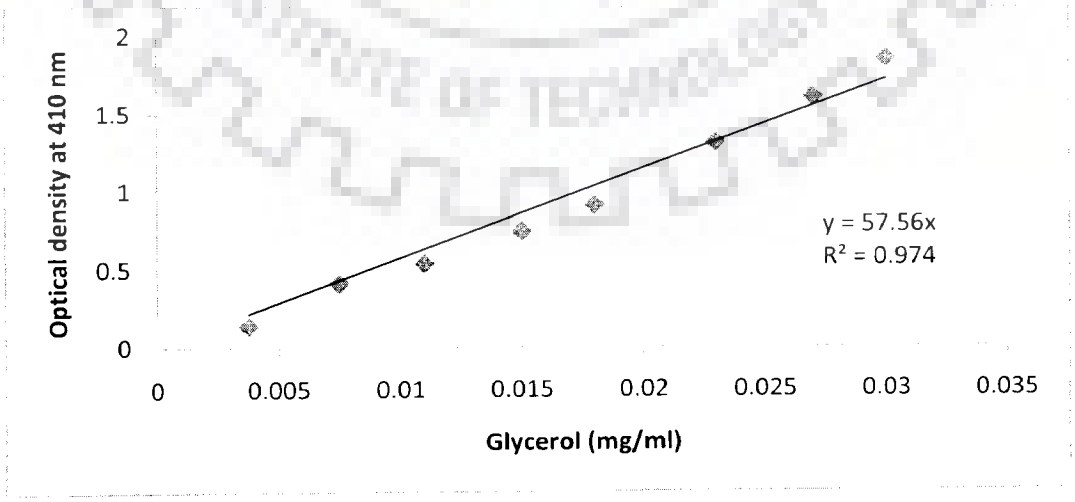

Fig 3.3 Calibration curve of glycerol

Glucose concentrations were measured by DNS method (Miller, 1959) while sucrose and starch were estimated after hydrolysis with 2 N HCl into glucose followed by addition of 2 N NaOH and then estimation of glucose by DNS method (Miller, 1959). For glucose estimation 1 ml of sample was mixed with 2 ml of DNS solution, further it was boiled in water bath for 5 min and subsequently optical density was measured at 540 nm spectrophotometrically after cooling at room temperature. Quantitative measurements of glucose were done from a standard curve between glucose concentration and optical density as shown in Fig 3.4. The composition of DNS solution used in the present study is listed in table 3.8.

Table 3.8 Composition of DNS solution for glucose estimation

Constituent	**Amount**
DNS (Dinitrosalicylic acid)	0.8 g
Phenol	0.16 g
Sodium sulphite	0.04 g
Sodium potassium tartarate	16 g
NaOH	0.8 g
Total volume	80 ml

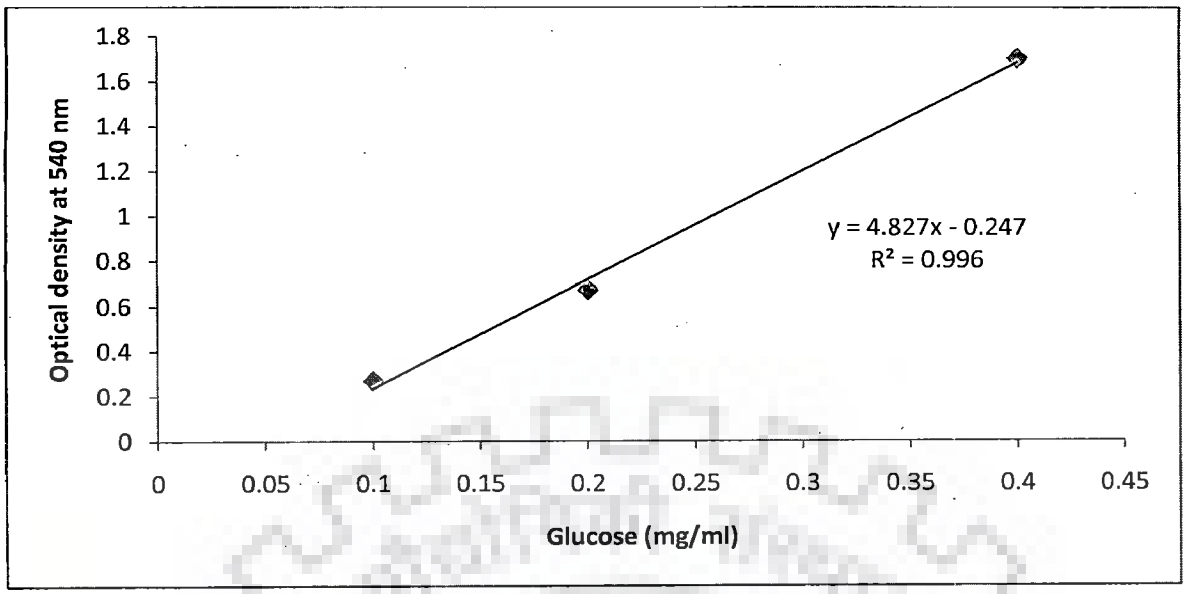

Fig 3.4 Calibration curve used for substrate analysis involving quantitative measurement of glucose

3.7.2 Quantitative analysis of Trehalose by enzymatic method

The concentration of trehalose in the cell extract was determined with enzyme trehalase method (Sigma-Aldrich) (Ruhal et al., 2011). The 100 µl of 0.012 U of enzyme from 5U (1 U can convert 1 µmole of trehalose to 2 µmole of glucose per min at 37°C) was added to the 200 µl of cell extract (in which trehalose has to be estimated) while for control 100 µl of sodium citrate buffer(1 mM, pH 5.5) was added instead of enzyme. This reaction mixture (pH 5.5) was kept over-night (12 h) at 37°C in a shaking incubator. Standard trehalose of known concentration (0.5 g/l) was also incubated separately with the samples to confirm the complete hydrolysis of trehalose by trehalase enzyme. The glucose formed after hydrolysis was quantified by DNS method (Miller, 1959).

The calibration curve for glucose estimation was prepared by adding 600 µl of DNS in known concentration of glucose solution (Fig 3.5) and glucose in samples was estimated by adding 600 µl DNS in samples with unknown concentration. Trehalose concentration was calculated as given by equation 1

$$\textbf{Trehalose}\left(\frac{\textbf{mg}}{\textbf{l}}\right) = \textbf{glucose}\left(\frac{\textbf{mg}}{\textbf{l}}\right) \times \frac{342}{2\times180} \times \frac{\textbf{volume of cell extract}}{\textbf{volume of culture broth}} \qquad (1)$$

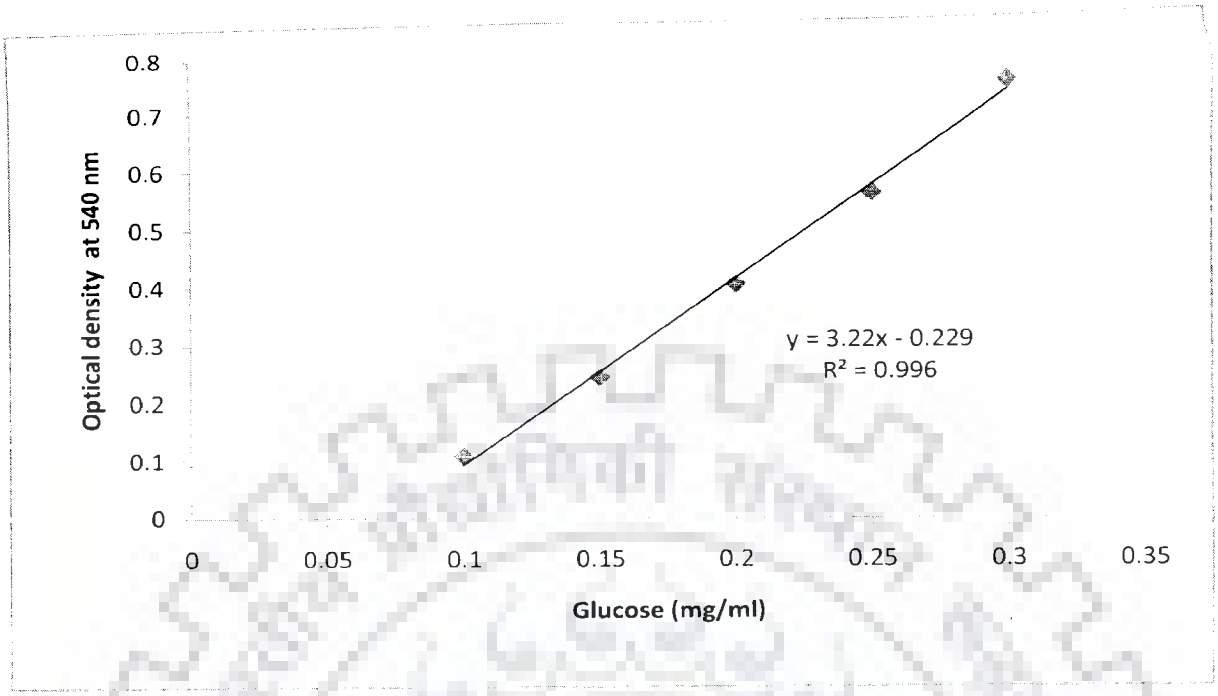

Fig 3.5: Calibration curve prepared for estimation of glucose for experiment involving glucose estimation after hydrolysis of trehalose by trehalase.

In equation (1), 342 and 180 are molecular weights of trehalose and glucose respectively. The equation is divided by two as trehalose is a disaccharide consisting of two molecules of glucose. Ten ml of cell culture broth was used for obtaining the cell pellet and the pellet was further boiled in one ml of 80% ethanol (v/v) for trehalose extraction followed by adjustment of volume to one ml with citrate buffer, hence in the equation (1), volume of cell extract was one ml while volume of culture broth was ten ml.

3.7.3 Quantitative analysis of maltose

The concentration of maltose in the cell extract was determined with enzyme maltase method. The 100 µl of 0.012 U of enzyme from stock 60 U (powder activity 100 U/mg solid) was added to the 200 µl of cell extract while for control 100 µl of sodium phosphate buffer (pH 7) was added instead of enzyme. This reaction mixture was kept over-night (12 h) at 37°C in a shaking incubator. Standard maltose of known concentration (0.5 g/l) was also incubated separately with the samples to confirm the complete hydrolysis of maltose by maltase enzyme. The glucose formed after hydrolysis was quantified by after adding 600 µl DNS solution. Maltose concentration was calculated as given by equation

$$\textbf{Maltose}\left(\frac{\textbf{mg}}{\textbf{l}}\right) = \textbf{glucose}\left(\frac{\textbf{mg}}{\textbf{l}}\right) \times \frac{\textbf{342}}{\textbf{2} \times \textbf{180}} \times \frac{\textbf{volume of cell extract}}{\textbf{volume of culture broth}}$$

Maltose was presented as maltose per gram of dry weight.

3.7.4 Quantitative analysis of glycogen

Glycogen was determined by enzyme amyloglucosidase (Sigma-Aldrich) as described by Seibold et al. 2007. In brief 10 ml of broth was harvested and centrifuged at 12,000 rpm for 10 mins at 4°C; bacterial cells obtained as pellet were washed twice with 0.85% saline and were used for glycogen determination while supernatant was discarded. The cell extract for determination of glycogen was obtained as described above in potassium acetate buffer (pH 4.2). The glycogen was determined as follows- 200 µl of cell extract was taken and 100 µl of enzyme (amyloglucosidase of concentration 2 mg/ml, Sigma Aldrich) was added, for control 200 µl of cell extract along with 100 µl of potassium acetate buffer was engaged for 3 h. The glucose was determined by adding 600 µl DNS solution (Miller, 1959) using total 300 µl reaction mixture. Glycogen was calculated by subtracting glucose concentration in cell extract treated with enzyme amyloglucosidase and cell extract without enzyme. Glycogen is presented as amount of glucose released per gram of dry weight.

3.7.5 Quantitative analysis of UDP-glucose and glucose-6-phosphate

UDP-glucose concentration was measured as Duan et al. 2008 while glucose-6-phosphate as by Lee et al. 1987. Briefly for measuring UDP-glucose concentration a standard curve was prepared by adding known concentration of UDP-glucose which was carried out as follows: final reaction mixture of one ml was prepared by mixing 40 µl of 26 µM NAD^+, 100 µl of UDP-glucose solution in potassium phosphate buffer of pH 6.8 and reaction was started after adding enzyme UDP-glucose dehydrogenase (0.05 U, Sigma-Aldrich) and immediately optical density at 340 nm was measured continuously until constant value was obtained (10 mins). NAD^+ has maximum absorption at 340 nm, therefore absorption were taken at 340 nm. Calibration curve is shown in Fig 3.6. For quantitative analysis of UDP-glucose in cell extract, 100 µl of cell extract was added as described above instead of known concentration of UDP-glucose and by comparing with standard curve the concentration of UDP-glucose in cell extract was calculated. Glucose-6-phosphate was also measured in similar manner that of UDP-glucose while NADP was used instead of NAD and enzyme Glucose-6-phosphate dehydrogenase was used instead of UDP-glucose dehydrogenase. Calibration curve prepared for glucose-6-phosphate measurement is shown in Fig 3.7.

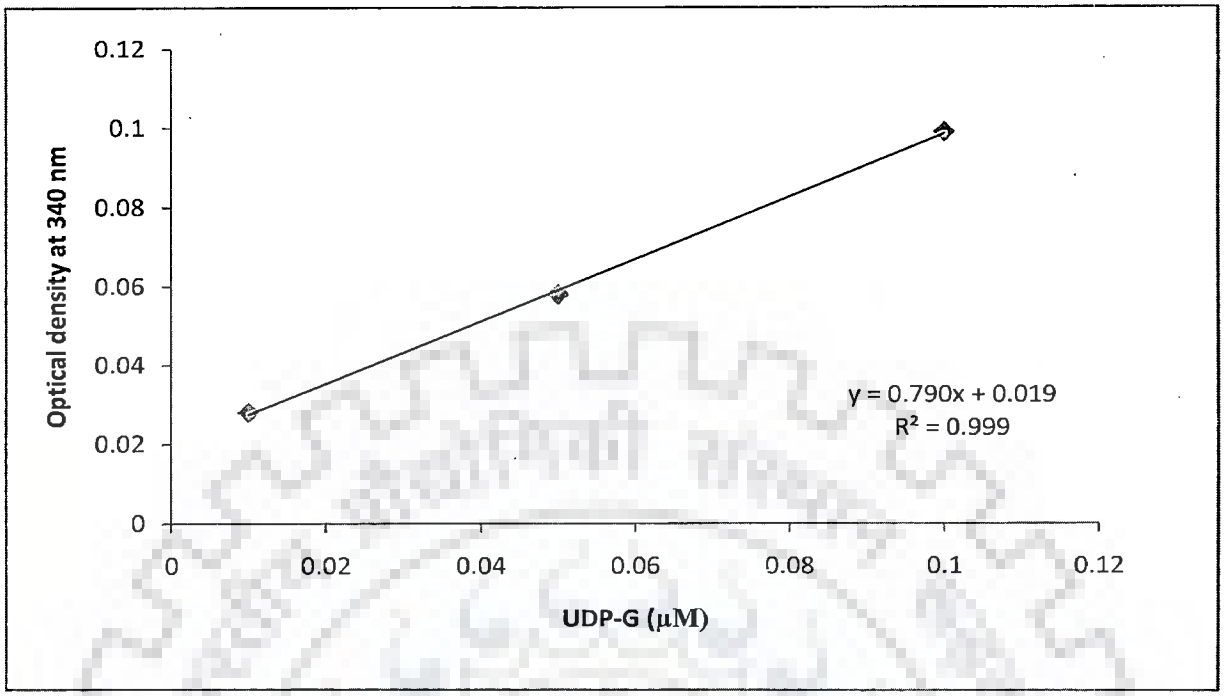

Fig 3.6 Calibration curve for UDP-glucose concentration and optical density at 340 nm

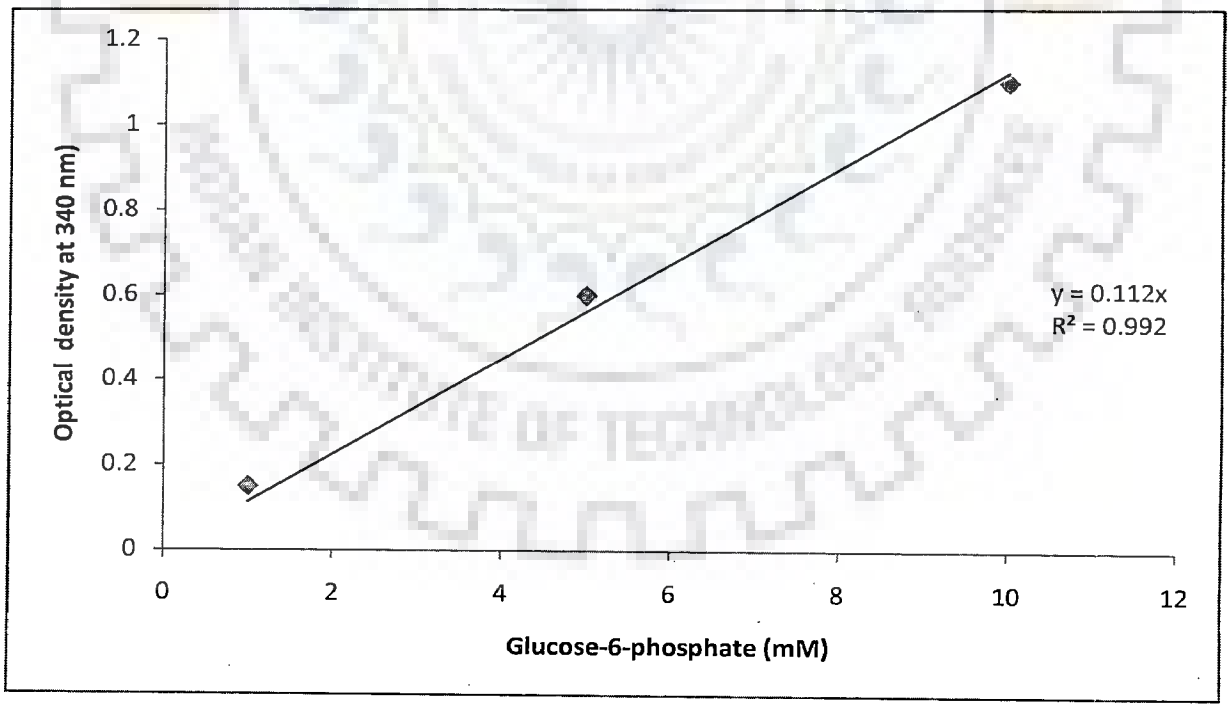

Fig 3.7 Calibration curve for quantitative measurement of glucose-6-phosphate.

3.7.6 HPLC analysis of organic acid and trehalose and GC analysis for methanol

Lactic acid and propionic acid concentrations were determined by HPLC method using a PL-Hi- Plex H column and an UV-Vis detector at 260 nm wavelength. The mobile phase used was 5 mM H_2SO_4 and flow rate was 1 ml/min. The concentrations of propionic and lactic acid were determined using standard plots obtained with pure propionic acid and lactic acid. The HPLC profiles of propionic acid and lactic acid are presented as fig 3.8 (a, b & c). Trehalose was also analysed by HPLC method (Section 4.1 & 4.2 of result and discussions) using a PL-Hi- Plex H column and RI detector with water as mobile phase at a flow rate of 0.8 ml/min. The trehalose chroamatogram and corresponding peak in cell extract are shown in fig 3.8 d & e.

Methanol was detected by gas chromatography (Dani manufactures) using sol-gel wax capillary column (0.25 x 20 x 0.12 mm) with FID detector and nitrogen as a carrier gas. The concentrations of methanol were determined using standard plots obtained with analytical grade chemicals (fig 3.8 (f)). Methanol was detected in untreated crude glycerol (fig 3.8 g) and but it was absent in treated crude glycerol (after autoclave) (fig 3.8 (h)). Standard graphs used for quantitative analysis of propionic acid, lactic acid, methanol and trehalose are shown in fig 3.9 a, b, c and d respectively.

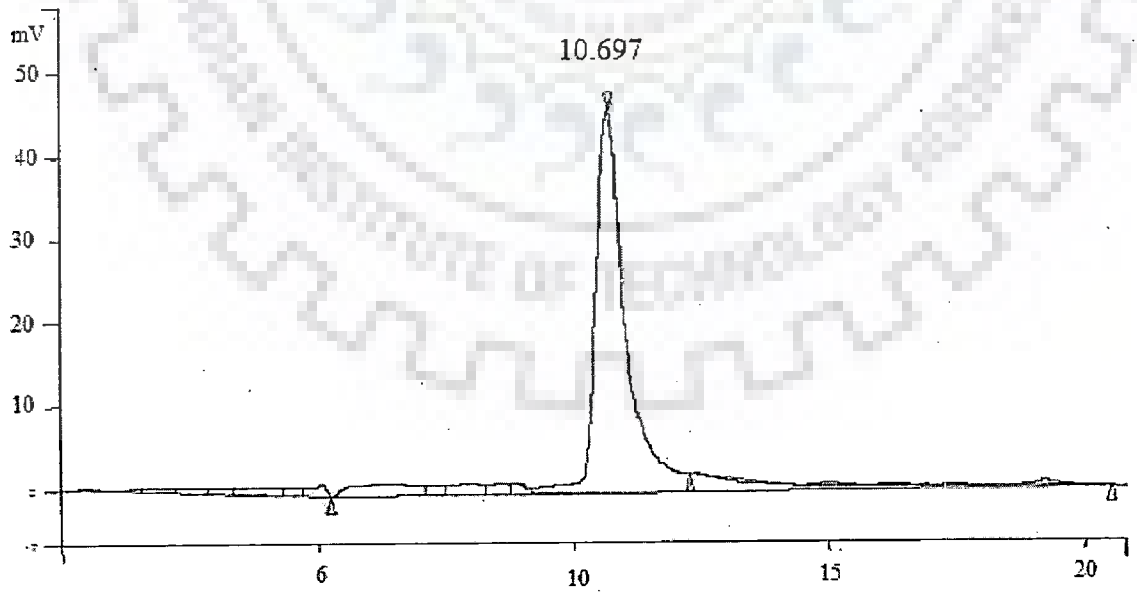

Fig 3.8 (a) HPLC chromatogram of standard chemical of lactic acid.

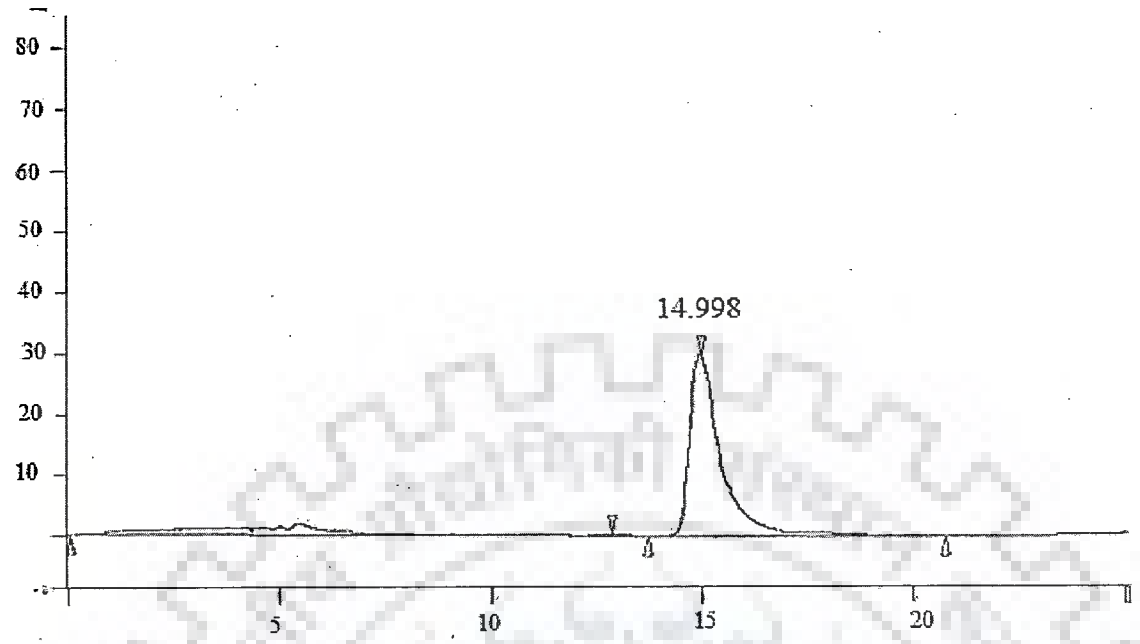

Fig 3.8 (b) HPLC chromatogram of standard chemical of propionic acid

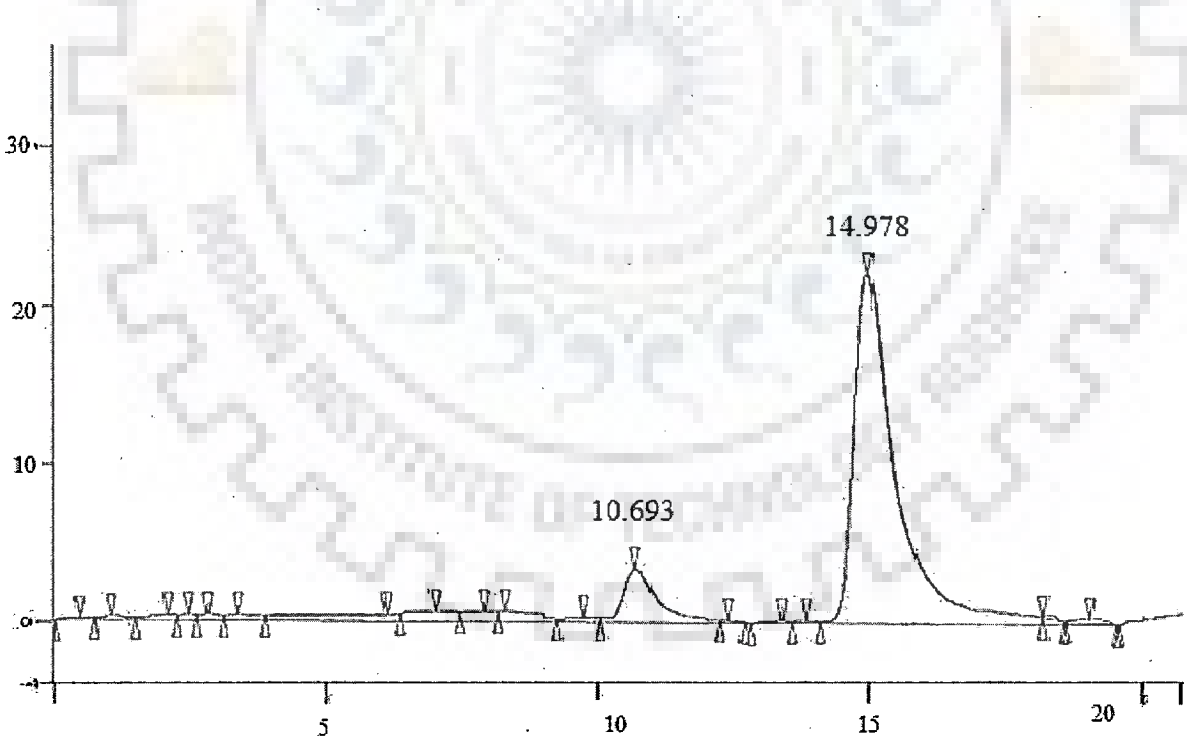

Fig 3.8 (c) HPLC chromatograms of propionic and lactic acid in fermentation broth of culture medium.

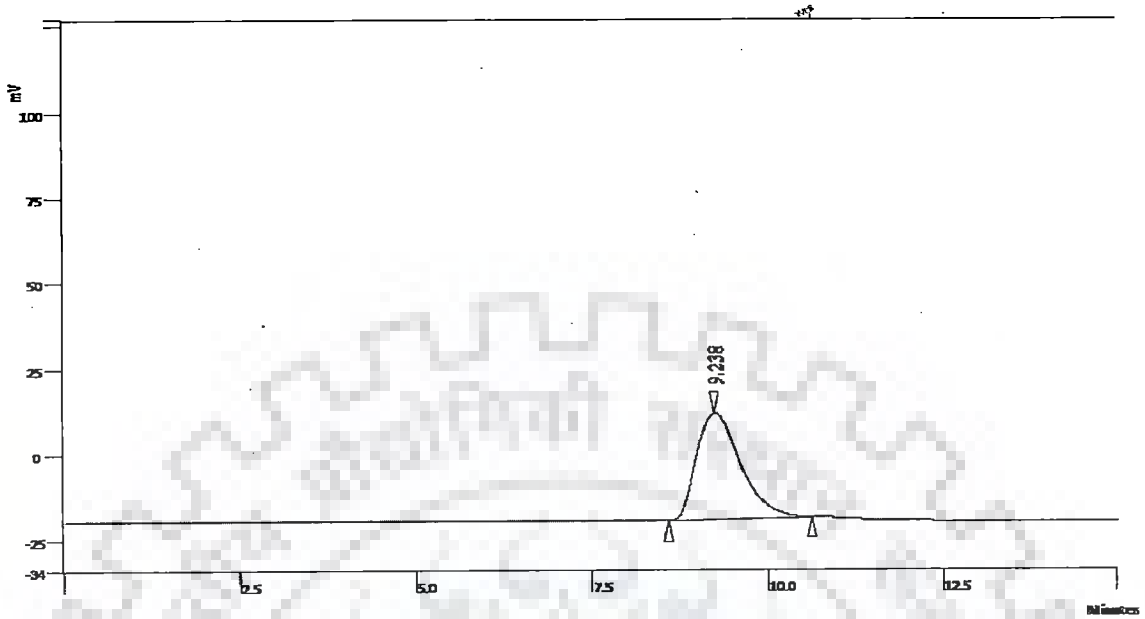

Fig 3.8 (d) HPLC chromatogram of standard chemical trehalose

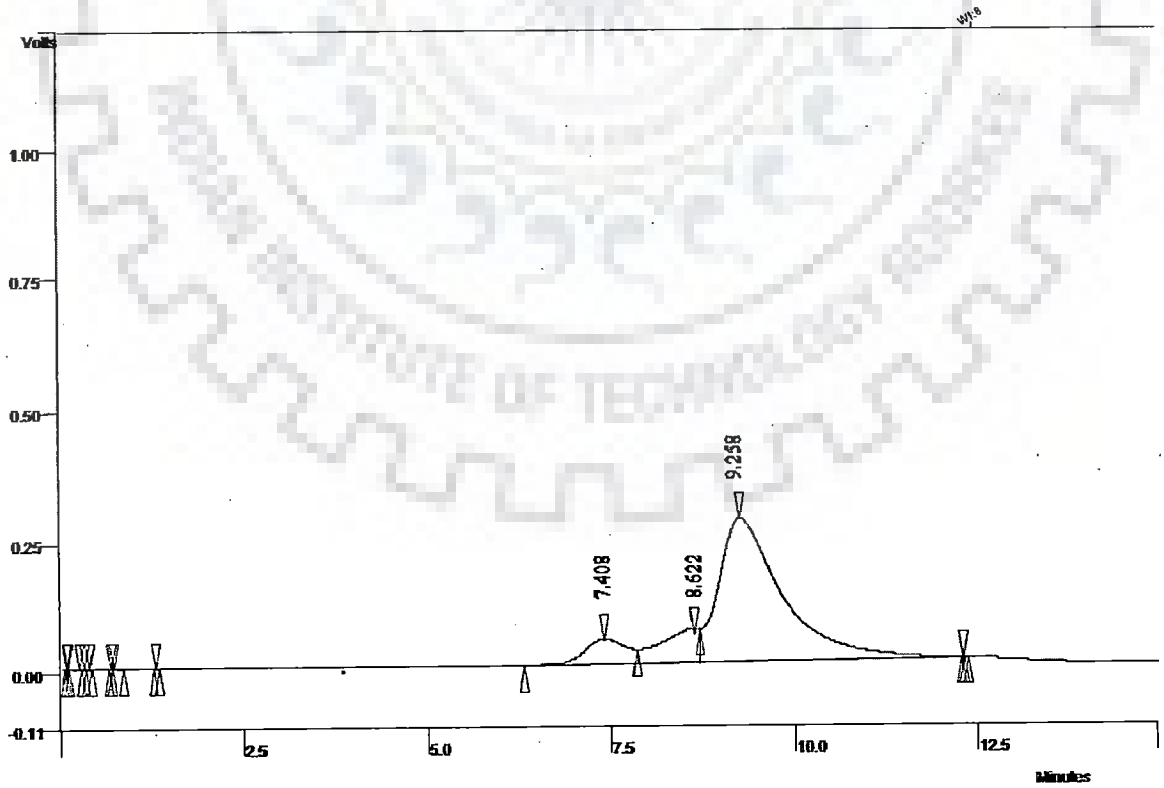

Fig 3.8 (e) HPLC chromatogram of trehalose in intracellular cell extract

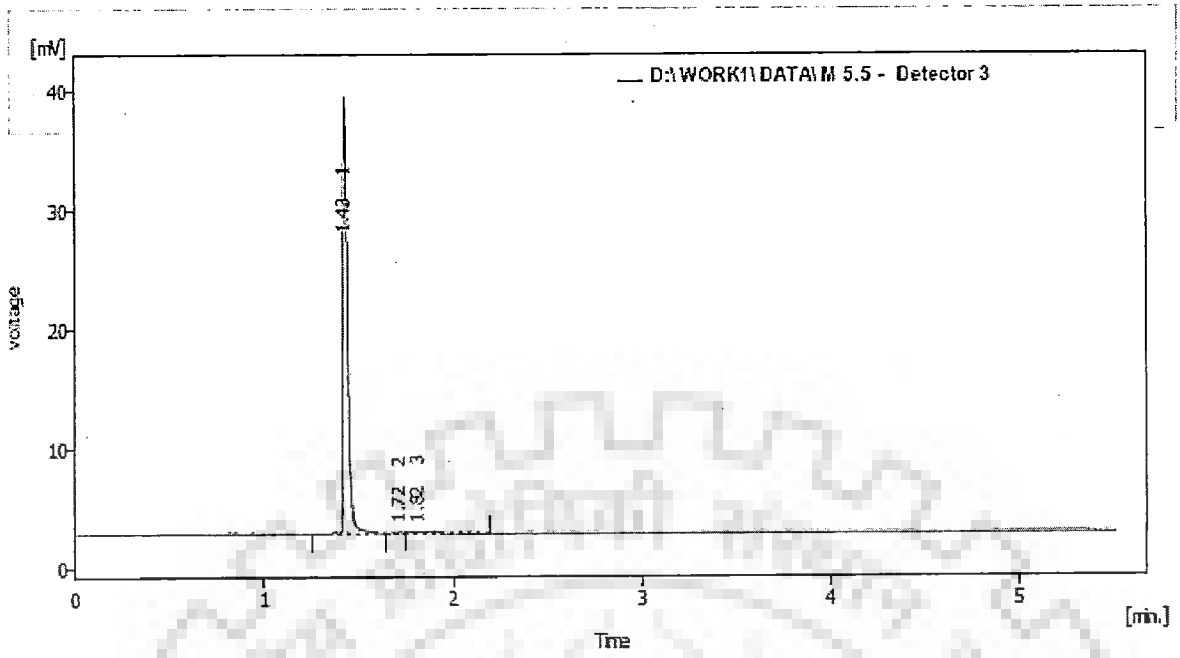

Fig 3.8 (f) GC chromatogram of standard methanol

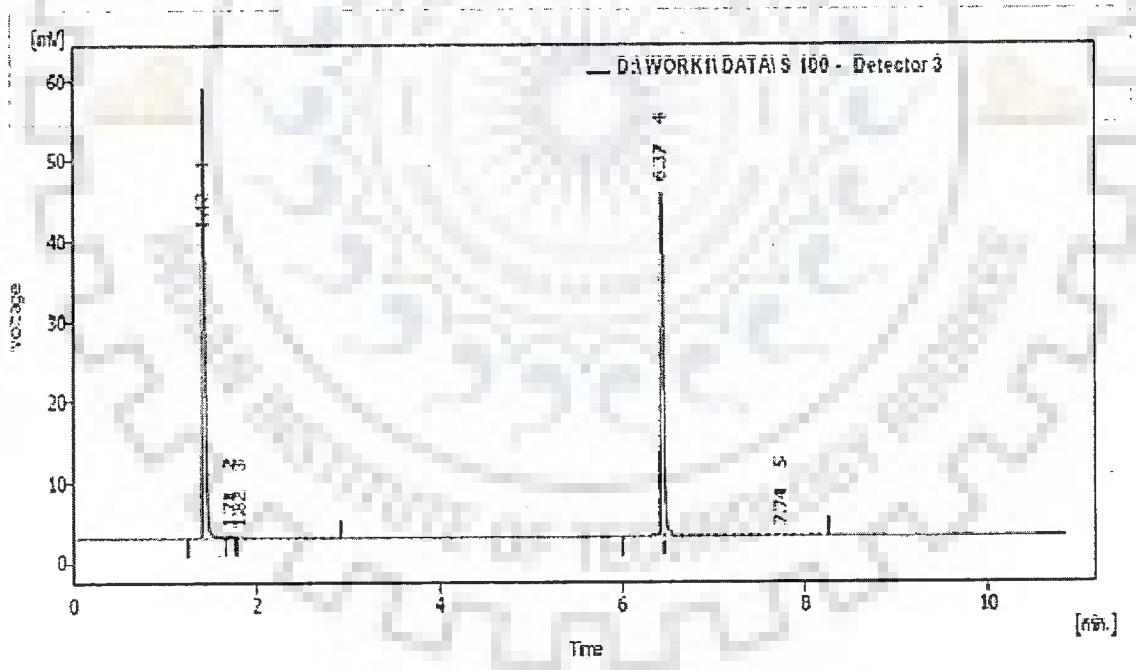

Fig 3.8 (g) GC chromatogram of methanol in untreated crude glycerol (injected after dilution)

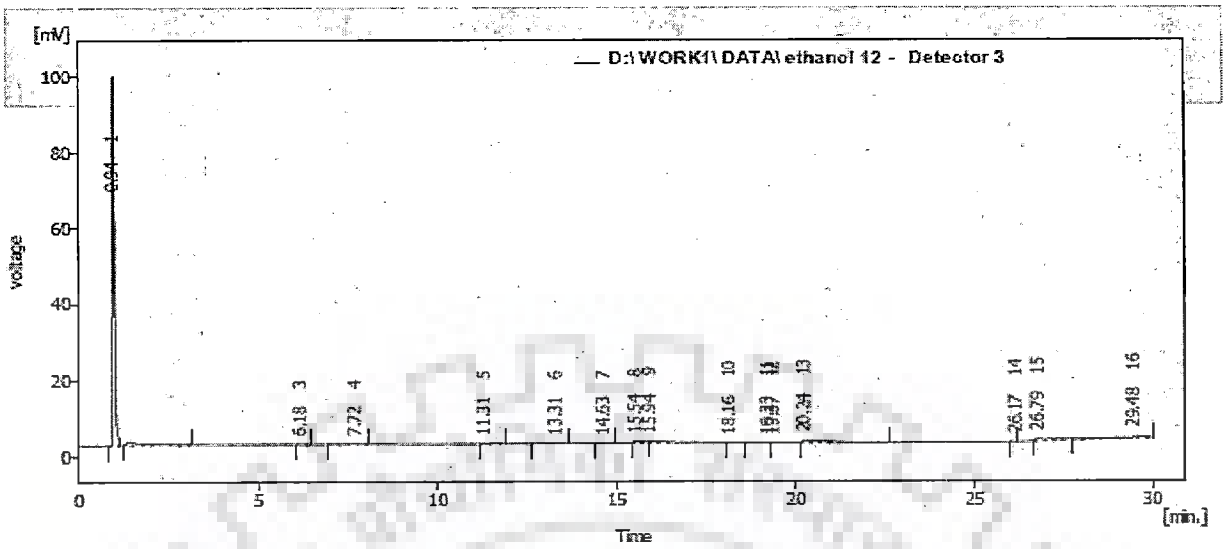

Fig 3.8 (h) No peak of methanol in chromatogram of treated crude glycerol (after autoclave) (injected after dilution)

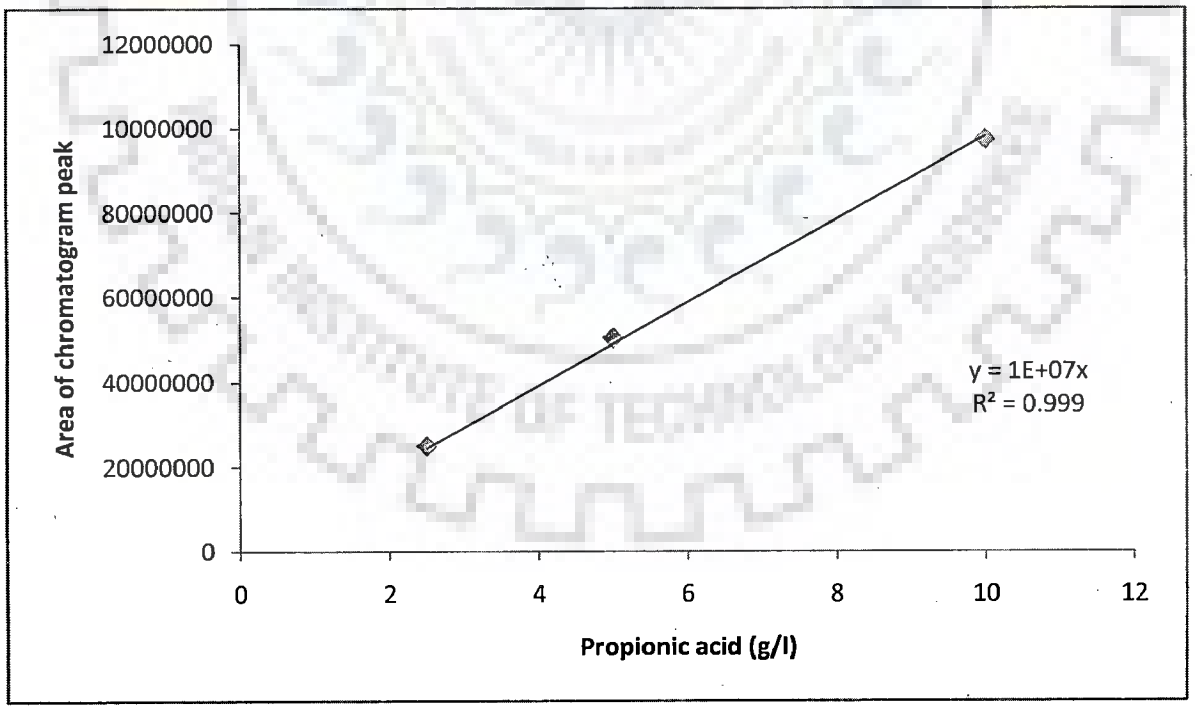

Fig 3.9 (a) Calibration curve of HPLC peak area with respect to three different known concentrations of standard chemicals of propionic acid (analytical grade).

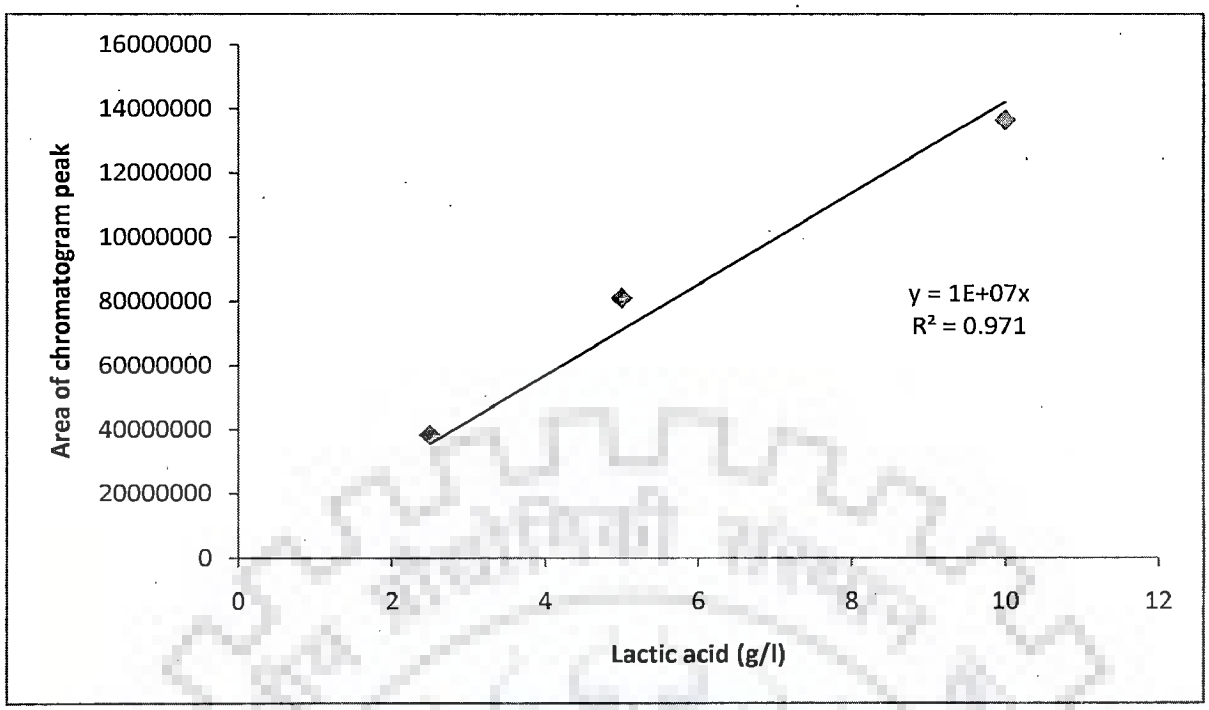

Fig 3.9 (b) Calibration curve of HPLC peak area with respect to three different known concentrations of standard chemicals of lactic acid (analytical grade).

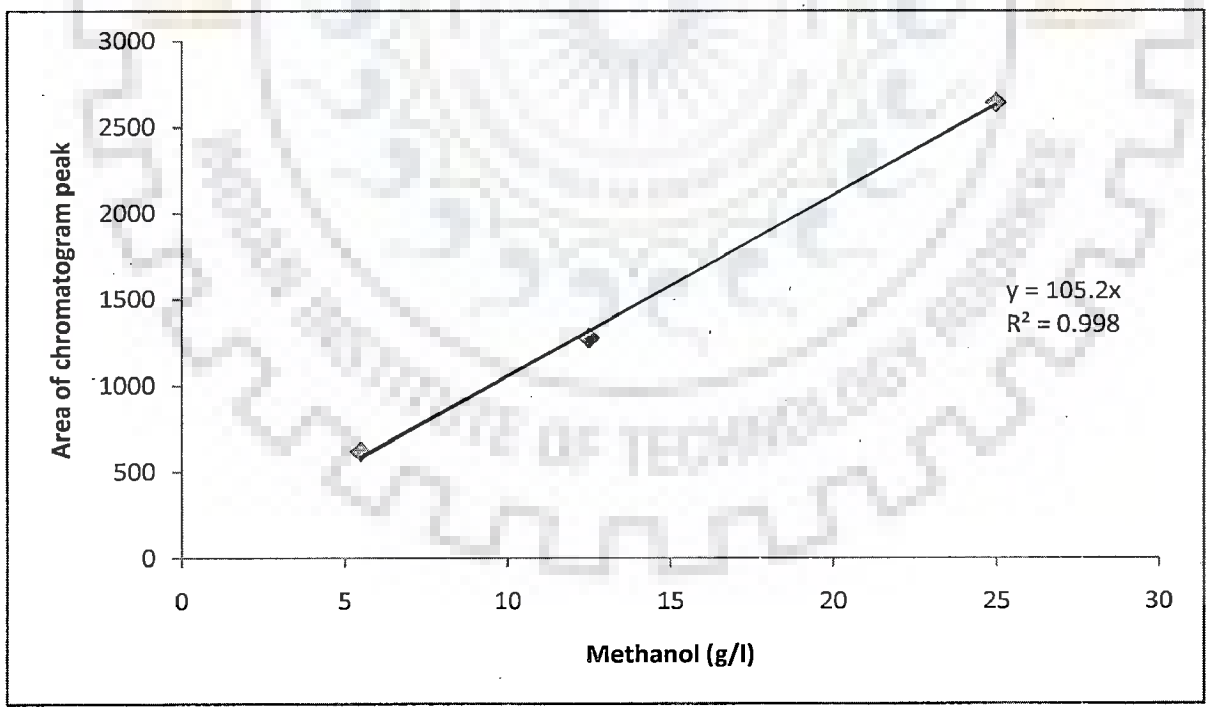

Fig 3.9 (c) Calibration curve of GC peak area with respect to three different known concentrations of standard chemicals of methanol (analytical grade).

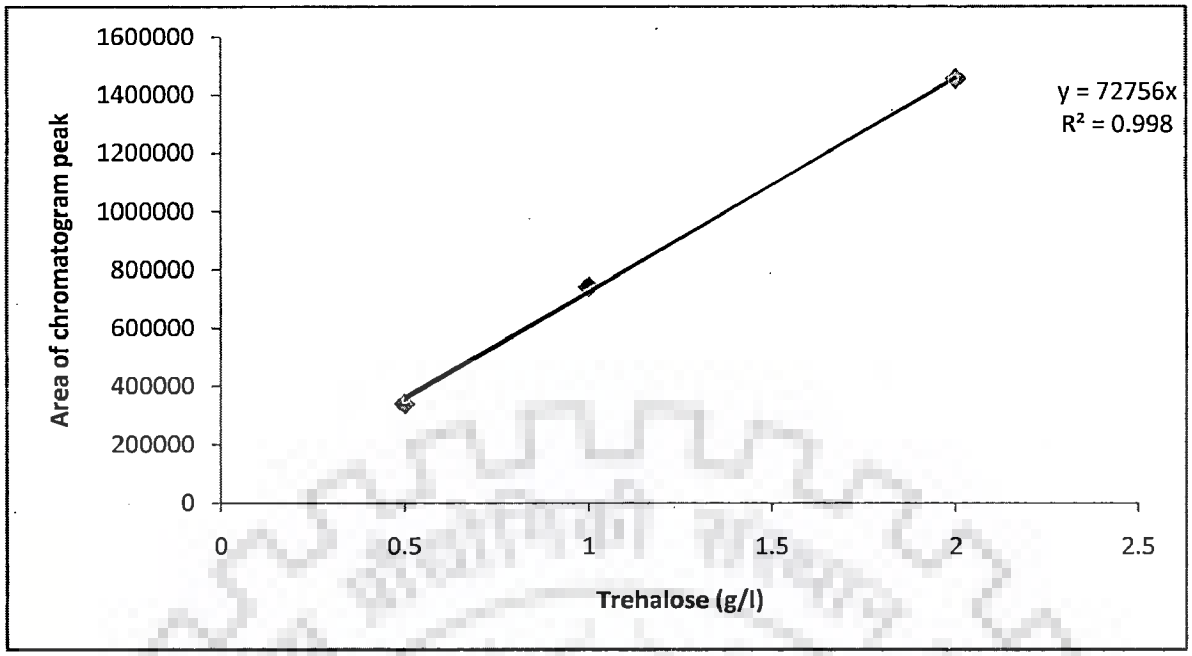

Fig 3.9 (d) Calibration curve of HPLC peak area with respect to three different known concentrations of standard chemicals of trehalose (analytical grade).

3.7.7 Biomass quantification

Samples collected at regular interval were centrifuged at 12,000 rpm and 4°C and the washed collected pellet was suspended in saline solution followed by taking optical density of the suspension at 600 nm. Dry weight (cell biomass) was calculated from a standard plot, between dry weight and optical density (OD) of different dilutions of cell suspensions: in brief 100 ml of fermentation broth was centrifuged at 12,000 rpm and 4°C for 15 mins and the resulting pellet was suspended in 10 ml of saline solution (0.85% NaCl); 3 ml of this suspension was kept at 105°C for over-night and dry weight was determined while remaining 7 ml was used to make different dilutions of cell suspensions and consequently corresponding optical density of these suspensions were taken at 600 nm. Finally a standard plot was prepared by plotting optical density against cell concentration as shown in fig 3.10.

3.7.8 Analysis of KCl by measuring K^+ ion by Atomic absorption spectroscopy

The mineral (potassium) was measured at the appropriate instrumental conditions using an atomic absorption spectrophotometer (AAS) (Avanta Grade M, GBC Scientific Equipment) and was quantified by standard curves made from standard KCl solution. Potassium cathode lamp was used with flame type air acetylene. The range for detection

was 0.1 to 2 ppm at wavelength of 766 nm. Sensitivity of measurement was 0.01 ppm. Standard graph is shown in Fig 3.11.

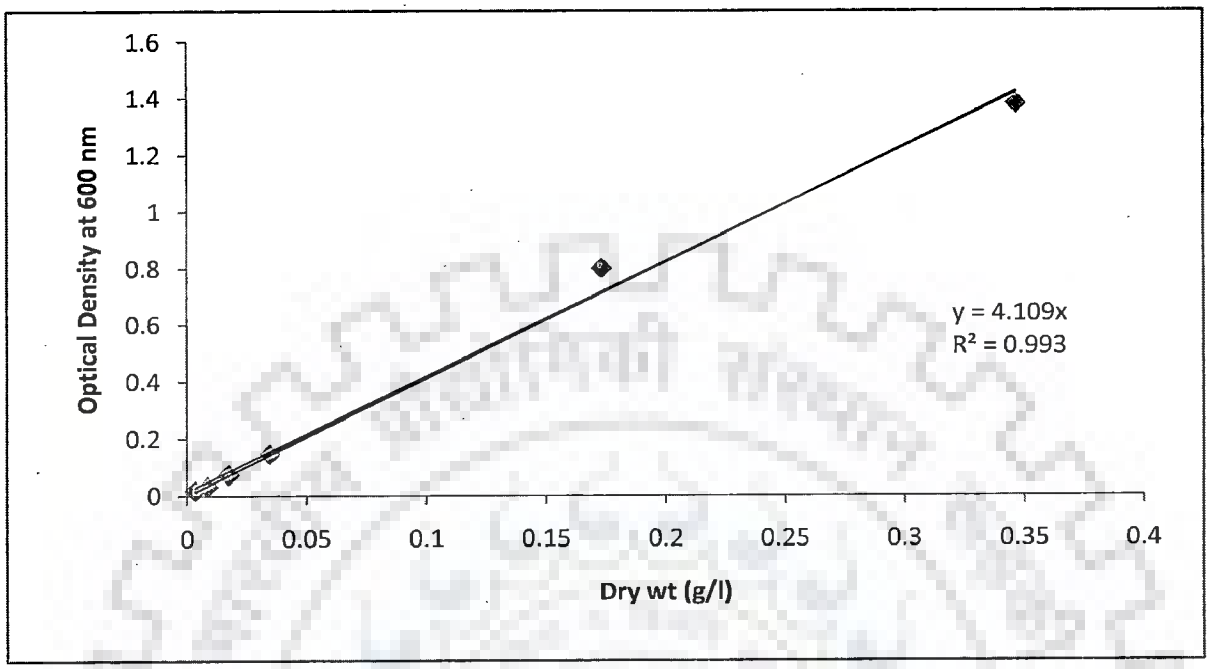

Fig 3.10 Calibration curve for biomass estimation

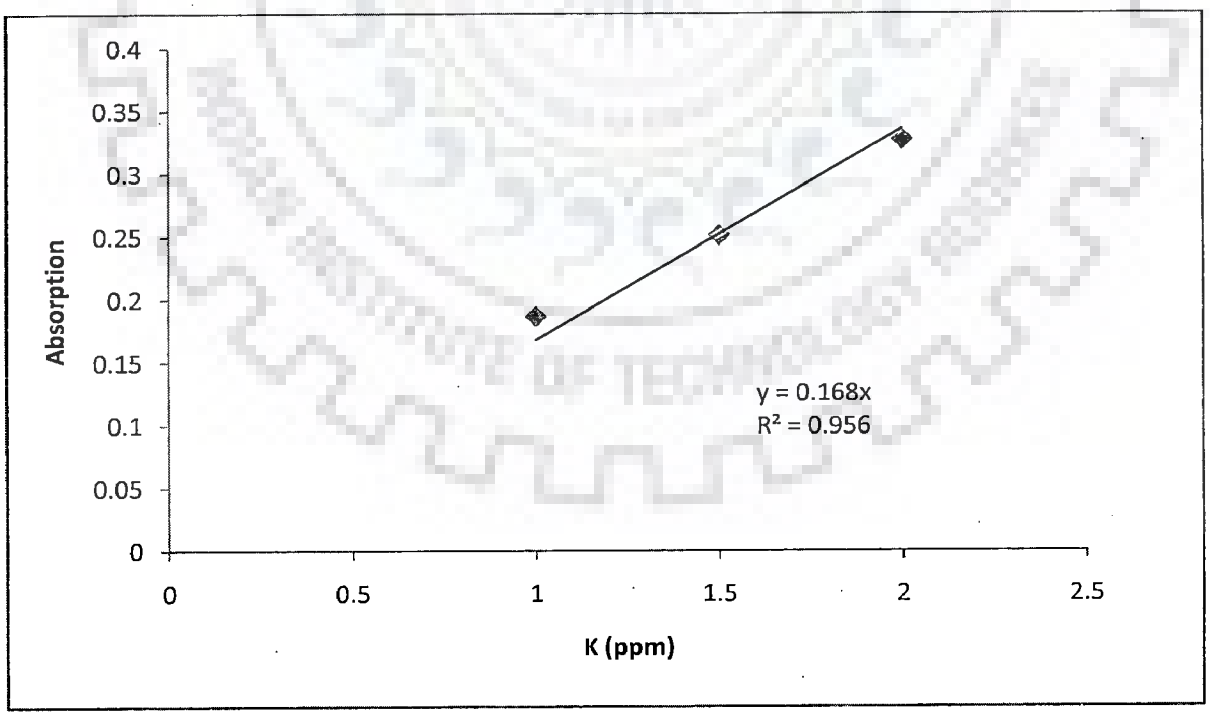

Fig 3.11 Calibration curve of atomic absorption spectroscopy for KCl estimation.

3.8 Determination of yield of trehalose, propionic acid and lactic acid, Specific growth rate, biomass productivity, substrate uptake rate.

3.8.1 Trehalose yield

Trehalose was estimated by enzyme trehalase (Sigma-Aldrich) method. The yields of trehalose were calculated with respect to substrate consumed (Y_{ts}) and biomass formed (Y_{tx}) as it was an intracellular product. The calculation is shown as equation 2 and 3

$$\mathbf{Ytx} = \frac{\text{trehalose } (\frac{mg}{l})}{\text{dry weight of biomass } (\frac{g}{l})} \quad (2)$$

$$\mathbf{Yts} = \frac{\text{trehalose } (\frac{mg}{l})}{\text{substrate consumed } (\frac{g}{l})} \quad (3)$$

Trehalose, biomass dry weight and substrate concentration were determined as mentioned in previous sections.

3.8.2 Propionic acid and lactic acid yield

Similarly, yields of propionic acid (Y_{pa}) and lactic acid (Y_{la}) were calculated as shown in equation 4 and 5

$$\mathbf{Ypa} = \frac{\text{propionic acid } (\frac{g}{l})}{\text{substrate consumed } (\frac{g}{l})} \quad (4)$$

$$\mathbf{Yla} = \frac{\text{lactic acid } (\frac{g}{l})}{\text{substrate consumed } (\frac{g}{l})} \quad (5)$$

3.8.3 Specific growth rate determination, biomass productivity determination, substrate uptake rate determination

Specific growth rate was calculated as the rate of increase in cell mass per unit biomass, substrate uptake rate is the substrate consumed by cells per unit time and the rate of cell productivity was calculated as the biomass produced in g/l per unit of time while glycerol conversion was calculated in percentage by dividing substrate consumed by the initial substrate ([(final substrate consumed)/initial substrate concentration]*100).

3.9 Measurement of in-vitro enzyme activities

The cell extract was used as the source of enzyme for various enzyme assays which were done at 37°C with final reaction mixture volume of one ml (if taken different then mentioned below). The enzyme assays for trehalose-6-phosphate synthetase (OtsA), trehalose-6-phosphatase were done using methods as described by Cardoso et al., 2007 with slight modification as described below. Phosphoglucomutase, phosphoglucose isomerase as described by De Geest and De Vuyst 2000. UDP-glucose pyrophosphorylase was determined as described by Duan X et al 2008. Pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase activity was determined as by Padilla et al. 2004. UDP-glucose dehydrogenase activity was determined as by Schiller J.G. et al 1973. Specific activities are expressed as nanokatal/g of biomass and one katal is defined as one mole of substrate converted per second under specified conditions. For the enzyme reaction involving measurement of NAD/NADP formation or disappearance (Phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase) was measured at 340 nm at 37°C (extinction coefficient used at absorbance 340 nm is 6.3 x 10^3 M^{-1} cm^{-1}). All the specific activities were done in triplicate with three independent experiments and activities were expressed as mean value and standard deviation. All the enzymes and chemicals used were of Sigma-Aldrich. For control, heat deactivated (10 min) cell extract was taken.

Phosphoglucoisomerase activity was determined in reverse direction with reaction mixture containing sodium phosphate buffer (50 mM, pH 6.8), $MgCl_2$ (5 µmole), NADP (0.4 µmole), glucose-6-P dehydrogenase (0.01 ml of 180 U/ml) and cell extract(100 µl). The reaction was initiated by adding of fructose-6-Phosphate (2.5 µmol).

Glucose-6-Phosphate dehydrogenase and gluconate-6-Phosphate dehydrogenase were determined in a reaction mixture containing Tris buffer (pH 7.5, 100 mM), DTT (1 mM), $MgCl_2$ (1 mM), NADP (1 mM) and cell extract (100 µl). The reaction was started by addition of Glucose-6-Phosphate (2 mM).

Phosphoglucomutase enzyme activity was measured in a reaction mixture of glycyl-glycine buffer (179 mM, pH7.4), NADP (0.67 µmole), glucose 1, 6-diphosphate (0.02 µmole), $MgCl_2$ (30 µmole), L-Cysteine (43 µmole), glucose-6-phosphate dehydrogenase (1U) and cell extract (100 µl). The reaction was initiated by adding 5 µmol of Glucose-1-Phosphate.

UDP-glucose/ADP-glucose/GDP-glucose pyrophosphorylase enzyme activities were measured in a reaction mixture containing NADP (1.4 mM), $MgCl_2$ (4 mM), UDP-glucose (1 mM), glucose 1,6-diphosphate (10 µM), phosphoglucomutase (66 mU), glucose 6-phosphate dehydrogenase (220 mU), Tris buffer (75 mM, pH 7.0) and cell extract in a final volume of 0.5 ml. Reaction started with the addition of UDP-glucose

Trehalose-6-phosphate synthetase (OtsA) assay was done as described by Cardoso et al 2007 with modification. Reaction mixture containing acetate buffer (50 mM, pH5.5), $MgCl_2$ (10mM), Glucose-6-phosphate (5 mM), UDP-glucose (5 mM) were prepared. The reaction mixture was incubated for one h at 37°C. Pi released was determined with Ames method spectrophotometrically (Ames, B.N., 1966). OtsB (Trehalose-6-phosphate phosphatase) was determined in MES buffer (50 mM, pH 6.5), 10 mM $MgCl_2$ and Trehalose-6-phosphate. Reaction mixture was incubated for one h at 37°C and Pi determined as above. For control heat deactivated cell extract was taken.

UDP-glucose dehydrogenase was assayed with reaction mixture of one ml which consists of UDP-glucose (5 µmole), NAD (0.5 µmole), glycyl-glycine buffer (0.1 µmole, pH 8.7) and cell extract. Reaction started with the addition of UDP-Glucose.

Trehalose degrading enzymes were also determined. Trehalase was determined as follows- the cell extract so obtained was used for Trehalase activity in a 300 µl total reaction mixture having 200 µl of 2 mM trehalose and 100µl of crude extract. For control, heat inactivated crude extract was used. Reaction mixture was incubated for 12 hs at 37°C. Glucose was estimated by DNS method (Miller, 1959). Unit of enzyme is expressed in micromole of glucose liberated per min under the assay conditions. For TreS activity as described by Cardoso et al 2007, in brief reaction mixture consist of phosphate buffer (50 mM pH 5.2), Trehalose/Maltose (5 mM). Reaction was assayed in both directions by incubating at 37°C for one h and trehalose and maltose so formed were estimated enzymatically by Trehalase and Maltase. Trehalose phosphorylase as described by Cardoso et al 2007.

3.10 Multivariate analyses

3.10.1 Principle component analysis

PCA were done using matlab after normalising variables. Princomp function of Matlab was used for PCA.

3.10.2 Cluster analysis

Cluster analysis was done using Minitab software

3.11 Chemical mutagenesis and screening, isolation of mutant

25 ml of culture broth was taken from mid exponential growth phase of *P. shermanii* NCIM 5137. It was centrifuged and bacterial cell obtained in pellet were suspended in 5 mM sodium phosphate buffer of pH 7.1 under 4°C conditions. For chemical mutagenesis 8% EMS was dissolved in 5 mM phosphate buffer (pH 7). The bacterial cell was dissolved in the solution of 8% EMS and incubated for 45 mins at 37°C. After incubation cell pellet was washed thrice with $Na_2S_2O_3$ to neutralize the effect of mutagen. Further different dilutions of bacterial cell (10^{-1} - $10^{-8)}$ were incubated in plates with glucose as carbon source and no NaCl. From the agar plates, single colony were further selected and replica plated on agar plates with glucose as carbon source and 1%, 2%, 3%, 4% and 8% NaCl, simultaneously with plates containing 0.01% and 0.05% SDS and plates with no SDS and NaCl. Colonies which didn't grow or weakly grow on NaCl plates and SDS plates were selected for trehalose production study.

Two well known species of dairy *Propionibacterium*, mainly shermanii and freudenreichii were procured from each centre. These strains included

(a). *Propionibacterium shermanii* MTCC 1371

(b). *Propionibacterium freudenreichii* MTCC 1950

(c). *Propionibacterium shermanii* NCIM 5137

(d). *Propionibacterium freudenreichii* NCIM 2111

Trehalose accumulation studies were carried out with all these strains in similar experimental conditions and osmotic stress was provided in the media by addition of 1.5% NaCl. In all these strains, the effect of osmotic stress was evident as growth was slow in comparison to non stress condition and higher trehalose accumulation was also observed in osmotic stress condition. In *P. shermanii* MTCC 1371, effect of osmotic stress was clear from biomass growth profile in stress and non-stress conditions (Fig 4.1 A). Trehalose was detected in osmotic stress where-as no trehalose was found in non-stress condition (table 4.1). Thus the stress response of this strain was in the form of trehalose accumulation (known as compatible solute). Trehalose accumulation under osmotic stress in stationary phase was 32-35 mg/g of biomass (Table 4.1) where as no trehalose was detected in non-stress condition. The effect of osmotic stress on physiology can also be observed from slow growth in osmotic stress condition in comparison to non-stress condition.

In *P. shermanii* NCIM 5137, biomass growth was slower in osmotic stress while trehalose accumulation was detected even in non stress condition (Fig 4.1 B, Table 4.1). The trehalose accumulation in late exponential phase was 60 mg/g of biomass in osmotic stress condition (table 4.1). Interestingly, trehalose accumulation was detected in non-stress condition and it was 15 mg/g of biomass; it indicates that this strain has normal capability of trehalose accumulation. In fact, the trehalose accumulation was four fold higher in osmotic stress condition as compared to non-stress condition (table 4.1). Accumulation of trehalose in non-stress condition is advantageous as it can be exploited for commercial production with minimal osmotic stress condition. Altogether, amongst the strains from NCIM and MTCC of species shermanii, *P. shermanii* NCIM 5137 was more suitable for trehalose production study.

The effect of osmotic stress on growth of *P. freudenreichii* MTCC 1950 and *P. freudenreichii* NCIM 2111 were minimal as compared to *P. shermanii* strains of MTCC and NCIM (Fig 4.1 C & D). In *P. freudenreichii* MTCC 1950, trehalose was detected only in osmotic stress while in *P. freudenreichii* NCIM 2111 trehalose accumulation was observed in both osmotic stress and non-stress conditions (table 4.1). In *P. freudenreichii* MTCC 1950, trehalose accumulation was 32-37.5 mg/g of biomass under osmotic stress while in *P. freudenreichii* NCIM 2111, 27 mg/g of biomass of trehalose was detected under similar experimental conditions. In *P. freudenreichii* NCIM 2111, 8 mg/g of biomass of trehalose was detected in non-stress condition (table 4.1). All the experiments were done at 30 °C.

The screening study shows that trehalose accumulation is widespread in all the procured strains of *Propionibacterium* while it seems that trehalose accumulation is the primary defence strategy under stress condition. From the comparison of trehalose content of all these four strains studied under the influence of osmotic stress, it was concluded that highest trehalose accumulation was observed in *P. shermanii* NCIM 5137 (60 mg/g of biomass) (table 4.1). Since, *P. shermanii* NCIM 5137 was capable of accumulating higher trehalose under osmotic and non-stress conditions, hence it was considered most suitable strain for further study. The *P. shermanii* has renomenclatured as *P. freudenreichii* ssp *shermanii* (Thierry et al., 2011).

Chapter 4

Results and Discussion

Chapter 4

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Trehalose is a non reducing sugar ubiquitously distributed in nature. It is well known for its protective role against various stresses and reported to have nutraceutical value (Arguelles, 2000). The study of trehalose accumulation in microbes is significant, as it is a physiological response of microbes against various stresses, especially under osmotic stress. The study of trehalose metabolism under osmotic stress can also provide insight on metabolic regulation associated with trehalose biosynthesis. Trehalose production capability in any industrial microbes also imparts stress tolerance against various stresses encountered during commercial bio production processes (Carvalho et al., 2011). Recently, it was also reported that ethanol tolerance in *S. cerevisiae* is also related to trehalose accumulation (Li et al., 2009). Similarly, recombinant strain of *Lactococcus lactis* accumulating higher trehalose was more stress tolerant (Carvalho et al., 2011). Thus there are widespread interests on trehalose accumulation study in various microorganisms. The commercial applications of trehalose as food additives, enzyme stabilizer, cell & organ preservative, ingredient of cosmetic product and probable medical uses creates common interest on its commercial production (Kidd et al., 1994; Colaco et al., 1992; Eroglu et al., 2000; Higashiyama, 2002). The conventional process of producing trehalose using *Saccharomyces cerevisiae* has a relatively low production yield and has been replaced by an enzymatic conversion process (Maruta et al., 1995). However, the advantage of microbial based processes is its ability to efficiently utilize agricultural and industrial wastes as substrate for the production of valuable metabolites like trehalose (Li et al., 2011). In particular, trehalose accumulation is also known in various strains of dairy *Propionibacterium* (Cardoso et al., 2004). In fact, trehalose accumulation in *P. freudenreichii* was reported as defense strategy against osmotic, oxidative and acidic stresses (Cardoso et al., 2007). OtsAB pathway (trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase) for trehalose synthesis and TreS (trehalose synthase) for catabolic pathway was already proposed in *P. freudenreichii* (Cardoso et al., 2007). Detail information about trehalose biosynthesis pathway in dairy *Propionibacterium* is limited as complete genome sequence of this genus is just recently published (Falentin et al., 2010). Various applications of dairy *Propionibacterium* based on its physiology and metabolism (Thierry et al., 2011; Hugenschmidt et al., 2010) has also been proposed.

Based on these observations we devised our strategy to get detailed insight into trehalose metabolism in *Propionibacterium shermanii* under osmotic stress condition and finally proposed the suitability of crude glycerol as carbon source for economic production of trehalose together with propionic acid (Ruhal et al., 2011).

4.1. Screening of procured strains for higher trehalose production under osmotic stress in static flask conditions

Trehalose is a non-reducing disaccharide distributed widely in dairy *Propionibacterium* (Cardoso et al. 2004). Unfortunately, detailed insight to trehalose bio-synthesis in dairy *Propionibacterium* is very limited. In fact, information regarding regulation of trehalose biosynthesis in other microbes is restricted to OtsAB, TreYZ and TreS. In recent years, the research field of dairy *Propionibacterium* have gradually increased and these include studies about the various probiotic potentials, the synthesis of bifidogenic growth factor, vitamins, conjugated linoleic acid (CLA) and the potential of *Propionibacterium* as protective cultures (Thierry et al., 2011). Thus trehalose production in dairy *Propionibacterium* will be advantageous due to its GRAS nature and possibility of obtaining other product along with trehalose. So an attempt was made to study the trehalose accumulation in locally available dairy *Propionibacterium*.

For the screening of suitable strain for trehalose accumulation study, four major available strains were procured from two major culture collection centres, MTCC Chandigarh and NCIM Pune India. These strains were reported to be isolated from dairy based fermented food. In preliminary study, growth of cultures was carried out in media as described elsewhere (Cardoso et al., 2004). It was already known that *Propionibacterium* produces trehalose under stress conditions; hence potential of trehalose accumulation of these collected strains was determined under osmotic stress condition. All experiments for screening were carried out in static flask culture condition (material and methods). The growth of biomass and trehalose accumulation were studied in media containing 20 g/l of glucose with and without osmotic stress. For trehalose quantification and comparison of trehalose content, cells from stationary phase (mainly early stationary phase) were harvested and comparative analysis of trehalose yield based on biomass (since trehalose was intracellular) were made.

Two well known species of dairy *Propionibacterium*, mainly shermanii and freudenreichii were procured from each centre. These strains included

(a). *Propionibacterium shermanii* MTCC 1371

(b). *Propionibacterium freudenreichii* MTCC 1950

(c). *Propionibacterium shermanii* NCIM 5137

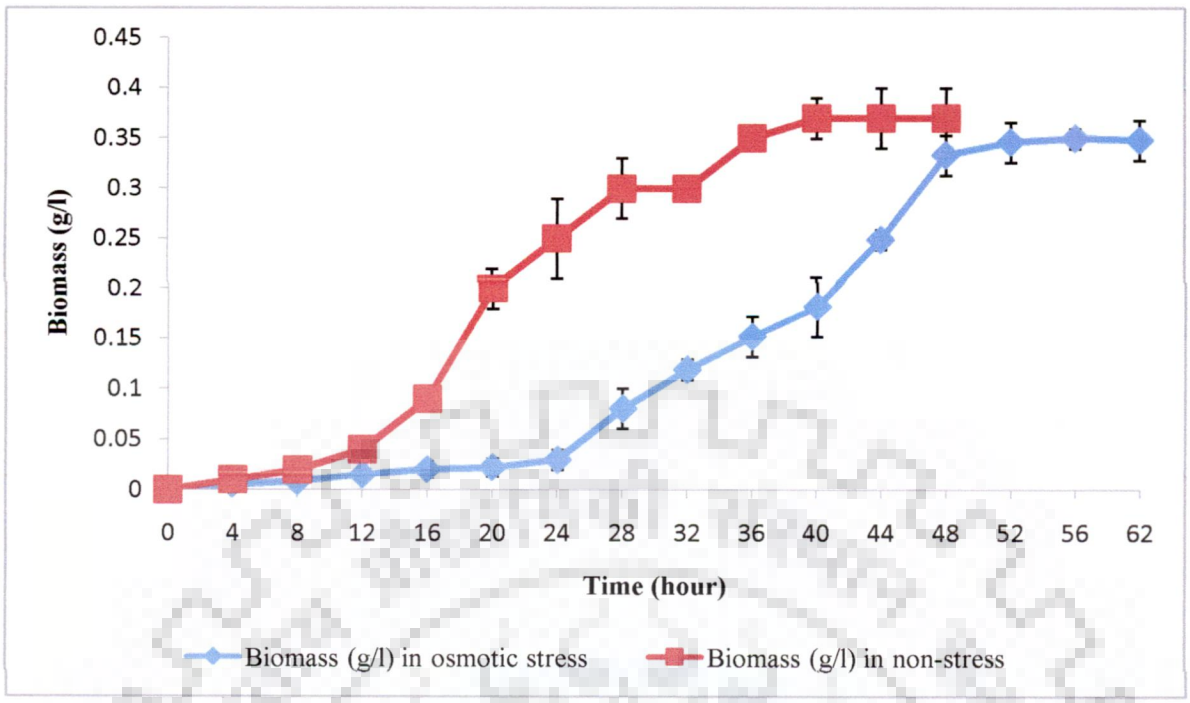
(d). *Propionibacterium freudenreichii* NCIM 2111

Trehalose accumulation studies were carried out with all these strains in similar experimental conditions and osmotic stress was provided in the media by addition of 1.5% NaCl. In all these strains, the effect of osmotic stress was evident as growth was slow in comparison to non stress condition and higher trehalose accumulation was also observed in osmotic stress condition. In *P. shermanii* MTCC 1371, effect of osmotic stress was clear from biomass growth profile in stress and non-stress conditions (Fig 4.1 A). Trehalose was detected in osmotic stress where-as no trehalose was found in non-stress condition (table 4.1). Thus the stress response of this strain was in the form of trehalose accumulation (known as compatible solute). Trehalose accumulation under osmotic stress in stationary phase was 32-35 mg/g of biomass (Table 4.1) where as no trehalose was detected in non-stress condition. The effect of osmotic stress on physiology can also be observed from slow growth in osmotic stress condition in comparison to non-stress condition.

In *P. shermanii* NCIM 5137, biomass growth was slower in osmotic stress while trehalose accumulation was detected even in non stress condition (Fig 4.1 B, Table 4.1). The trehalose accumulation in late exponential phase was 60 mg/g of biomass in osmotic stress condition (table 4.1). Interestingly, trehalose accumulation was detected in non-stress condition and it was 15 mg/g of biomass; it indicates that this strain has normal capability of trehalose accumulation. In fact, the trehalose accumulation was four fold higher in osmotic stress condition as compared to non-stress condition (table 4.1). Accumulation of trehalose in non-stress condition is advantageous as it can be exploited for commercial production with minimal osmotic stress condition. Altogether, amongst the strains from NCIM and MTCC of species shermanii, *P. shermanii* NCIM 5137 was more suitable for trehalose production study.

The effect of osmotic stress on growth of *P. freudenreichii* MTCC 1950 and *P. freudenreichii* NCIM 2111 were minimal as compared to *P. shermanii* strains of MTCC and NCIM (Fig 4.1 C & D). In *P. freudenreichii* MTCC 1950, trehalose was detected only in osmotic stress while in *P. freudenreichii* NCIM 2111 trehalose accumulation was observed in both osmotic stress and non-stress conditions (Table 4.1). In *P. freudenreichii* MTCC 1950, trehalose accumulation was 32-37.5 mg/g of biomass under osmotic stress while in *P. freudenreichii* NCIM 2111, 27 mg/g of biomass of trehalose was detected under similar experimental conditions. In *P. freudenreichii* NCIM 2111, 8 mg/g of biomass of trehalose was detected in non-stress condition (Table 4.1).

The screening study shows that trehalose accumulation is widespread in all the procured strains of *Propionibacterium* while it seems that trehalose accumulation is the primary defence strategy under stress condition. From the comparison of trehalose content of all these four strains studied under the influence of osmotic stress, it was concluded that highest trehalose accumulation was observed in *P. shermanii* NCIM 5137 (60 mg/g of biomass) (table 4.1). Since, *P. shermanii* NCIM 5137 was capable of accumulating higher trehalose under osmotic and non-stress conditions, hence it was considered most suitable strain for further study. The *P. shermanii* has renomenclatured as *P. freudenreichii* ssp *shermanii* (Thierry et al., 2011).



4.1 (A) Growth pattern of *P. shermanii* MTCC 1371 under non-stress and osmotic stress conditions in static flask culture

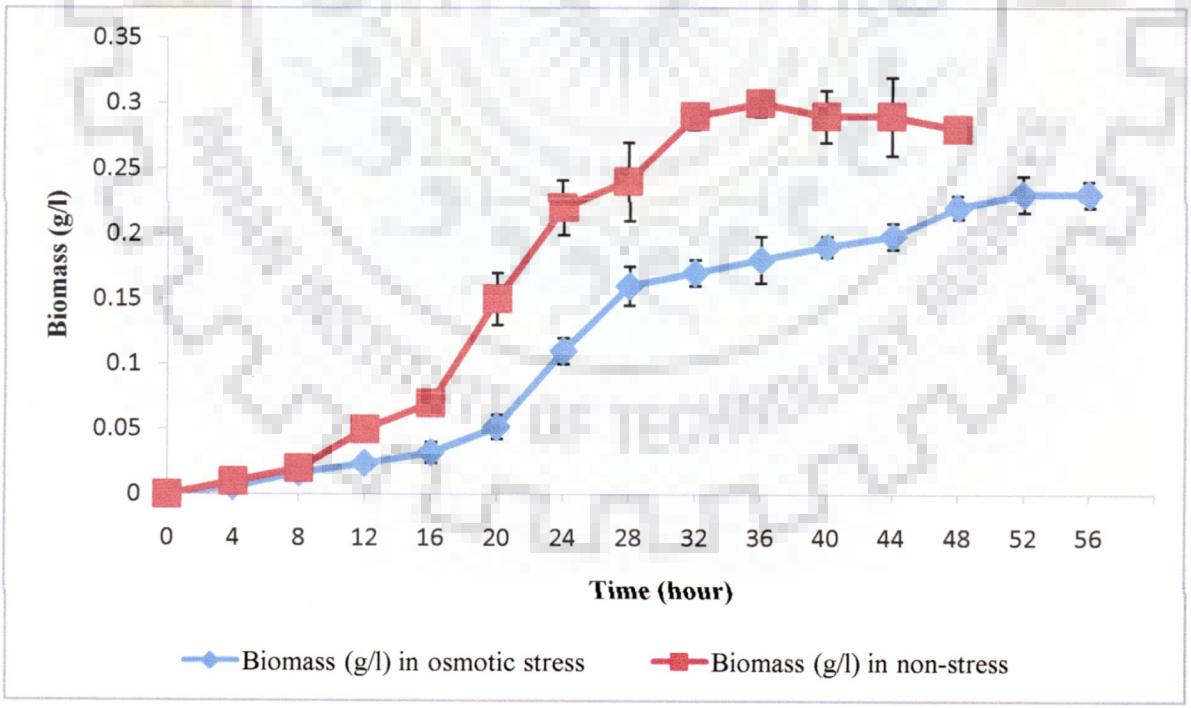


Fig 4.1 (B) Growth pattern of *P. shermanii* NCIM 5137 under non-stress and osmotic stress conditions in static flask culture

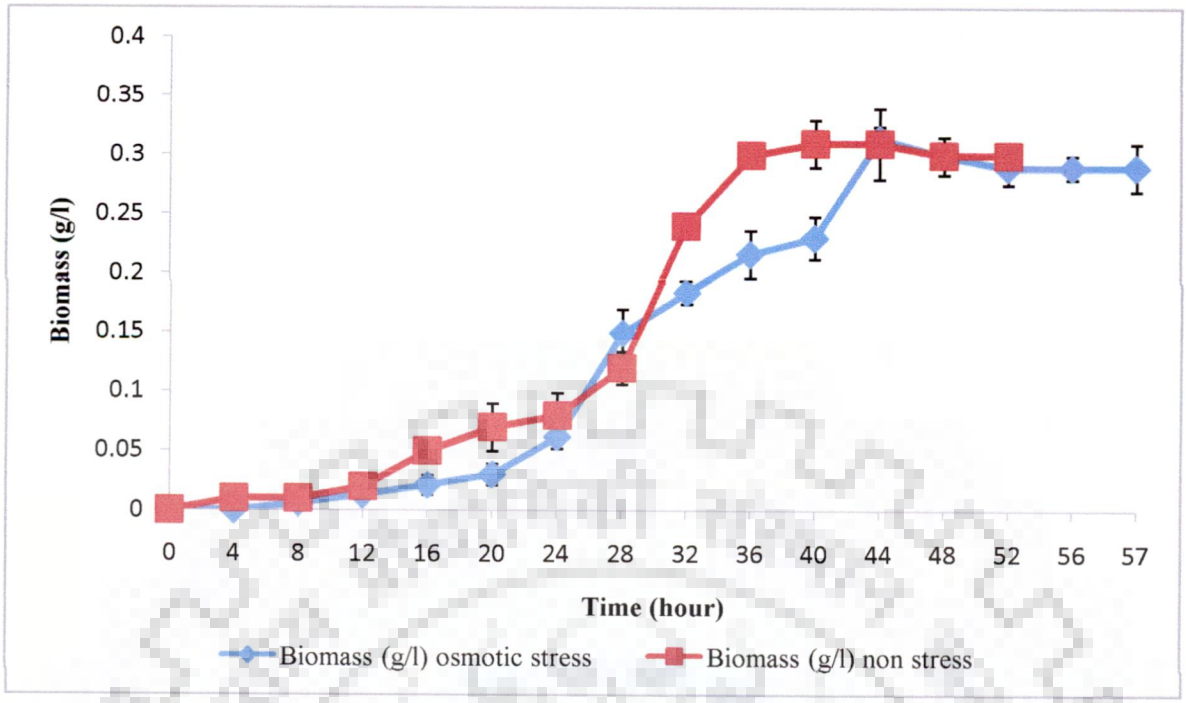


Fig 4.1 (C) Growth pattern of P. *freudenreichii* MTCC 1950 under non-stress and osmotic stress conditions in static flask culture

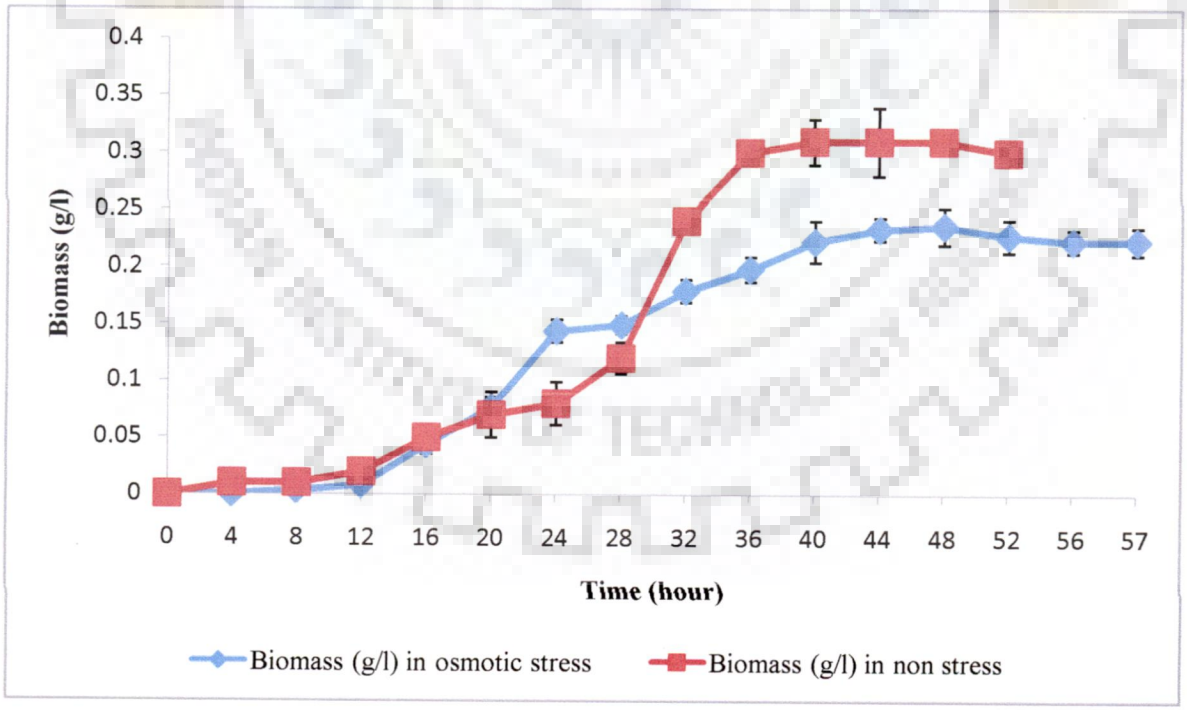


Fig 4.1 (D) Growth pattern of *P. freudenreichii* NCIM 2111 *Propionibacterium* under non-stress and osmotic stress conditions in static flask culture

Table 4.1: Comparison of trehalose yield (mg/g of biomass) in osmotic and non stress conditions in different strains of dairy *Propionibacterium*

Strain	Osmotic stress	Non-stress
P. freudenreichii MTCC 1950	37±4	0
P. shermanii MTCC 1371	35±2	0
P. freudenreichii NCIM 2111	40±3	8±1
P. shermanii NCIM 5137	60±5	15±2

4.2. Effects of inorganic or organic nitrogen source, temperature, pH, osmotic stress and carbon source on trehalose accumulation in *P. shermanii* NCIM 5137

In previous studies with *P. freudenreichii*, significant variations in trehalose accumulation were observed with environmental conditions like pH, osmotic stress and carbon source (Cardoso et al., 2004). Therefore, it was planned to investigate the influences of these parameters on trehalose biosynthesis in *P. shermanii* NCIM 5137. Hence, we studied effects of temperature, pH, osmotic stress and nitrogen source on trehalose accumulation in *P. shermanii* NCIM 5137.

4.2.1. Effects of inorganic and organic nitrogen sources on trehalose accumulation in *P. shermanii* NCIM 5137

The principle difference between trehalose and other compatible solutes (glutamate, proline) is absence of nitrogen in its chemical structure. Therefore, to examine the effect of nitrogen source on trehalose yield, an experiment was carried out with inorganic nitrogen source (ammonium citrate) in a chemically defined media and compared the trehalose production with complex media (yeast extract, tryptone and peptone). A media without nitrogen source was also used but no growth was observed.

In a chemically defined media, the maximum trehalose yield achieved with respect to biomass was equivalent to that obtained with complex medium (93 mg/g of biomass) (Fig 4.2 & 4.3). Similarly, final trehalose yield at stationary phase was 15 mg/g of biomass (glucose as carbon source in both medium). The study with chemically defined media also indicates that trehalose synthesis in *P. shermanii* NCIM 5137 is de-novo. However, the growth in chemically defined media was slower than with complex media. Thus all further studies were carried out with complex media. Likewise, in *Corynebacterium* during total absence of nitrogen trehalose was the only detectable compatible solute and indicated that nitrogen have no influence on trehalose accumulation (Wolf et al., 2003). Furthermore, it seems that *P. shermanii* NCIM 5137 also possesses catabolic machinery for trehalose, since trehalose content was decreasing with growth after initial increase at mid-exponential phase.

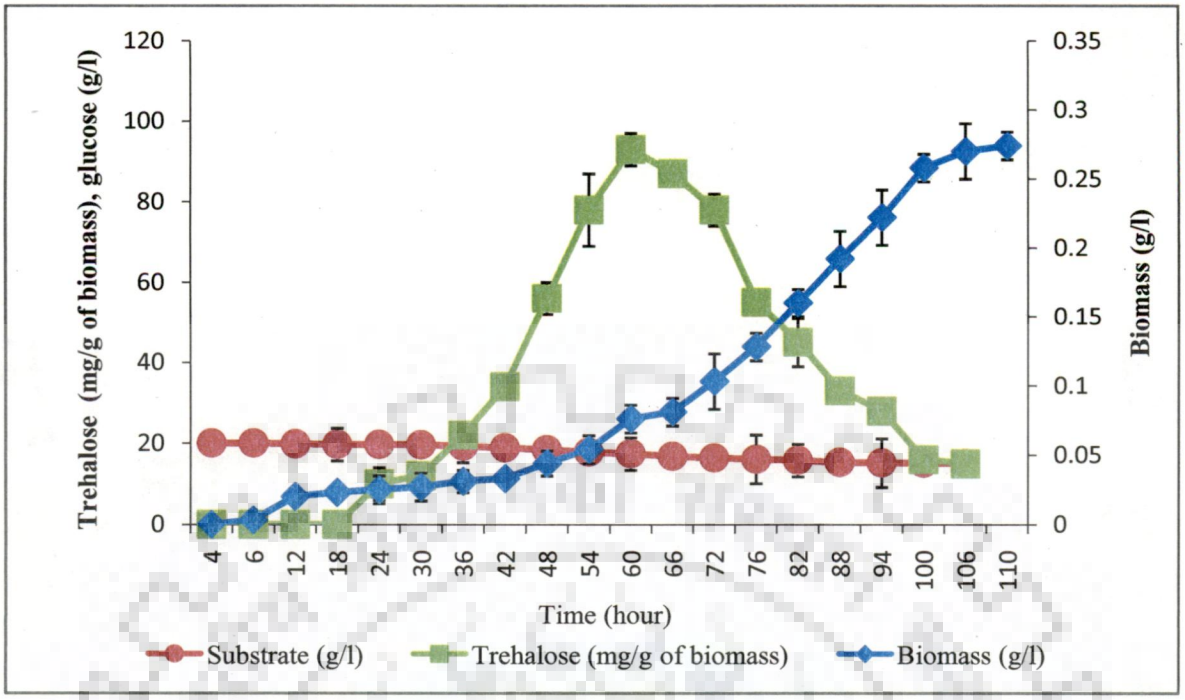


Fig 4.2 Trehalose yield, residual substrate concentration and biomass growth profiles with chemically defined media (source of inorganic nitrogen source) in static flask condition (non-stress) at 30°C

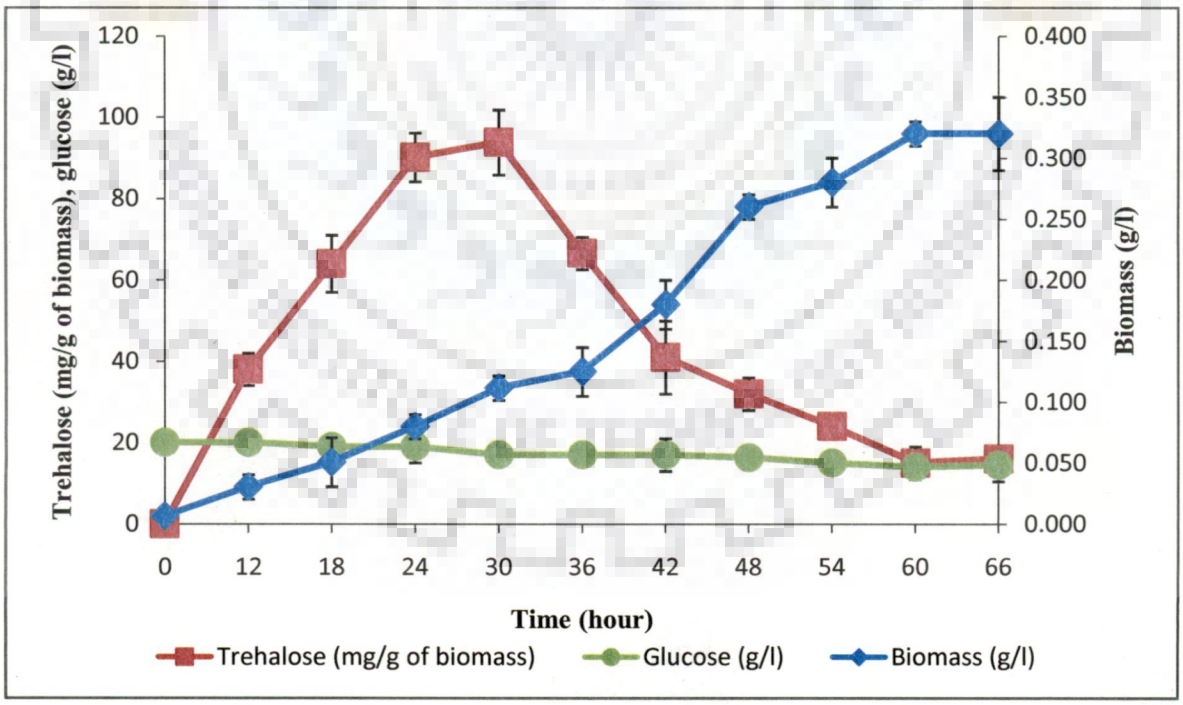


Fig 4.3 Trehalose accumulation residual substrate concentration and biomass growth profiles with complex medium (source of organic nitrogen source) in static flask condition (non-stress) at 30°C

4.2.2. Effect of temperature on trehalose accumulation in *P. shermanii* NCIM 5137

Effect of temperature on trehalose yield was evaluated by static flask culturing at temperatures of 45°C and 20°C other than 30°C (optimum temperature of growth of *P. shermanii* NCIM 5137). These higher and lower temperatures didn't have any beneficial effect on trehalose accumulation in *P. shermanii* NCIM 5137. In fact, no growth was observed at 20°C while decrease in maximum trehalose yield was observed at 45°C (32 mg/g of biomass) as compared to 30°C (92 mg/g of biomass) (fig 4.3 & 4.4). Previously, in *P. freudenreichii*, no effect of higher temperature on trehalose accumulation was reported while lowering of temperature didn't have any beneficial effect on higher trehalose yield (Cardoso et al., 2004). Thus in all further study, 30°C was used as incubation temperature. Thus, it seems that *P. shermanii* NCIM 5137 may have different defence mechanism against temperature stress.

4.2.3. Effect of carbon source on trehalose yield in *P. shermanii* NCIM 5137

The effect of carbon source was studied under controlled conditions of pH and temperature using batch reactor. Dissolved oxygen concentration was maintained at 5-10% of saturation of O_2 by purging nitrogen gas for 0-24 h. Similar to previous reported study (Cardoso et al., 2004) three carbon sources were chosen including glucose, lactose (glycolytic carbon source) and lactic acid (gluconeogenic carbon source). The highest trehalose accumulation in substrate lactose was 101 mg/g of biomass (fig 4.5); while in substrate glucose it was 45 mg/g of biomass (fig 4.6) and in lactic acid 38 mg/g of biomass (fig 4.7). Final biomass achieved in substrate glucose, lactic acid and lactose were 4.4 g/l, 3.69 g/l and 3.36 g/l respectively (fig 4.5, 4.6 and 4.7). There was complete substrate consumption in all the carbon sources in batch reactor study. Final trehalose yield in glucose, lactose and lactic acid were 15, 18 and 0 mg/g of biomass (fig 4.5, 4.6 and 4.7). Comparison of various fermentation parameters indicated that maximum specific growth rate was 0.09 h^{-1} in lactose, 0.12 h^{-1} in lactic acid and 0.2 h^{-1} in glucose. The highest substrate consumption rate were 0.25 g l^{-1} h^{-1}, 0.3 g l^{-1} h^{-1} and 0.34 g l^{-1} h^{-1} in substrates lactose, lactic acid and glucose respectively while highest biomass production rate were 0.037 g l^{-1} h^{-1}, 0.035 g l^{-1} h^{-1} and 0.066 g l^{-1} h^{-1} in lactose, lactic acid and glucose respectively. Other major products in the entire carbon source were lactic acid and propionic acid, while only propionic acid was the product when lactic acid was used as carbon source (fig 4.5, 4.6 and 4.7). The minimum trehalose yield in lactate probably be

due to low efficiency of gluconeogenesis while prompt availability of glucose moieties when lactose or glucose were used as carbon source seems to be determinant for the efficient accumulation of trehalose (Cardoso et al., 2004).

4.2.4. Trehalose accumulation under osmotic stress in *P. shermanii* NCIM 5137 in static flask conditions

In the previous section (4.1), it was already observed that under osmotic stress trehalose production was enhanced in *P. shermanii* NCIM 5137. Therefore, in the present study, trehalose accumulation under osmotic stress was again evaluated in static flask with the objective of monitoring trehalose content at regular interval of time with substrate glucose at incubation temperature 30°C. The maximum trehalose yield in osmotic stress was 122 mg/g of biomass while in non-stress 92 mg/g of biomass was obtained (fig 4.8 & 4.9). In fact, the effect of osmotic stress was also apparent from the final trehalose yield achieved at stationary phase and it was 44 mg/g of biomass as compared to 15 mg/g of biomass in non-stress condition (fig 4.10).

In addition, it was found that the level of trehalose was higher in the initial exponential phase both in osmotic stress and non-stress conditions while it starts degrading in late exponential phase. This suggested that the mechanism of protection might involve minimizing the damage caused to cells, which possibly require the presence of trehalose in vivo of the cell; this hypothesis is supported by the results obtained in yeast where a strong correlation between trehalose content and stress resistance has been demonstrated for a variety of stresses (Li et al., 2009). Similarly, the trehalose concentration was about six fold increased and trehalose becomes the predominant compatible solute of *C. glutamicum* under osmotic stress conditions (Morbach & Kramer, 2005).

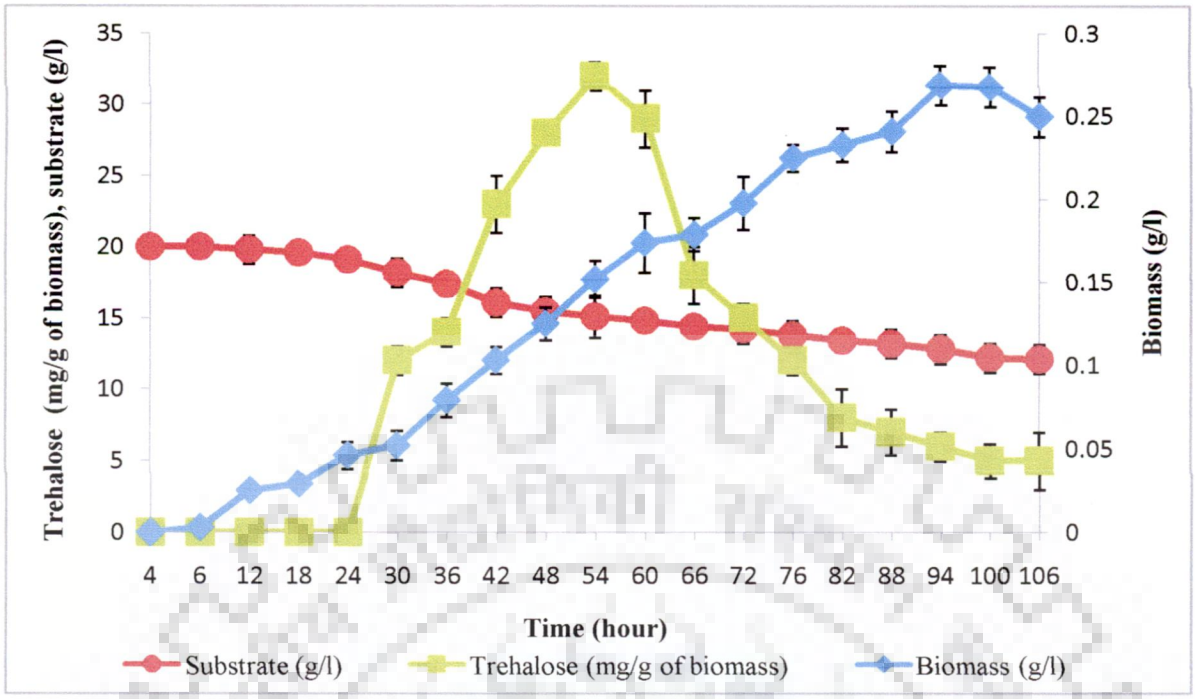


Fig 4.4 Effect of higher temperature (45°C) on trehalose accumulation residual substrate concentration and biomass growth profiles of in *P. shermanii* NCIM 5137 in static flask culture

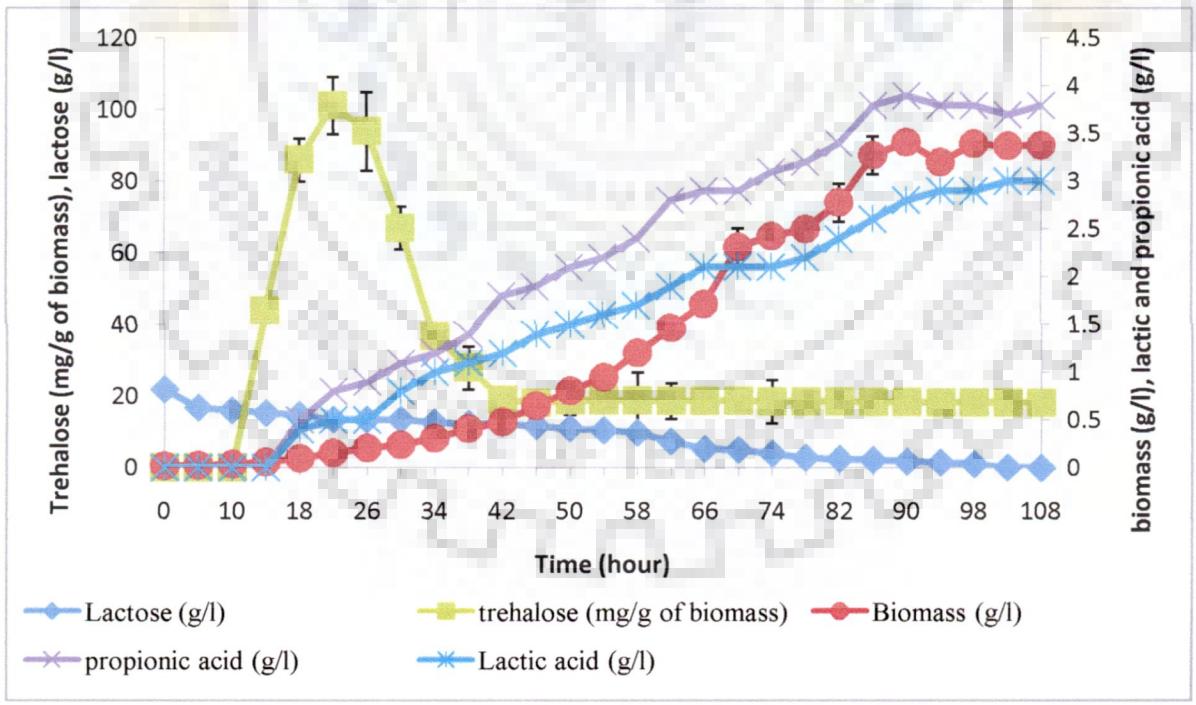


Fig 4.5 Trehalose yield, biomass growth profile, residual substrate concentration, lactic and propionic acid production profiles in carbon source lactose in batch reactor with *P. shermanii* NCIM 5137

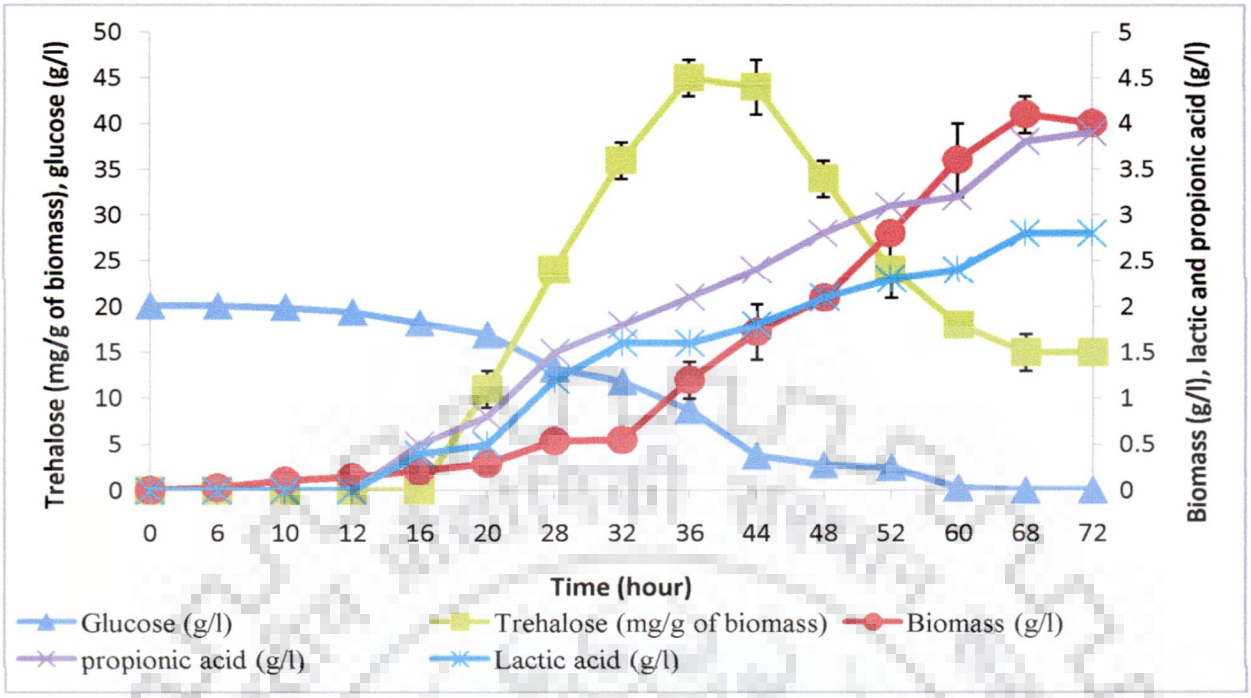


Fig 4.6 Trehalose yield, biomass growth profile, residual substrate concentration, lactic and propionic acid production profile in carbon source glucose in batch reactor with *P. shermanii* NCIM 5137

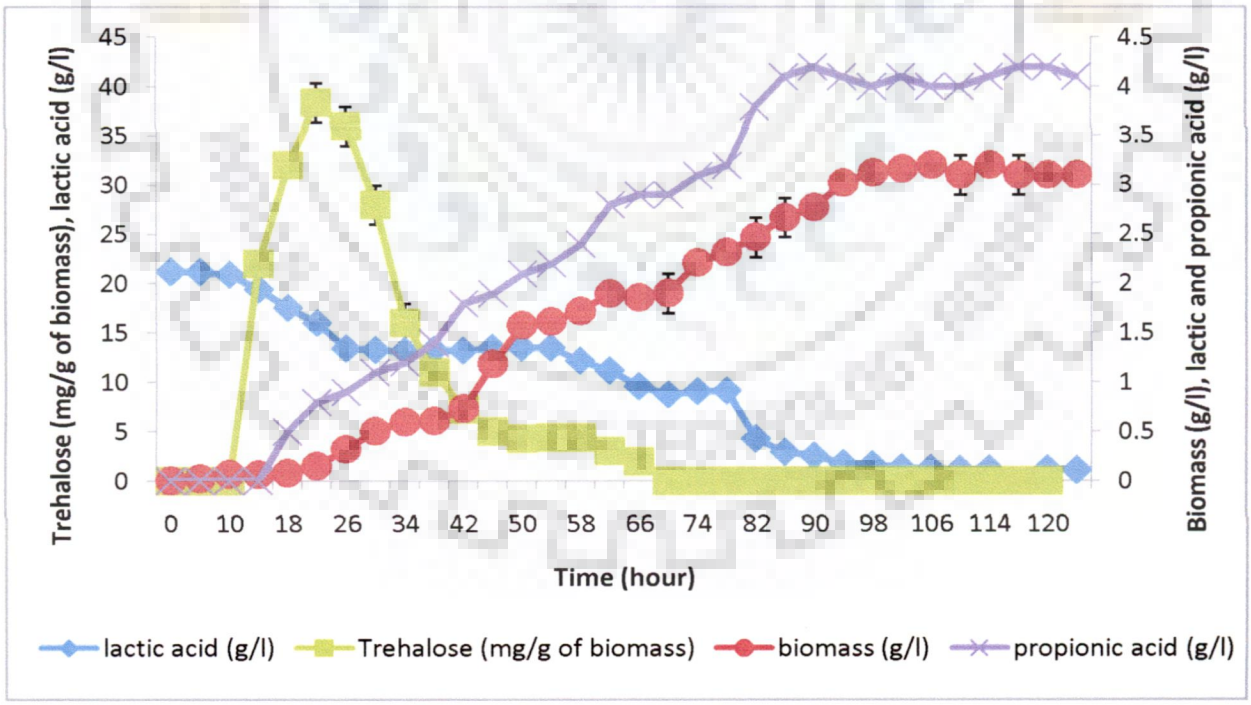


Fig 4.7 Trehalose yield, biomass growth profile, residual substrate concentration and propionic acid production profiles in carbon source lactic acid in batch reactor with *P. shermanii* NCIM 5137

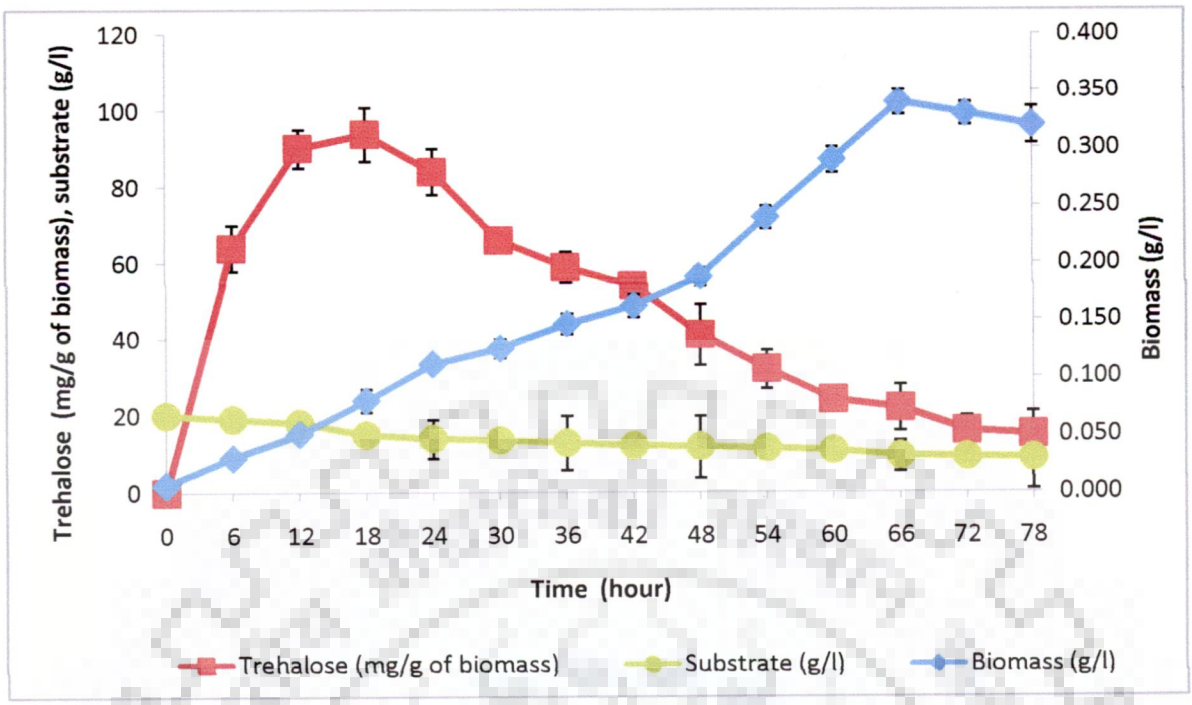


Fig 4.8 Trehalose yield, residual substrate concentration and biomass growth profiles under non stress condition in static flask condition.

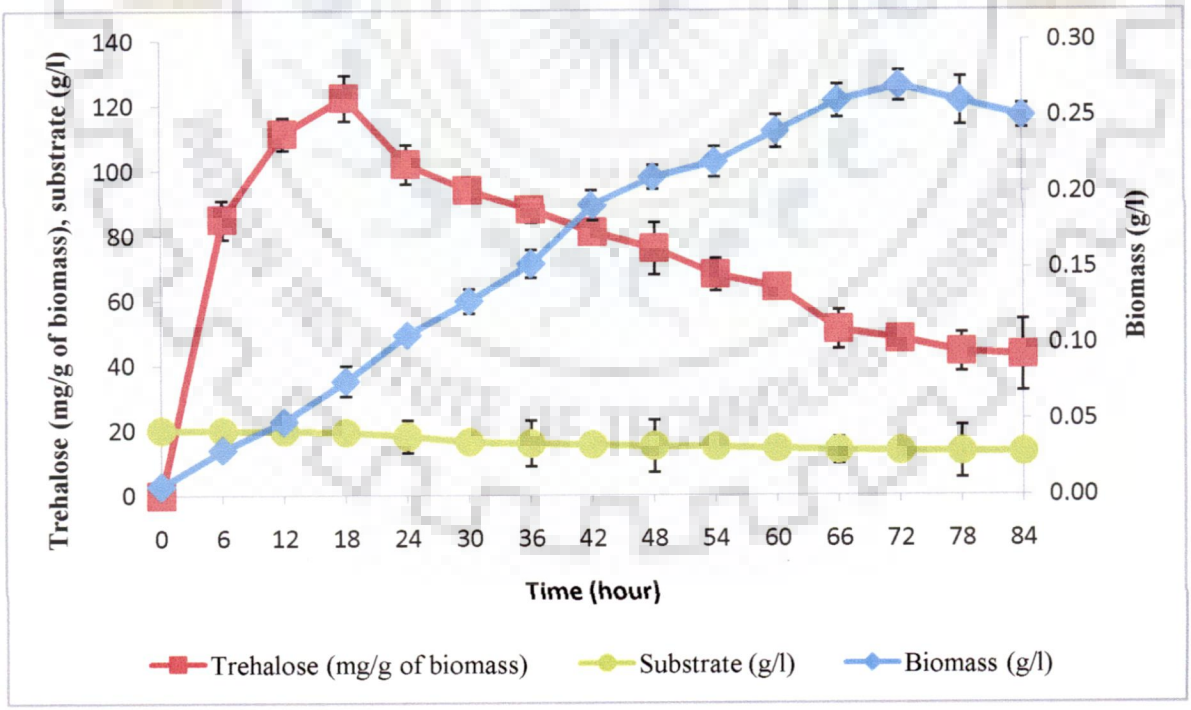


Fig 4.9 Trehalose yield, residual substrate concentration and biomass growth profiles under osmotic stress condition in static flask condition

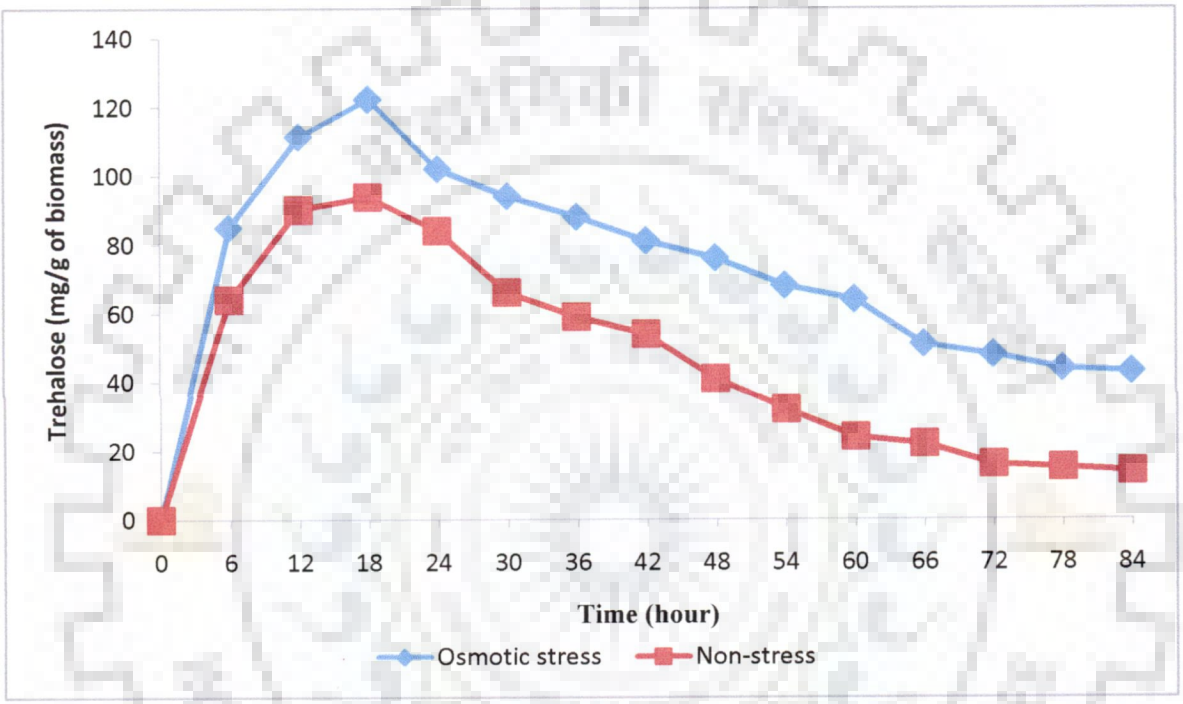


Fig 4.10 Comparison of trehalose accumulation in non-stress and osmotic stress conditions in static flask culture

4.2.5. Batch reactor studies of osmotic, oxidative and pH stress on trehalose accumulation

As the beneficial effect of osmotic stress was evident from static flask study hence influence of osmotic stress was also studied under controlled conditions of pH and dissolved oxygen. In batch reactor, biomass concentration achieved was much higher than in static flask condition (approx 10 times) while complete substrate consumption was also achieved within 80 hours. Osmotic stress was given in the form of 1.5% NaCl and glucose was used as carbon source. Trehalose accumulation started early in growth phase (from 12 hour) in osmotic stress (Fig 4.11) while in non-stress it started after 28 hour (Fig 4.12). Under osmotic stress, maximum trehalose yield was 160 mg/g of biomass in early exponential phase in comparison to 45 mg/g of biomass in non-stress. It is worth noting that maximum trehalose yield achieved in batch reactor under non-stress condition was less than (two times lower) the yield obtained in static flask study. However, final trehalose yield achieved in non-stress condition was similar both in static flask and batch culture condition.

There was rapid degradation of trehalose with time in non stress condition, but trehalose yield was relatively stable (with respect to degradation) during osmotic stress condition (Fig 4.11 & 4.12). The effect of osmotic stress was apparent from the differences in various fermentation parameters in osmotic and non-stress conditions as given in Table 4.2. Specific growth rate, substrate consumption rate, biomass production rate and biomass yield were lower in osmotic stress condition in comparison to non-stress (Table 4.2). In batch reactor study approximately 100% of substrate consumptions were achieved in osmotic stress and non-stress conditions. Thus osmotic stress seems to have effect on the physiology of the bacterium. Since, oxidative stress also enhances trehalose in other microbes like yeast hence we checked combined effect of oxidative and osmotic stress by culturing bacteria under 50% of air saturation and extracellular salt (1.5% NaCl), but no enhancement of trehalose yield was observed (Fig 4.12 B). Similarly, no effect of lower pH (5.5) was observed on trehalose yield (Fig 4.12 C).

Table 4.2 Comparison of fermentation parameters (highest) in osmotic stress and without osmotic stress in batch reactor with glucose as carbon source, pH 6.8 and 5% air saturation

Parameter	**Osmotic stress**	**Non-stress**
Specific growth rate, μ (h^{-1})	0.12±0.01	0.2±0.02
Substrate consumption rate, Qs (g l^{-1} h^{-1})	0.29 ± 0.03	0.34±0.01
Biomass production rate, Qx (g l^{-1} h^{-1})	0.032± 0	0.066± 0
Biomass yield (g/g)	0.131±0.01	0.22±0.03
Trehalose (mg/g of biomass)	160±11	45±6
Substrate consumption (%)	100	100

In conclusion, it can be emphasized that effect of osmotic stress was more prominent for enhanced trehalose accumulation in *P. shermanii* NCIM 5137 (Table 4.3). Unfortunately, trehalose yield was lower in lactic acid (Table 4.3), hence search of some other cheap carbon source was continued further. There was no significant advantage of using lactose as substrate while effect of osmotic stress on trehalose accumulation in lactose media was not evaluated as it was already reported by Cardoso et al 2004 that there was decrease in yield with increase in osmotic stress in lactose media. Similarly, It was concluded that temperature, pH, aeration cannot be used as variables for enhancing trehalose yield in *P. shermanii* NCIM 5137.

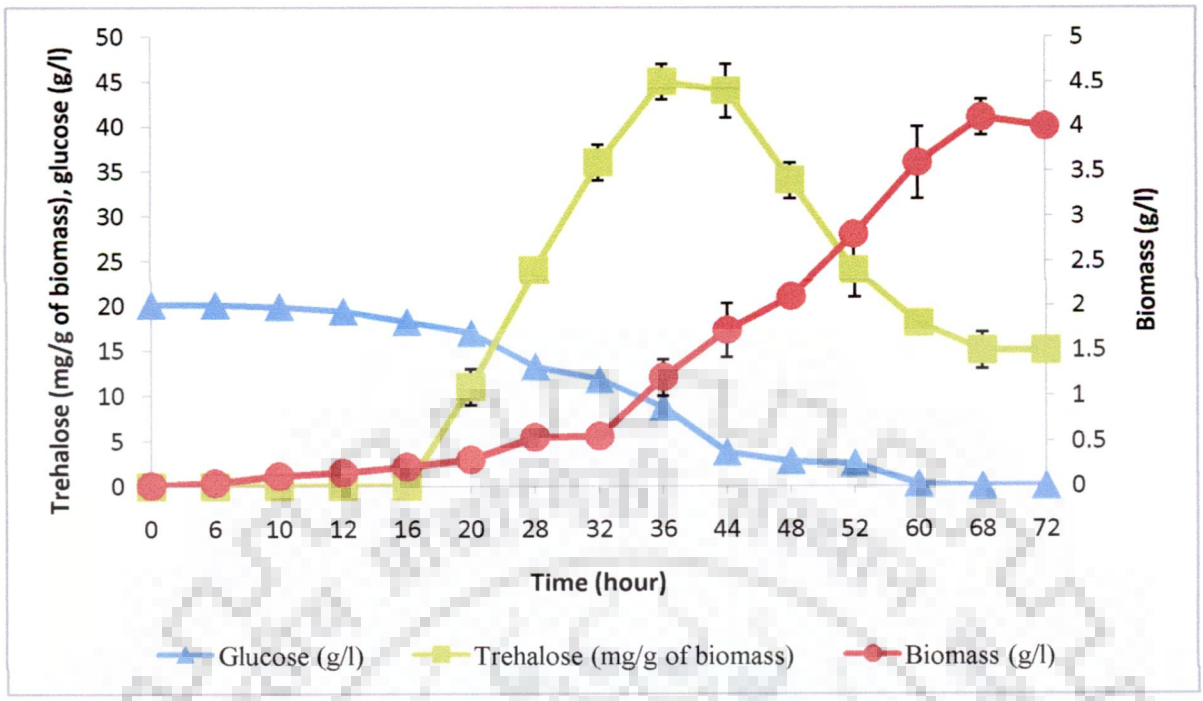


Fig 4.11 Trehalose yield, residual substrate concentration, biomass growth profiles under non-stress condition in batch reactor

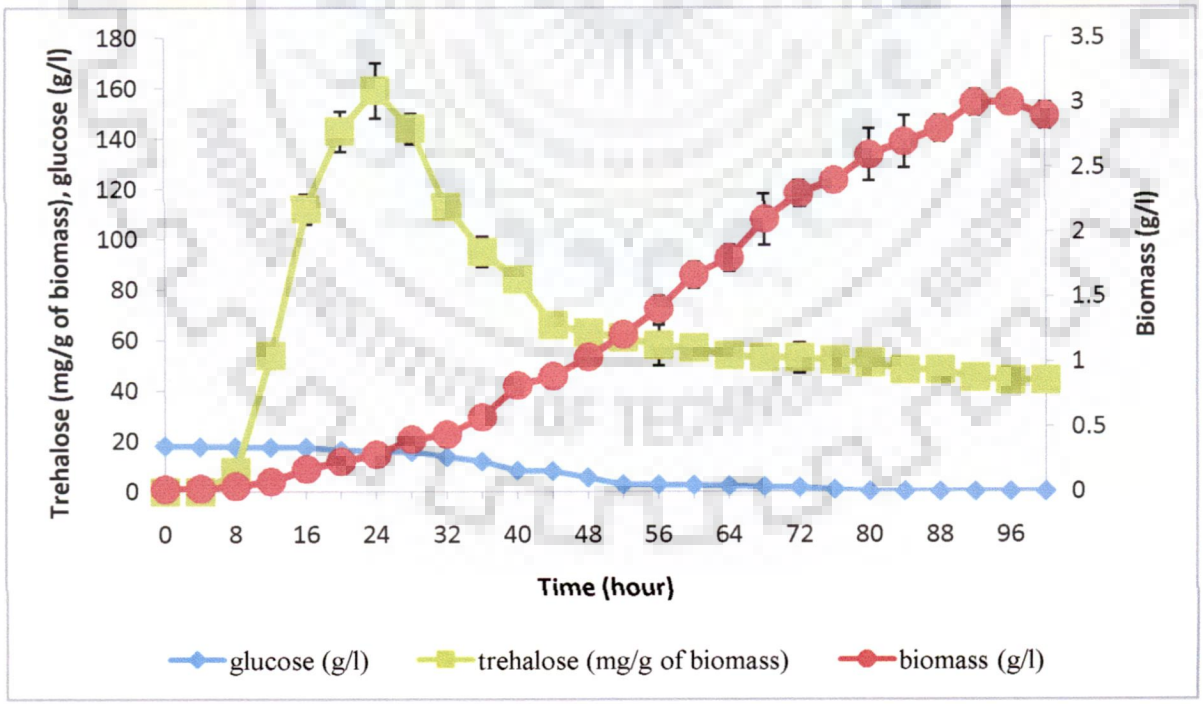


Fig 4.12 (A) Trehalose yield, residual substrate concentration, biomass growth profiles under osmotic-stress condition in batch reactor

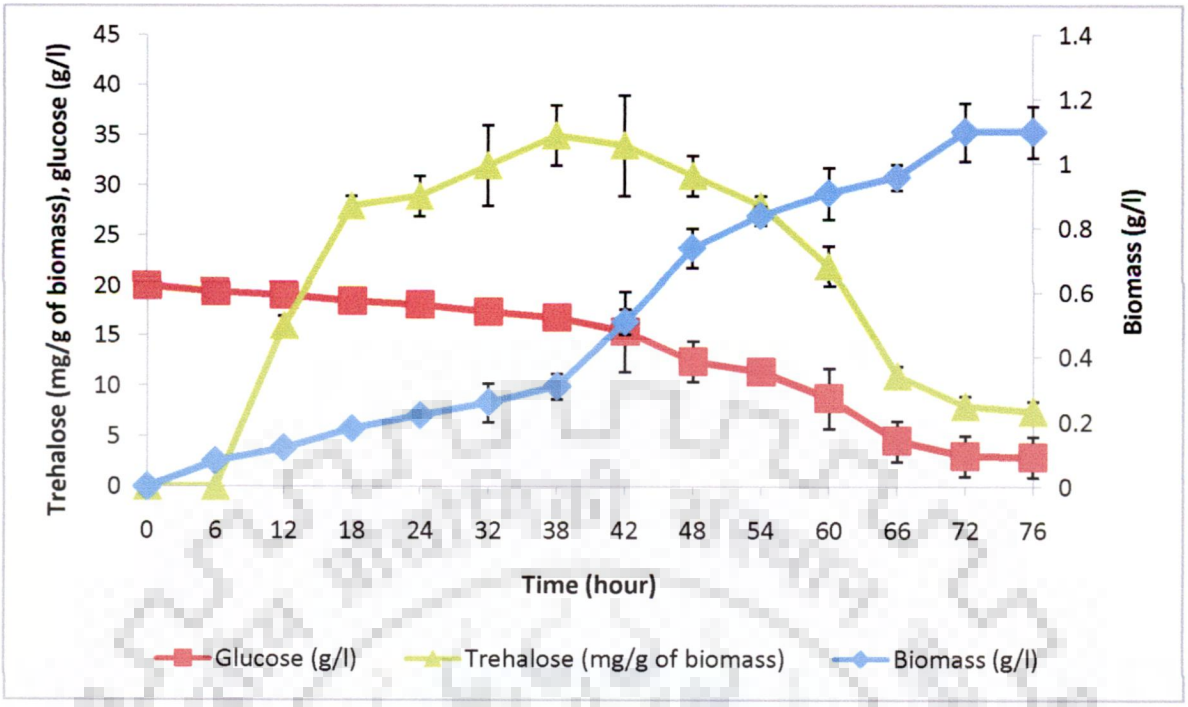


Fig 4.12 (B) Trehalose yield, residual substrate concentration, biomass growth profiles under osmotic (1.5% NaCl) and oxidative (50% air saturation) stress in batch reactor.

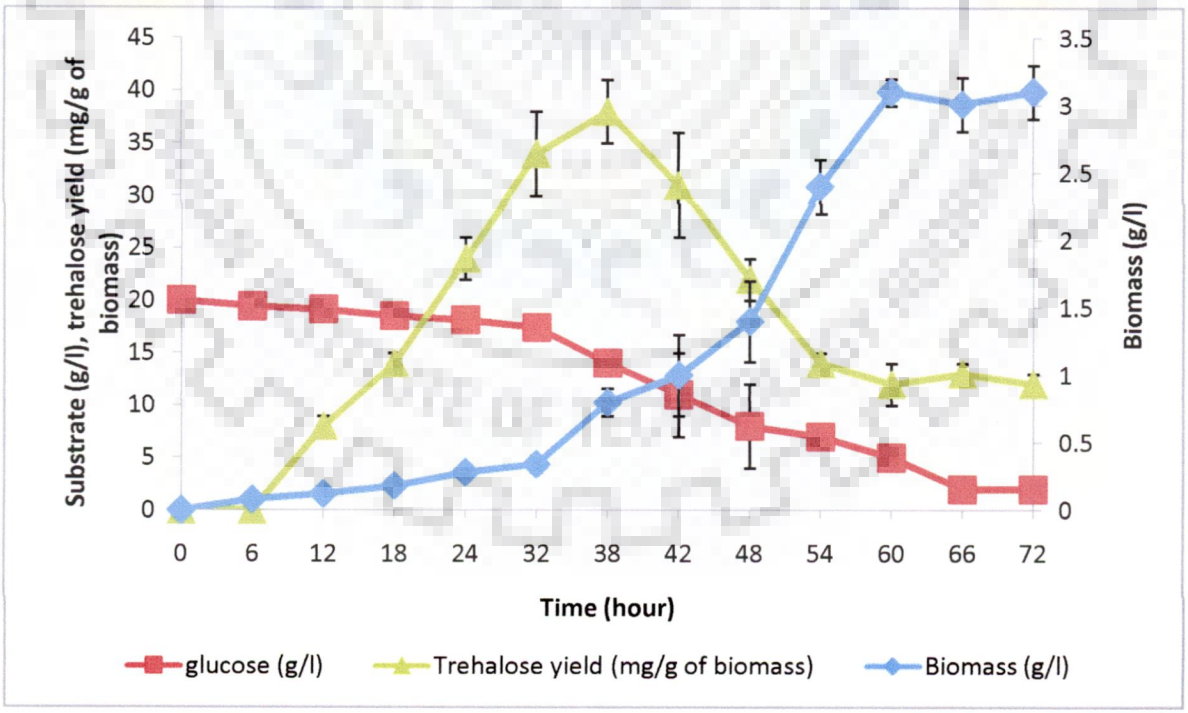
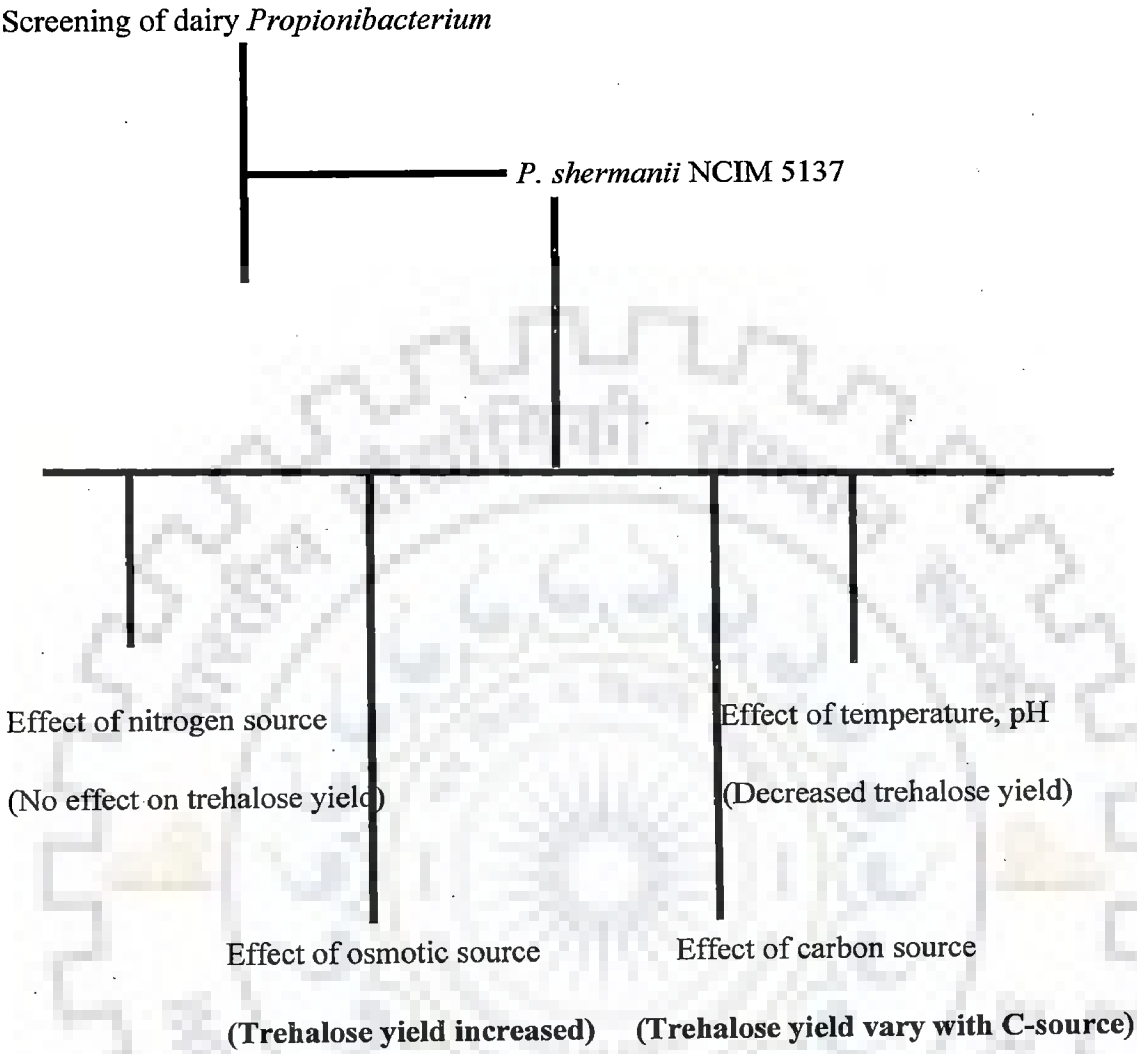


Fig 4.12 (C) Trehalose yield, residual substrate concentration, biomass growth profiles at pH 5.5 in batch reactor

Table 4.3: Comparison of trehalose yield in non-stress and osmotic stress (1.5% NaCl), different carbon source (glucose, lactose and lactic acid)

Carbon source	**Y_{tx} (maximum)**	**Y_{tx} (final)**
Non-stress (glucose)	45±4	15±1
Osmotic stress (glucose)	159±6	44±3
Lactose	101±4	18±2
Lactic acid	38±2	0

Thus major conclusions from section 4.1 and 4.2 have been shown in following diagram. In all further studies, media with glucose as carbon source was used as a control for evaluating effects various parameters on trehalose yield in *P. shermanii* NCIM 5137. Further, search for cheap carbon source was carried out with maltose, sucrose, starch and glycerol.


Screening of dairy *Propionibacterium*
P. shermanii NCIM 5137
Effect of nitrogen source
(No effect on trehalose yield)
Effect of osmotic source
(Trehalose yield increased)
Effect of temperature, pH
(Decreased trehalose yield)
Effect of carbon source
(Trehalose yield vary with C-source)

4.3. Effect of cheap carbon sources (sucrose, maltose, starch and glycerol) on intracellular accumulations of trehalose, maltose and glycogen in *Propionibacterium shermanii* NCIM 5137

In previous section it was observed that in carbon source has influence on trehalose yield, therefore it was planned to study effect of cheap carbon sources (sucrose, starch, maltose and glycerol) on trehalose yield. In addition, accumulations of maltose and glycogen were also studied as they are related to trehalose through TreS and TreYZ pathway (fig 4.13). In this section trehalose accumulation in *P. shermanii* NCIM 5137 was studied in different sugars including monosaccharide (glucose), disaccharides (maltose and sucrose), polysaccharide (starch) and other gluconeogenic carbon source like glycerol. Glycerol was chosen as suitable carbon source as there may be a possibility to use alternative cheap source of glycerol like biodiesel waste (source of crude glycerol). The intracellular glycogen and maltose accumulations were also determined along with trehalose in glucose, sucrose, glycerol and starch media. To understand the interrelationship between intracellular accumulations of these three carbohydrates (trehalose, glycogen and maltose) in four carbon sources a multivariate approach of cluster analysis was used which indicated more collinearity (90%) in glycogen and trehalose formations as compared to maltose accumulation (45%). Higher accumulation of trehalose from glycerol and close relationship between glycogen and trehalose formations in *Propionibacterium shermanii* is yet to be reported in literature. The determination of trehalose, maltose and glycogen was targeted since these two carbohydrates were linked to trehalose through two pathways of trehalose biosynthesis (TreS and TreYZ) as shown in fig 4.13. Although, TreYZ is not reported in the genome sequence of *P. freudenreichii* (Falentin et al., 2010). Overall objective of the present section was to describe the process of selecting suitable carbon source for trehalose production using *P. shermanii* NCIM 5137. Beside an effort was also made to find the correlation between trehalose, maltose and glycogen accumulation in four carbon source. In case of trehalose production with maltose, this interrelationship between trehalose, glycogen and maltose was not evaluated as maltose itself is the substrate for growth.

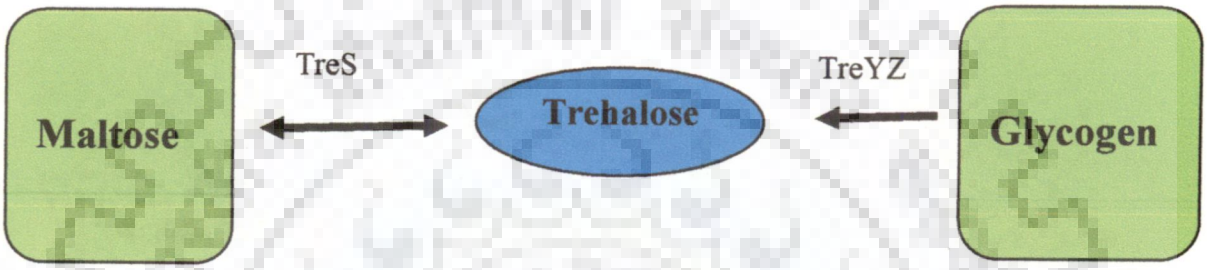


Fig 4.13 Trehalose synthesis is linked to maltose and glycogen through TreS and TreYZ

4.3.1. Trehalose accumulation in monosaccharide (glucose) and disaccharide (sucrose and maltose) sugars in static flask culture.

The study on influence of carbon sources on trehalose accumulation was performed with monosaccharide (glucose), disaccharide (maltose and sucrose) sugars. In carbon source glucose, the highest trehalose accumulation of 93.81 mg/g of biomass (fig. 4.14) was observed while maximum specific growth rate (μ) was found to be 0.105 h^{-1}. On the other hand maximum accumulation of trehalose was 170.4 mg/g of biomass when sucrose was used and it was 128.54 mg/g of biomass when maltose was used as carbon source (Fig. 4.15 & 4.16). Interestingly, trehalose accumulations were different in monosaccharide and disaccharide sugars and final trehalose accumulation in stationary phase was higher in both disaccharide sugars (85 & 55 mg/g of biomass in sucrose & maltose) as compared to glucose (15 mg/g of biomass). While trehalose yield in sucrose and maltose was higher in comparison to lactose (final yield 18 mg/g of biomass).

4.3.2. Trehalose accumulation in polysaccharide carbon sources (starch) in static flask culture

Maximum intracellular accumulation of trehalose in starch was 185 mg/g of biomass (Fig 4.17) which was approximately similar to that produced in disaccharides (sucrose, 170.47 g/g biomass). Final trehalose yield was 46 mg/g of biomass in stationary phase which was lower than with sucrose (85 mg/g of biomass), maltose (55 mg/g of biomass) but higher than glucose (15 mg/g of biomass). The bacteria utilised starch as amylase activity was observed in the medium. The previous reports on trehalose accumulation with different microorganisms in different starch including cassava starch and corn starch reported 220 mg/g of biomass trehalose (Chi et al., 2009). Thus trehalose production in *P. shermanii* NCIM 5137 from carbon source starch was not encouraging in comparison to previous reported strains.

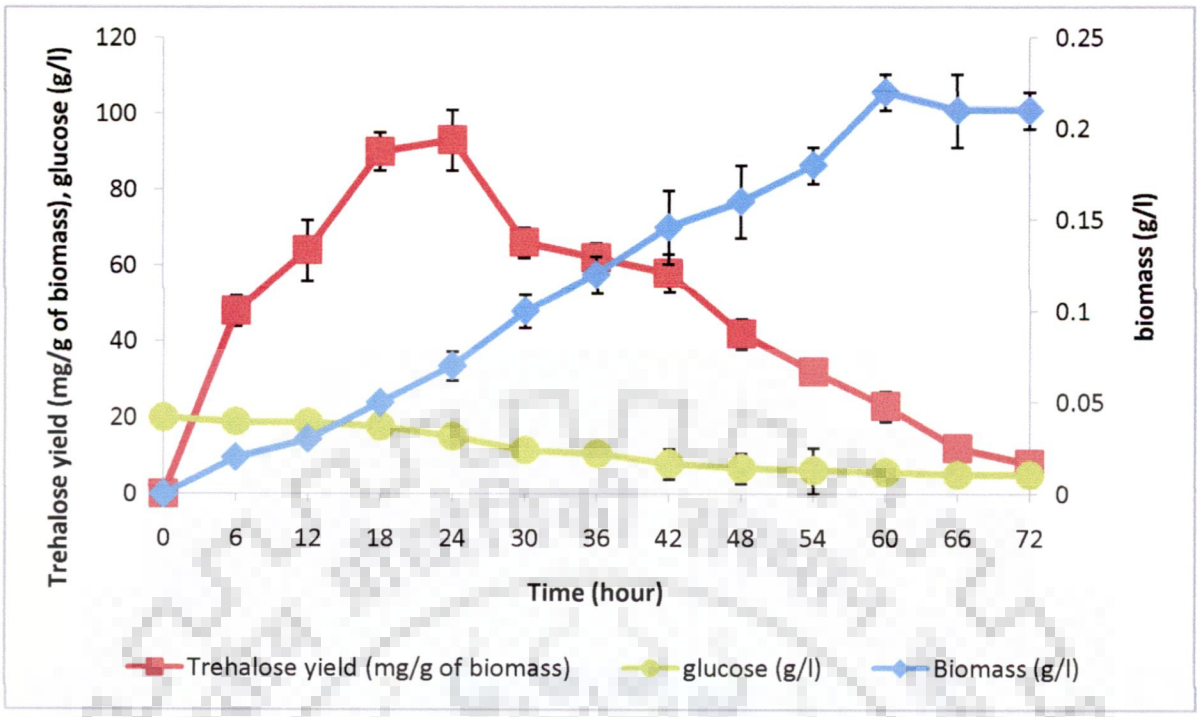


Fig 4.14 Trehalose yield, residual substrate concentration and biomass growth profiles with carbon source glucose in static flask condition.

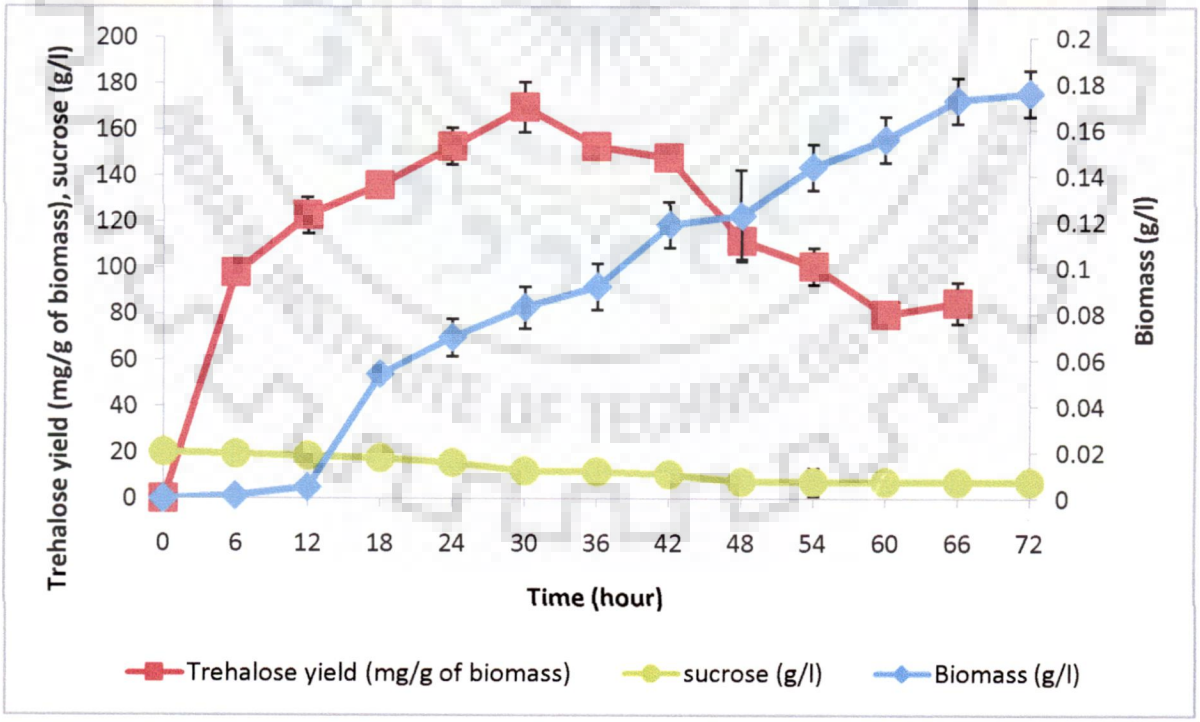


Fig 4.15 Trehalose yield, residual substrate concentration and biomass growth profiles with carbon source sucrose in static flask condition.

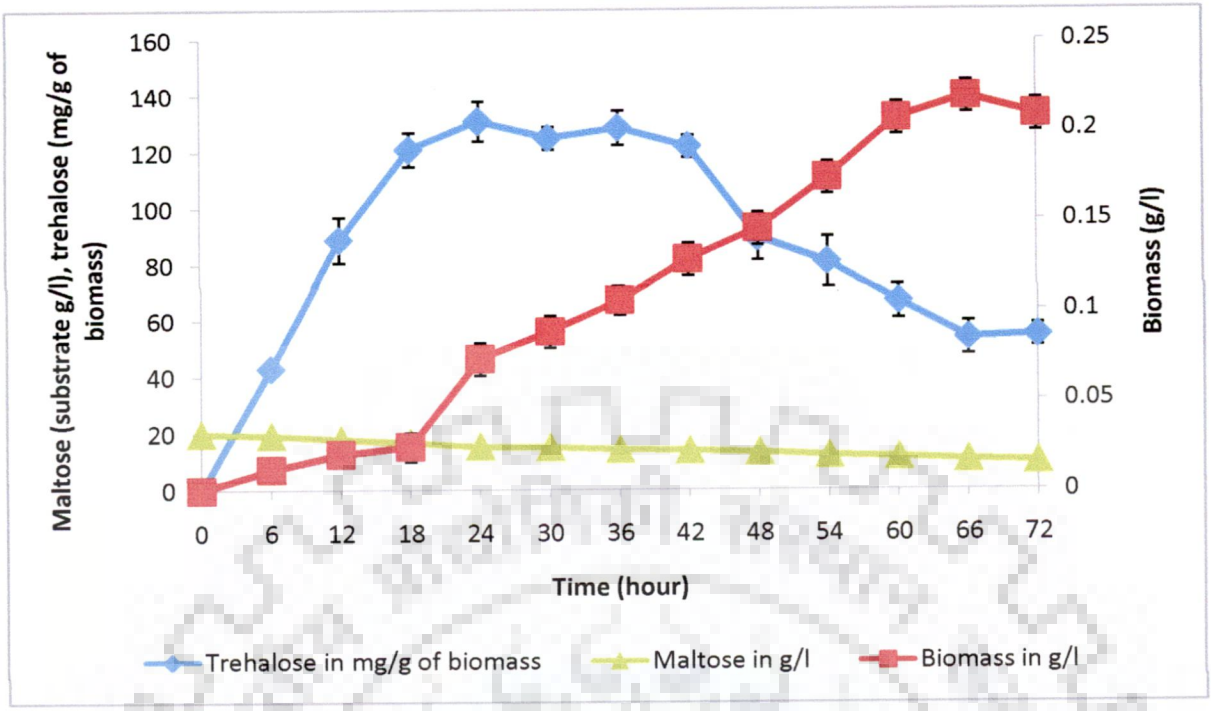


Fig 4.16 Trehalose yield, residual substrate concentration and biomass growth profiles with carbon source maltose in static flask condition

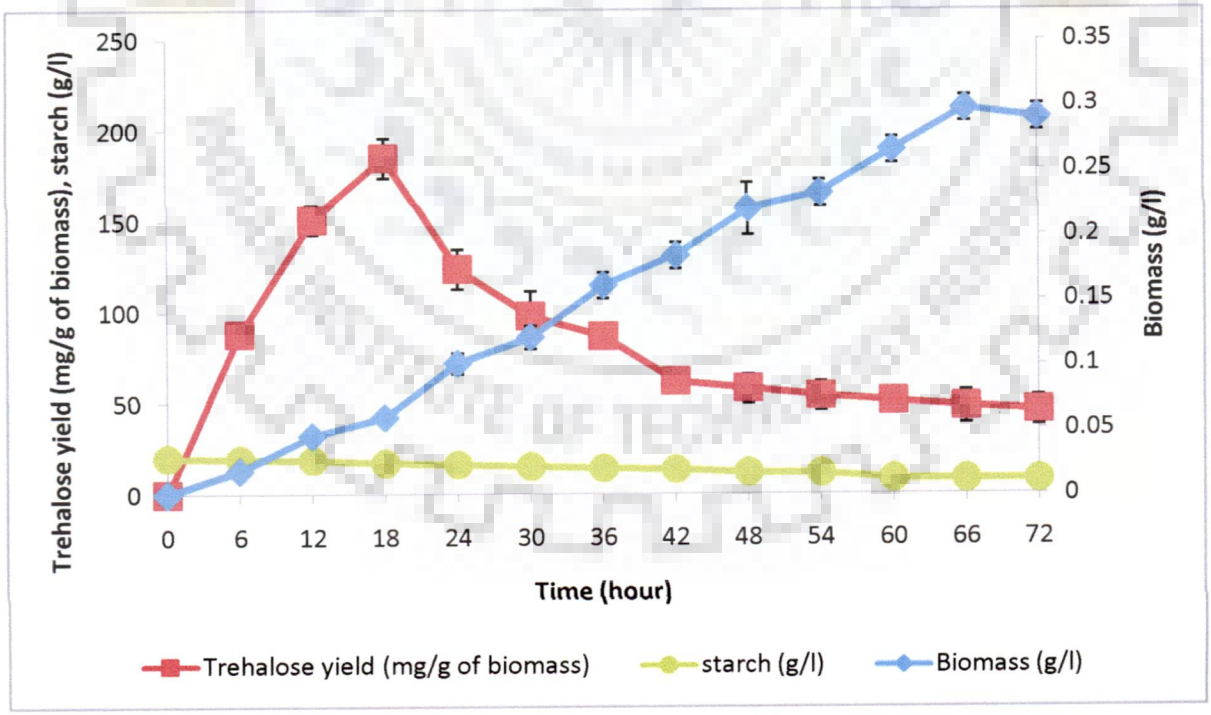


Fig 4.17 Trehalose yield, residual substrate concentration and biomass growth profiles with carbon source starch in static flask condition

4.3.3. Trehalose accumulation in gluconeogenic carbon source glycerol

Further the influence of other carbon source like glycerol on trehalose accumulation was also studied. Accumulation of trehalose in lactic acid media was not very encouraging, as growth rate was significantly slow and stationary phase was achieved after 300 hour (discussed in section 4.2). In case of glycerol media, stationary phase was achieved within 72 hours. While in glycerol, highest trehalose yield was 385 mg/g of biomass (Fig 4.18) and final trehalose yield of 89 mg/g of biomass was obtained. Therefore, comparison of trehalose yield in above cheap carbon sources illustrated that carbon source have effect on trehalose accumulation and it was found to be highest with glycerol in mid exponential phase. Trehalose accumulation or production from glycerol is not reported with any other microbes while reports of trehalose productions from sucrose, maltose and starch are already available in literature (Chi et al., 2000). But the suitability of any substrate is judged from the availability of its cheap source, like some wastes. Likewise, the biodiesel waste is a source of crude glycerol which can be used as a source of carbon for trehalose production. However, it is desirable to use biodiesel waste without any costly pre-treatment process.

4.3.4. Intracellular accumulation of trehalose, maltose and glycogen indifferent carbon sources (glucose, sucrose, starch and glycerol)

Among all the carbon sources, sucrose and glycerol were found to be most suitable due to high trehalose production (85 and 89 mg/g of biomass respectively) in comparison to glucose (15 mg/g of biomass) and can be utilized as low-cost carbon sources. Further the physiology of microorganism was evaluated with respect to biosynthesis of other related storage carbohydrate including glycogen and maltose. Glycogen is reported to accumulate in many microbes like *Corynebacterium* (Seibold et al., 2007) and *Mycobacterium* (Pan et al., 2008).

In the present study, along with trehalose, glycogen and maltose were analyzed simultaneously in *P. shermanii* grown in four carbon sources- glucose, sucrose, starch and glycerol as shown in Fig. 4.19-4.22. Maltose accumulation was highest in starch (250 mg (g of biomass)$^{-1}$) (Fig 4.19) followed by glucose 58 mg/g of biomass (Fig 4.20) while it was minimal in sucrose (18 mg/g of biomass) (4.21) and glycerol (5 mg/g of biomass) (fig 4.22), which indicates that in carbon source sucrose and glycerol the accumulation of maltose was not favoured in *P. shermanii.* Maximum glycogen accumulation was 34.43 mg/g of biomass in starch (Fig 4.19), 28.5 mg/g of biomass in substrate glucose (fig 4.20),

and 28.71 mg/g biomass in sucrose (fig 4. 21). In glycerol, highest glycogen accumulation was up to 139 mg/g biomass (fig 4.22). Like trehalose, glycogen and maltose contents of growing cell decreased in late exponential phase.

In order to understand the interrelationship between maltose, glycogen and trehalose accumulations a multivariate cluster analysis was performed on the basis of co linearity. The dendrogram of cluster analysis in different carbon sources (glucose, sucrose, soluble starch and glycerol) showed close similarity of 90% between trehalose and glycogen accumulation while maltose was similar to cluster of both of these sugars with about 45% similarity (Fig. 4.23) which gives inference that trehalose and glycogen accumulations are more closely related phenomenon in comparison to maltose formation.

Cluster analysis data showed close association between glycogen and trehalose accumulation and this was also supported in recent literature where they found relationship between trehalose and glycogen metabolism in *Mycobacterium* (Chander et al 2011). In *Corynebacterium* no glycogen was detected in gluconeogenic substrates like acetate and lactate (Seibold et al 2007) but here higher glycogen accumulation in substrate glycerol was observed. Although, in yeast, trehalose and glycogen accumulate not only upon carbon starvation but also under other stress conditions such as nitrogen or sulfur starvation, heat shock, or osmotic stress (Hottiger et al., 1987, 1989; Eleutherio et al., 1993; De Vergilio et al., 1994; Parrou et al., 1997; Mahmud et al., 2009). In *Corynebacterium* during hyper-osmotic shock there is a rapid degradation of intracellular glycogen and accumulation of trehalose through TreYZ pathway (Seibold and Eikmann 2007). TreYZ pathway is known in bacteria like *Mycobacterium* and *Corynebacterium* (Padilla et al 2004) but it was not found in *P. freudenreichii* (Cardoso et al 2007; Falentin et al., 2010). Both glycogen and trehalose require nucleotide sugar for their synthesis, hence probably some common enzyme providing this nucleotide sugar may have a role in developing a close relationship between glycogen and trehalose synthesis. Another important sugar obtained in our results was maltose. Maltose was formed by inter-conversion of trehalose by enzyme trehalose synthase (TreS) and was reported as degradation pathway of trehalose in *P. freudenreichii* (Cardoso et al 2007). Maltose accumulation was only 45% similar to trehalose and glycogen accumulations.

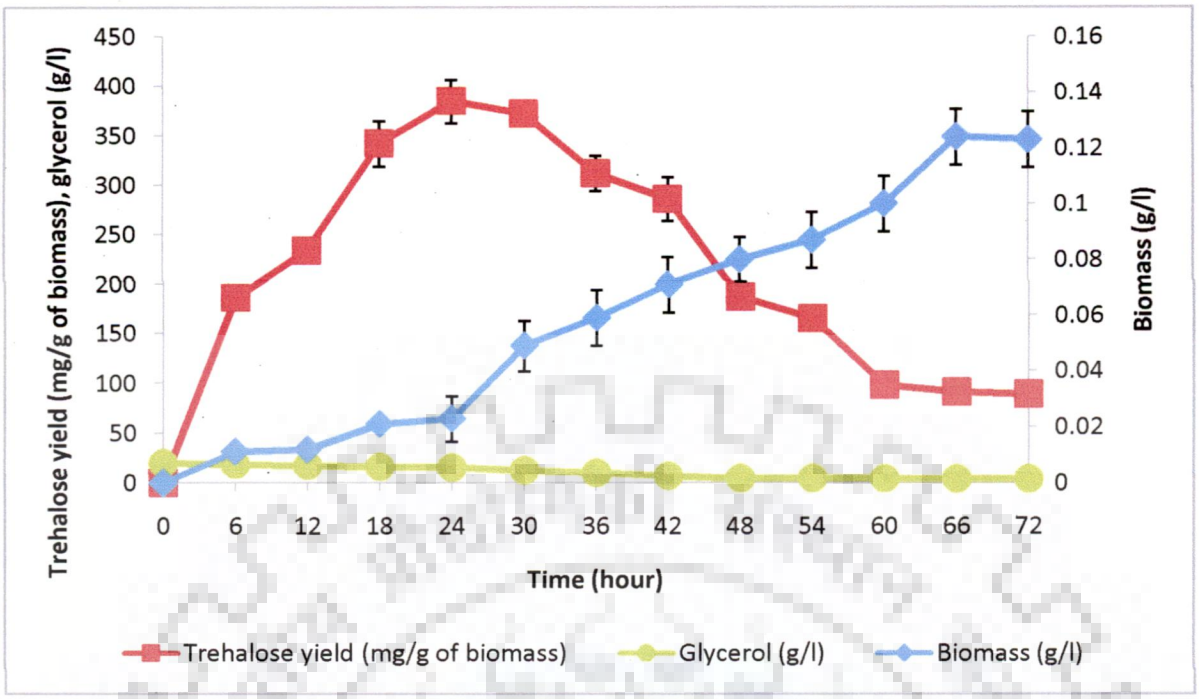


Fig 4.18 Trehalose yield, residual substrate concentration and biomass growth profiles with carbon source glycerol in static flask condition

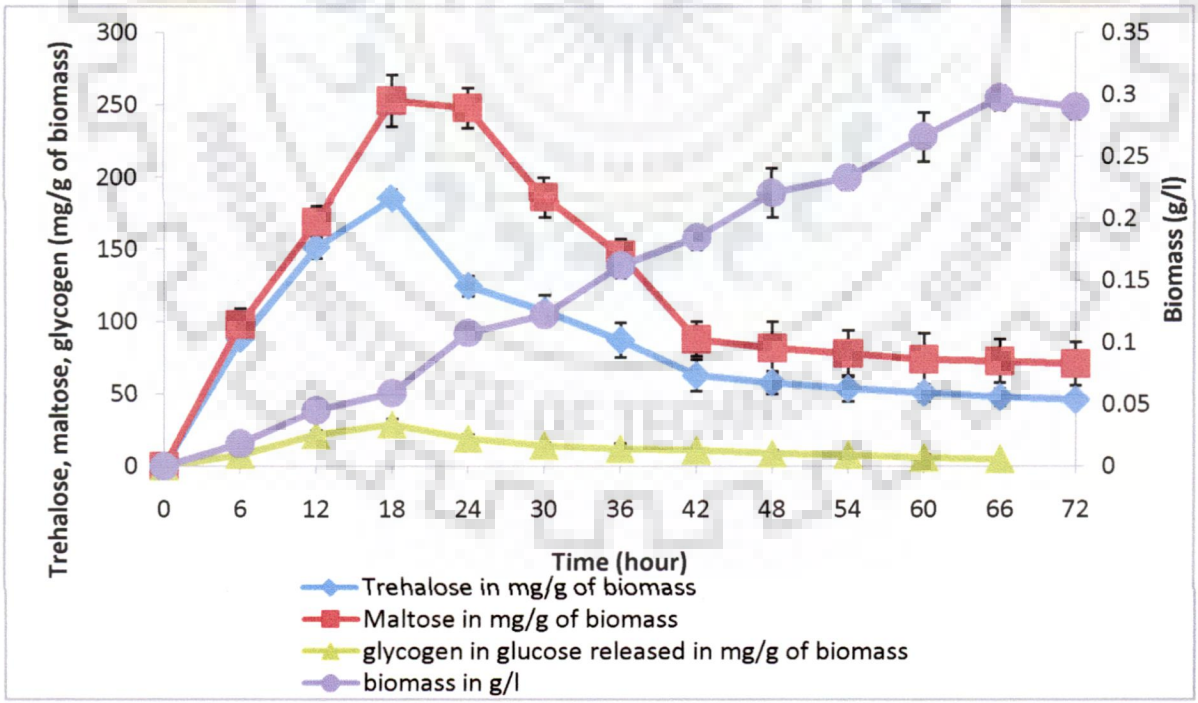


Fig 4.19 Trehalose, maltose and glycogen accumulations profiles with substrate soluble starch in static flask condition

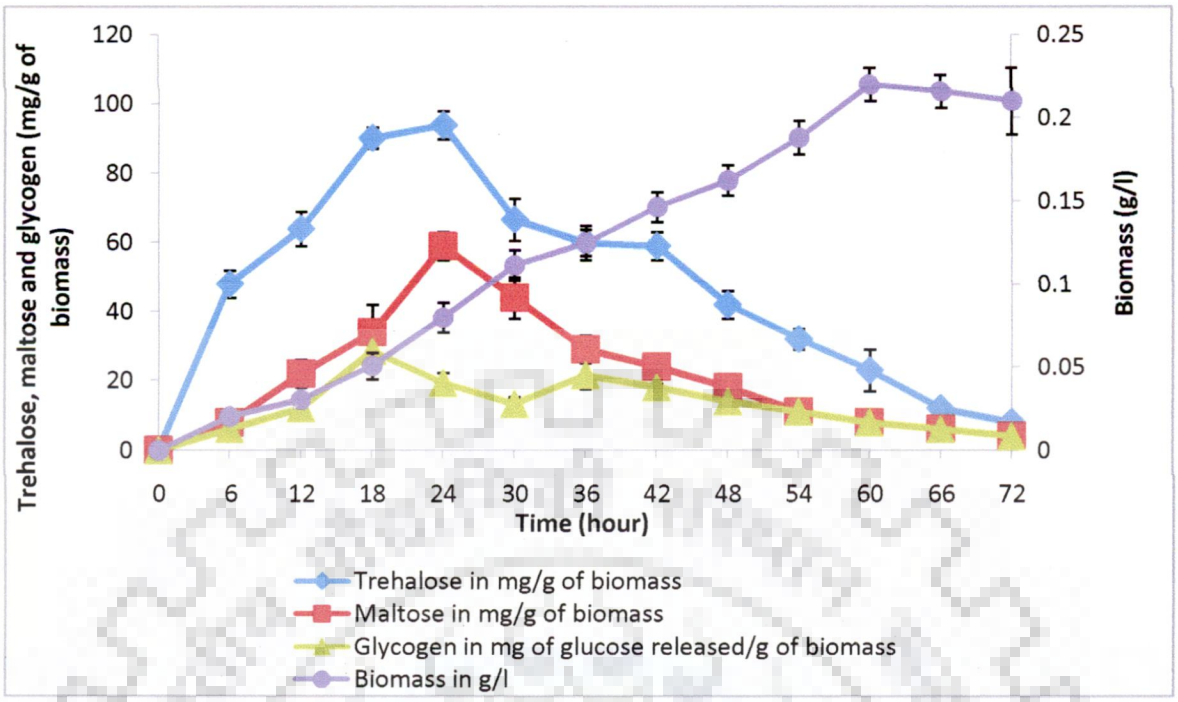


Fig 4.20 Intracellular trehalose, maltose and glycogen accumulations with carbon source glucose in static flask condition

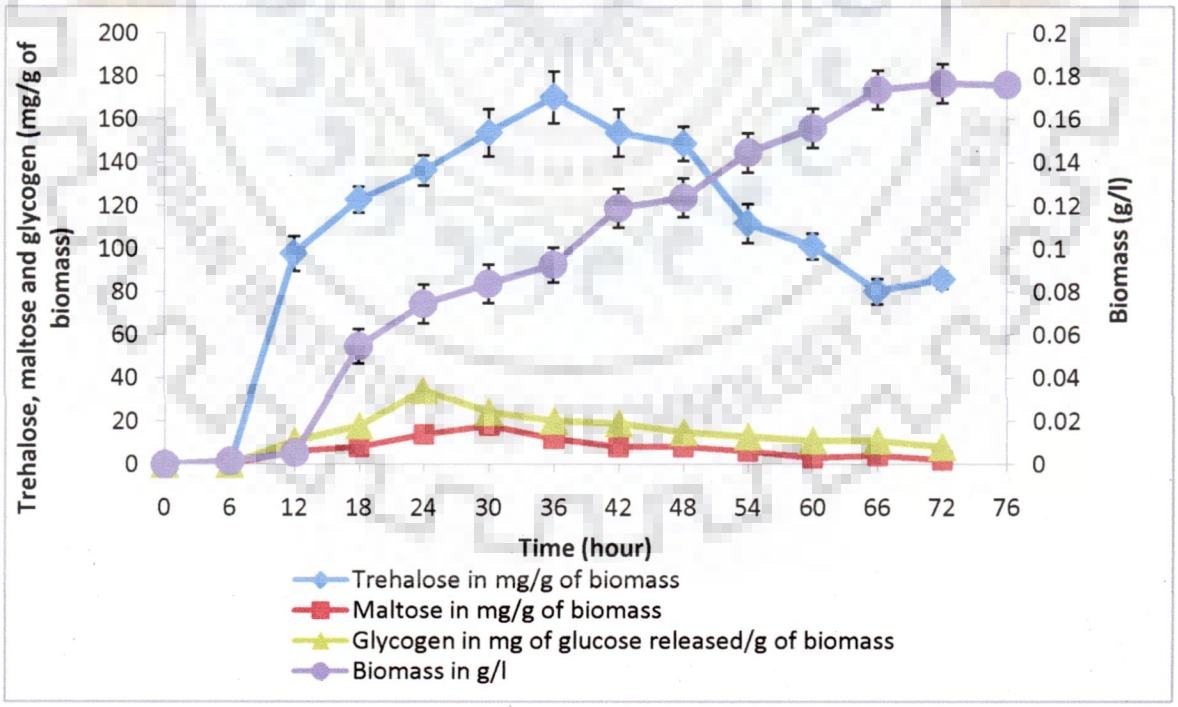


Fig 4.21 Intracellular trehalose, maltose and glycogen accumulation with carbon source sucrose in static flask condition

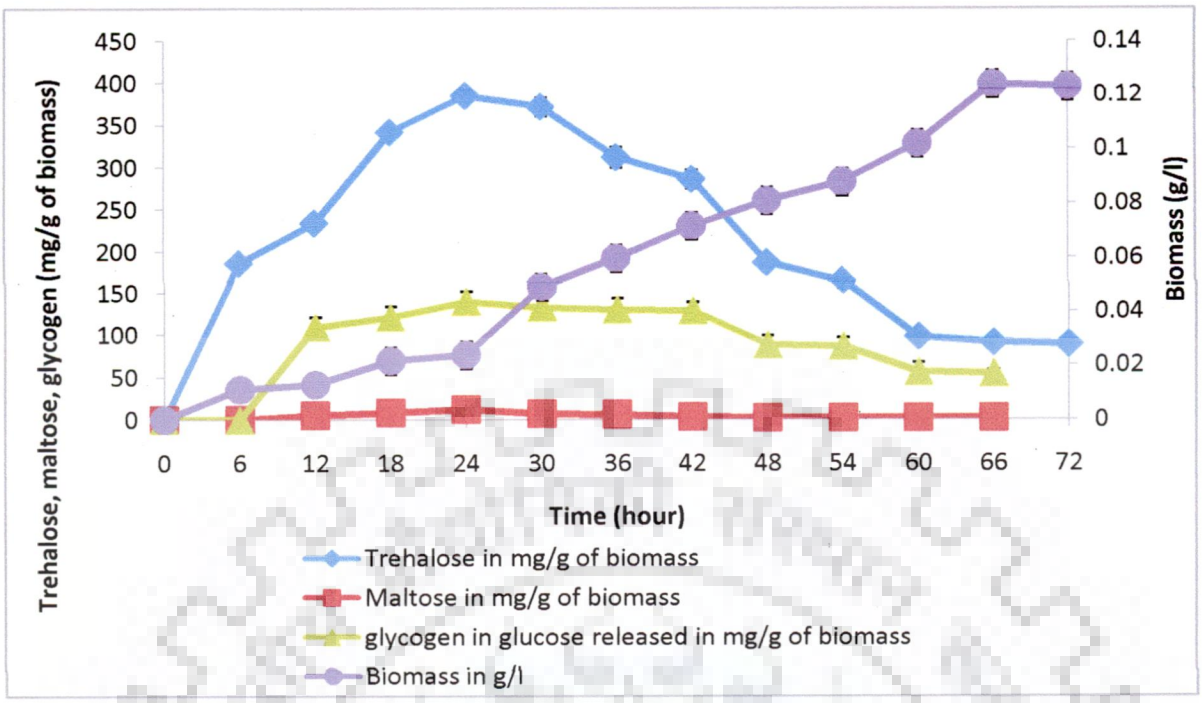


Fig 4.22 Intracellular trehalose, maltose and glycogen accumulation with carbon source glycerol in static flask condition

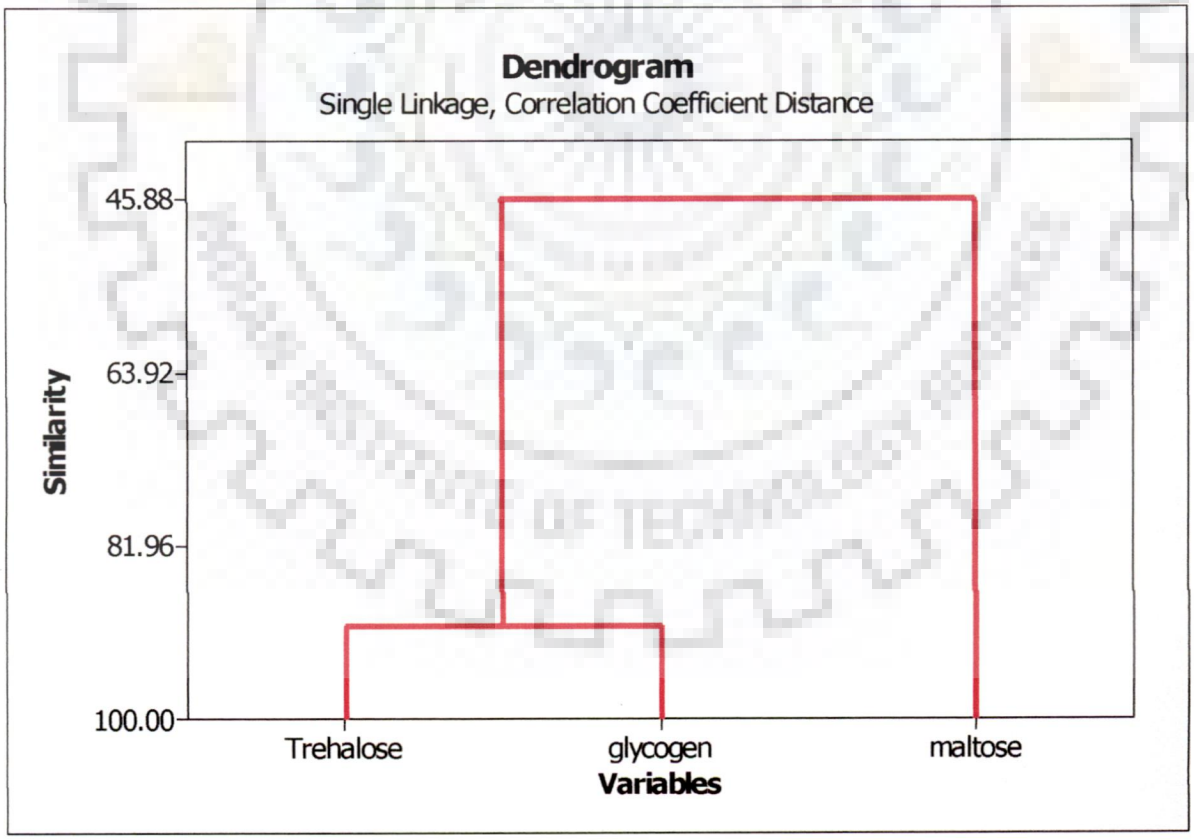


Fig 4.23 Cluster analysis of three storage carbohydrates in *P. shermanii* NCIM 5137 in substrates glucose, sucrose, starch and glycerol. Trehalose and glycogen accumulation was more similar phenomenon

In the present study accumulation of trehalose in different carbon sources in *P. shermanii* was investigated. Trehalose accumulation was enhanced with disaccharides (maltose, 128.54 mg/g of biomass; sucrose, 170.47 mg/g of biomass) in comparison to monosaccharide (glucose 93.81 mg/g of biomass) while it was maximum in gluconeogenic substrate glycerol (385 mg/g of biomass). In all carbon sources it was a general observation that trehalose accumulation was higher in early exponential phase but it decreased with growth which can be due to higher degradation or lower biosynthesis rate of trehalose. Another important aspect was that trehalose degradation with growth was low in carbon source maltose, sucrose and glycerol as compared to glucose. Trehalose production from various carbon sources like sucrose, maltose and starch is known in many microbes but accumulation of higher trehalose from glycerol is not reported in any microbe. Recently it was reported that antifungal property of *Propionibacterium* was enhanced by using glycerol as carbon source probably due to enhanced production of propionic acid (Lind et al., 2010). Further, cluster analysis data showed close association between glycogen and trehalose formations. Thus on comparison of trehalose yields in different carbon sources, glycerol and sucrose were found most suitable carbon source (table 4.4). However, maximum trehalose yield achieved was higher (2 fold) in glycerol as compared to sucrose medium. In case of sucrose decrease in trehalose accumulation with growth was less as compared to glycerol medium. It is already reported that sucrose is an inhibitor of trehalase enzyme which is responsible for catabolism of trehalose (Fleischmacher et al., 1980 Wisser et al., 2000). Thus it can be proposed that due to inhibition of trehalose (in presence of sucrose), degradation rate of trehalose in late exponential phase was less in sucrose medium as compared to glycerol media. Therefore, in the present study, glycerol was chosen for further study as it supports two fold higher trehalose yield in the early exponential phase. Thus effort should be made to arrest the decrease in trehalose yield with growth in glycerol media. However, it should also be mentioned that trehalose yield with respect to substrate consumed was higher with sucrose (1.66 fold) media as compared to glucose media (table 4.4). Thus, it was planned that by arresting the decrease in trehalose content with growth, yield can be improved in glycerol media.

Table 4.4 Trehalose yield in different carbon source (glucose, sucrose, starch and glycerol), Ytx (trehalose yield in mg/g of biomass), Yts (mg/g of substrate), "Max" represent maximum trehalose yield during its growth phase and "fin" represent final trehalose yield in early stationary phase

Carbon source	**Y_{tx} (max)**	**Yts (max)**	**Y_{tx} (fin)**	**Yts (fin)**
Glucose	93±2	2.37±0.4	12±1	0.154±0.03
Sucrose	171±6	5.1±0.8	85±3	1.2±0.1
Starch	185±8	3.89±0.3	46±2	1.13±0.2
Glycerol	385±11	2.8±0.4	89±4	0.72±0.1

4.4 Suitability of crude glycerol obtained from bio diesel waste for trehalose and propionic acid production

Since glycerol was chosen as suitable carbon source for higher trehalose accumulation in *P. shermanii* NCIM 5137 (Table 4.4), hence we investigated the possibility of using an alternative economic source of glycerol, in the form of biodiesel waste. Various microbial conversions of this crude glycerol into useful bio-products are already known (Pyle et al., 2008). Since propionic acid is produced from petroleum based substrates hence prospects for simultaneous productions of propionic acid was also evaluated. Crude glycerol is a by product of biodiesel manufacturing industries. This biodiesel waste also includes various impurities like methanol, soaps and various salts. Many thermo-chemical and biological methods are available for utilisation of crude glycerol for conversion into various useful products. In the present study, we had studied the suitability of this waste especially crude glycerol for microbial conversion (biological) into useful products like a non reducing sugar trehalose and organic acid like propionic acid. Hence, a study was conducted on trehalose and propionic acid productions from crude glycerol obtained from bio-diesel waste by a food microbe *Propionibacterium shermanii*. It was observed that crude glycerol obtained from biodiesel waste favoured higher trehalose and propionic acid productions as compared to pure glycerol. In crude glycerol media, trehalose yield based on substrate consumed was six times higher than with pure glycerol media. So effects of various fatty acids and salts which are generally present in crude glycerol obtained from bio-diesel waste on trehalose yield were studied. Amongst the impurities, fatty acids seems to have no beneficial effects on trehalose yield based on substrate consumed while the presence of KCl in the crude glycerol was probably responsible for achieving higher trehalose yield based on substrate consumed. Amongst the three concentrations (20 g/l, 10 g/l and 5 g/l) of crude glycerol studied for trehalose and propionic acid productions, 10 g/l was found to be optimum concentration for trehalose (final yield 131 mg/g of biomass, 43 mg/g of substrate) and propionic acid productions (0.63 g/g of substrate). The production of trehalose from crude glycerol is first time reported here and suitability of bio-diesel waste for production of trehalose along with propionic acid was clearly demonstrated (Ruhal et al., 2011).

4.4.1. Production of trehalose with 20 g/l of crude and pure glycerol

4.4.1.1. Static flask studies

Realizing the importance of trehalose as a nutraceutical, it was envisaged to explore glycerol as a carbon source for trehalose production using *P. freudenreichii subsp. shermanii*. Further, suitability of trehalose and propionic acid productions were assessed from an alternative cheap source of glycerol (biodiesel waste). Therefore, *P. freudenreichii subsp. shermanii* NCIM 5137 was cultured in a complex media with 20 g/l of pure glycerol as sole carbon source in static flask conditions to assess trehalose production. Similarly, crude glycerol was also used as sole carbon source in complex media under similar experimental conditions for culturing *P. shermanii* NCIM 5137The maximum and final trehalose yields with respect to biomass were 381 and 88 mg/g of biomass respectively. Unfortunately, the maximum and final trehalose yields with respect to substrate consumed were significantly lower (2.8 and 0.87 mg/g of substrate). Interestingly, with crude glycerol, the maximum trehalose yield achieved with respect to biomass was comparable to that obtained with pure glycerol but the final trehalose yield was enhanced by 40% (126 mg/g of biomass) (table 4.5, fig 4.24). In addition, there was a significant improvement in trehalose yield with respect to substrate consumed with crude glycerol media as compared to a media with pure glycerol. Maximum trehalose yield achieved with respect to substrate consumed was approximately forty times higher with crude glycerol (104 mg/g of substrate) to that obtained with pure glycerol (2.8 mg/g of substrate). Final trehalose yield with respect to substrate consumed were 15 mg/g of substrate and 0.87 mg/g of substrate in crude glycerol and pure glycerol media respectively. These results suggest that crude glycerol was a superior carbon source for the production of trehalose as compared to pure glycerol media.

4.4.1.2. Batch reactor studies

During static flask studies, complete substrate conversions were not achieved, which may be due to the decrease of pH of the broth during fermentation. Therefore, batch reactor studies were carried out with controlled conditions of dissolved oxygen, pH and temperature. In batch reactor, maximum trehalose yields obtained with respect to biomass were approximately similar with pure and crude glycerol media but final trehalose yield achieved was higher with crude glycerol (128 mg/g of biomass) as compared pure glycerol media (50 mg/g of biomass) (fig 4.25, table 4.6). As, the final biomass concentration

obtained with crude glycerol media was lower (2.03 g/l) as compared to that obtained with pure glycerol media (3.4 g/l), a comparison was made with respect to absolute trehalose concentration (mg/l) and it was observed that final trehalose concentration was 50% higher with crude glycerol media than with pure glycerol media. Perhaps, toxicities of impurities in crude glycerol media may be the reasons for obtaining lower biomass. Furthermore, final substrate conversions were 100% and 65% with pure and crude glycerol media respectively (table 4.6, fig 4.26). In case of pure glycerol media, under controlled conditions, the final trehalose yield achieved with respect to biomass (50 mg/g of biomass), decreased as compared to uncontrolled conditions (89 mg/g of biomass). It was already observed in the static flask study that the use of crude glycerol resulted in nearly 40 times improvement of the maximum trehalose yield with respect to substrate consumed as compared to pure glycerol media. In batch reactor study, it was observed that improvement in trehalose yield with respect to substrate consumed with crude glycerol media was not as significant as obtained in static flask study. Maximum trehalose yield with respect to substrate consumed with crude glycerol media was 110 mg/g of substrate whereas the final yield was 21 mg/g of substrate (table 4.6, fig 4.27). In pure glycerol media, maximum trehalose yield obtained with respect to substrate consumed was 17 mg/g and final yield was 8 mg/g of substrate (table 4.6). Comparison of kinetic parameters and productivity rates in pure and crude glycerol media indicated that the maximum specific growth rate, substrate uptake rate and volumetric rate of cell productivity were lower in crude glycerol media (table 4.6). Lower specific growth rate in crude glycerol media was probably due to presence of impurities in crude glycerol media.

Interestingly, propionic acid yield was found to be approximately two times higher with crude glycerol media as compared to pure glycerol media (table 4.6, fig 4.27). Moreover, higher propionic acid yield was accompanied with lower yield of lactic acid, and thus this process is commercially relevant and attractive. Maximum propionic acid yield of 0.5 g/g of substrate was reported in metabolically engineered strain of *Propionibacterium acidipropionici* (Zhang et al 2009) while in the present study, yield of 0.63 g/g of substrate was obtained. Altogether, advantage of using crude glycerol in the present study was higher yields of trehalose and propionic acid.

In summary, under controlled conditions with crude glycerol in batch reactor, propionic acid was major organic acid, as lactic acid yield was less than 0.1 g/g of substrate. However, further improvement in trehalose yield based on substrate consumed is a necessity for successful commercialization. So, an effort was made to understand the

reasons for achieving higher trehalose yield based on substrate consumed with crude glycerol media as compared to pure glycerol media.

Table 4.5 Maximum and final Trehalose yields with respect to biomass (Ytx, mg/g of biomass) and substrate (Yts, mg/g of substrate) in static flask culture conditions with pure and crude glycerol

Yield	**Crude glycerol**	**Pure glycerol**
Y_{tx} maximum	404±12	381±12
Y_{tx} final	126±11	89±3
Y_{ts} maximum	104±8	2.8±0.2
Y_{ts} final	15±2	0.72±0.04

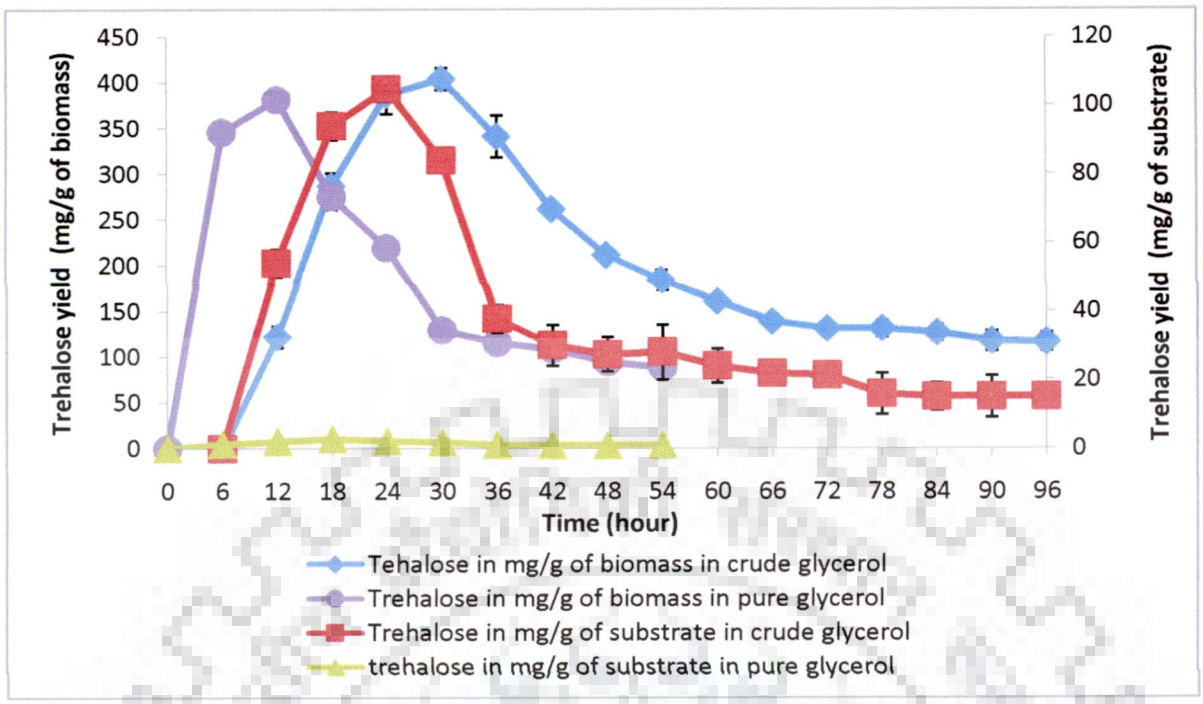


Fig 4.24 Comparison of trehalose yields with respect to biomass and substrate consumed in crude glycerol and pure glycerol in static flask culture conditions

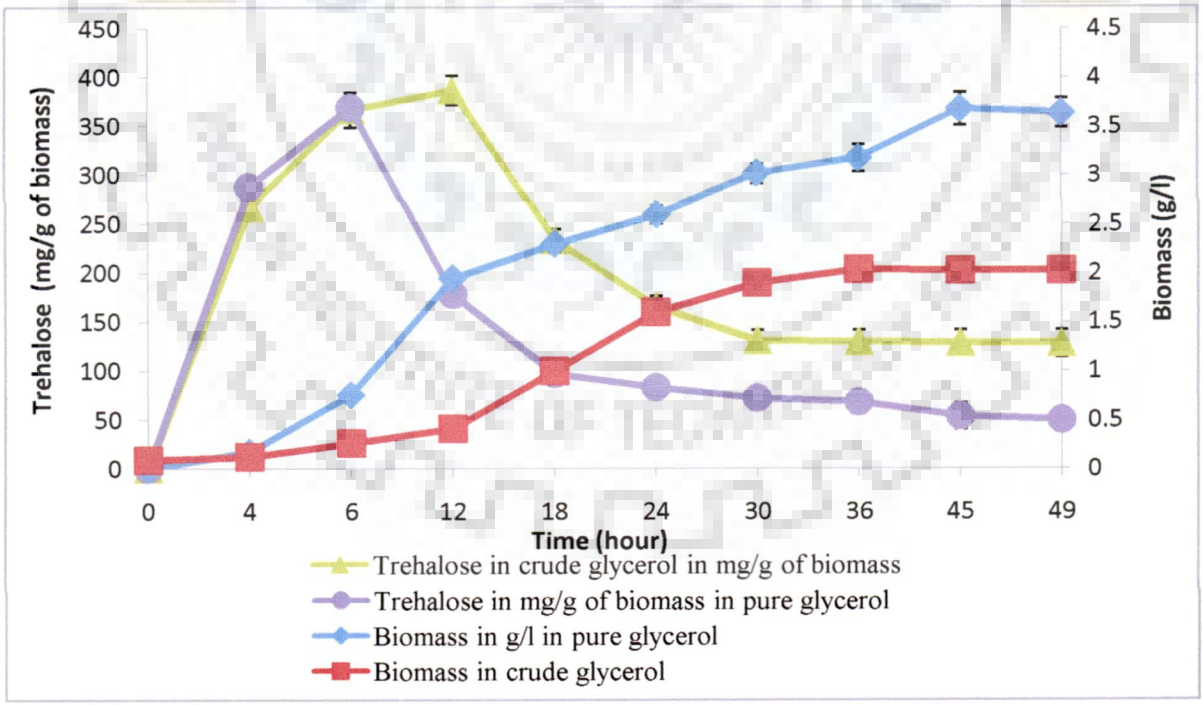


Fig 4.25: Trehalose in mg/g of biomass and biomass growth profile with crude glycerol and pure glycerol in batch fermenter

Table 4.6 Comparison of various parameters and yields with 20 g/l of crude and pure glycerol as substrate in batch reactor

Parameters	**Crude glycerol (20g/l)**	**Pure glycerol (20 g/l)**
Y_{tx} (mg/g of biomass)	128±3	52±4
Y_{ts} (mg/g of substrate)	21±2	8±0.8
Y_{pa} (g/g of substrate)	0.63±0.03	0.31±0.03
Y_{la} (g/g of substrate)	0.07±0.0	0.065±0.0
Trehalose concentration (mg/l)	26±3	17±4
Final biomass (g/l)	2.03±0.5	3.4±0.8
Maximumμ(hr^{-1})	0.69±0	1.4±0
Maximal substrate uptake rate ($gl^{-1}h^{-1}$)	0.2±0	1.2±0
Volumetric rate of cell productivity ($g\ l^{-1}\ hr^{-1}$)	0.07±0	0.16±0
Final glycerol conversion (%)	65±1	100

Where,

*Y*tx and *Y*ts are trehalose yields with respect to biomass and substrate consumed and the method of calculation is as described in material and methods

Y_{pa} and Y_{la} are yields of lactic acid and propionic acid respectively with respect to substrate consumed

Specific growth rate (m) is defined as the rate of increase in cell mass per unit biomass

Substrate uptake rate is the substrate consumed by cells per unit time and the rate of cell productivity is the biomass produced in g/l per unit of time

Glycerol conversion (%) was calculated in percentage by dividing substrate consumed by the initial substrate:

Glycerol conversion (%) = [(20-final substrate)/20]*100

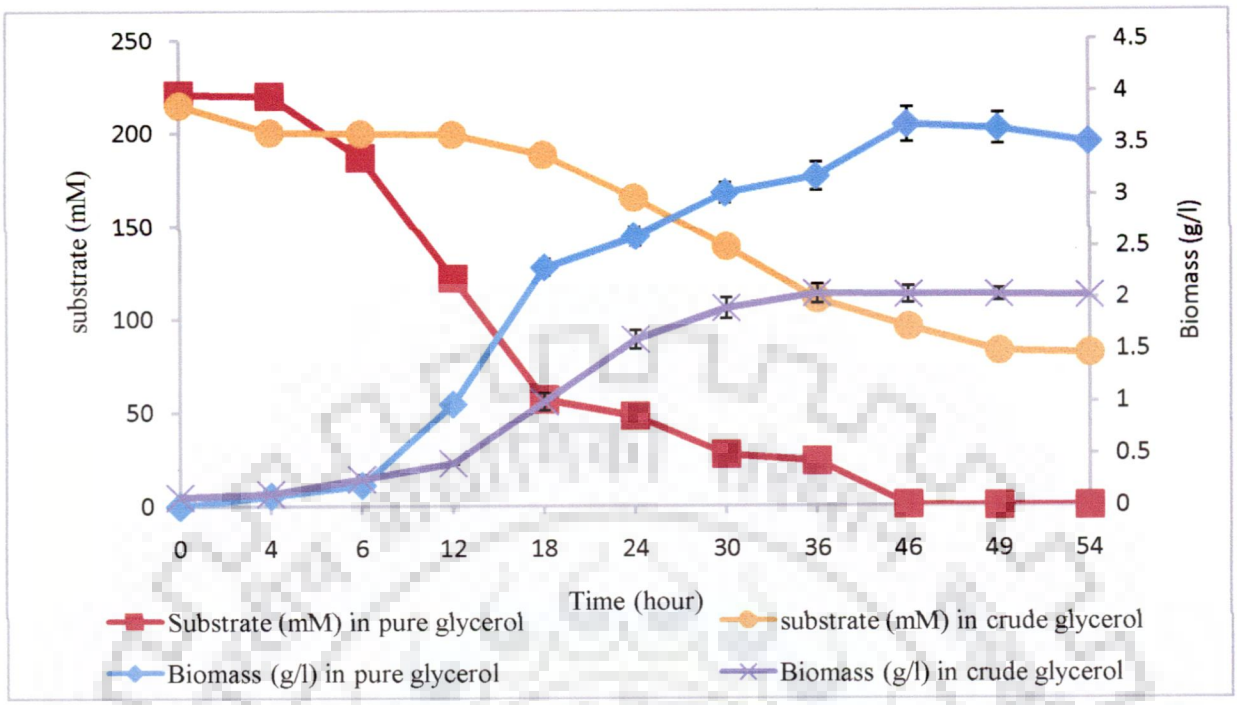


Fig 4.26 Biomass growth and residual substrate concentration profiles with pure and crude glycerol in batch fermenter

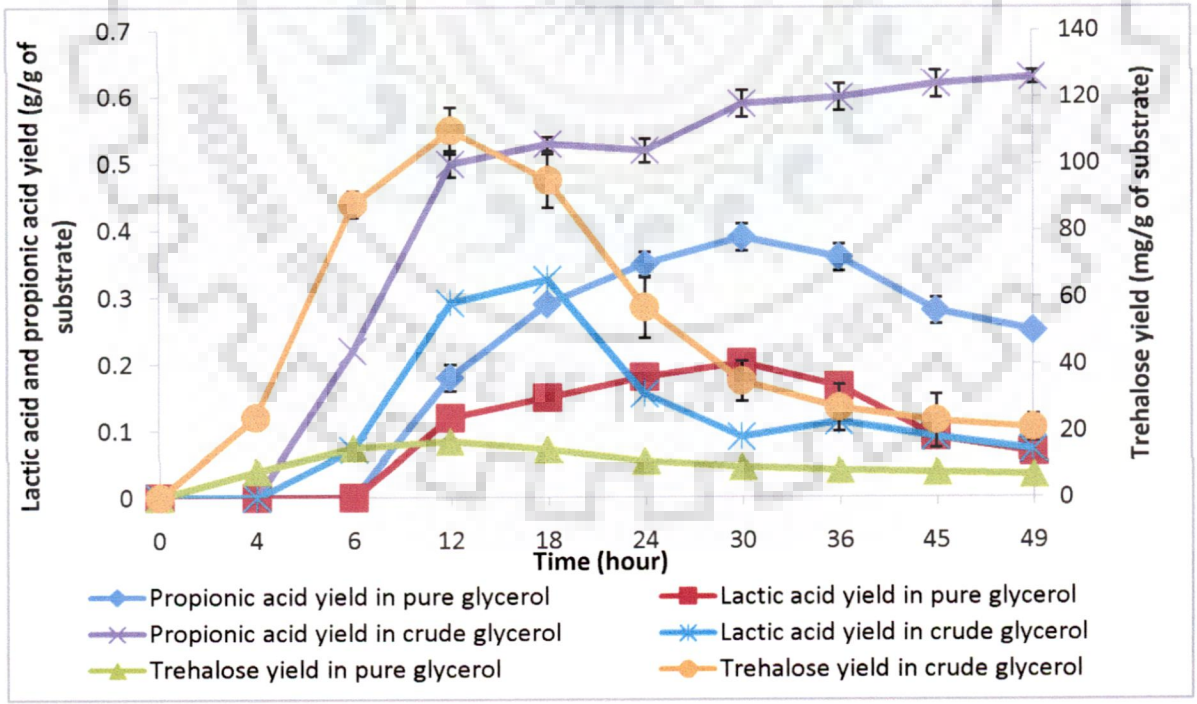


Fig 4.27 Comparison of yields of propionic acid, lactic acid and trehalose in pure and crude glycerol in batch fermenter

4.4.2. Effect of impurities of crude glycerol on trehalose production

Trehalose yield achieved with respect to substrate consumed was higher in crude glycerol media and thus it can be predicted that certain components in crude glycerol, are probably responsible for enhancement of trehalose production. Soaps and methanol are major impurities in crude glycerol (Pyle et al., 2009). Methanol is used during trans-esterification reaction while soaps are produced due to the reaction of fatty acids with alkali (KOH). During pre-treatment, methanol was completely removed by autoclaving (confirmed by GC analysis) hence study concerning the effect of methanol on trehalose yield was not carried out (materials and methods section). Soaps react with HCl to form free fatty acids and KCl during pre-treatment of crude glycerol with HCl. Hence, other major impurities were fatty acids and KCl. To evaluate the apparent synergism between impurities of crude glycerol and trehalose yield, an experiment was envisaged, in which a chemically defined media was used along with selective individual impurity. The effect of particular impurity was evaluated by comparing trehalose yields obtained with and without an impurity in the chemically defined media. In the chemically defined media, pure glycerol was used as a carbon source.

4.4.2.1. Effect of fatty acids on trehalose yields (based on substrate & biomass)

The impact of individual fatty acid was assessed after supplementation of 0.5% of different fatty acids separately. Oleic acid, linoleic acid, palmitic acid and stearic acid were reported to be major fatty acids found in crude glycerol (Pyle et al., 2009). It was observed that addition of tween 80 was necessary to reduce the turbidity of the culture medium containing glycerol; hence the effect of 0.2% tween 80 was also studied separately under similar experimental conditions. Surprisingly, there were no significant effects of different fatty acids and tween 80 on trehalose yield based on substrate consumed while it seems that it marginally reduced the final trehalose yield based on biomass (fig 4.28 & 4.29). Thus it can be predicted that there was no effect of individual fatty acid on trehalose production. It can be further concluded that the presence of individual fatty acid in crude glycerol was not responsible for achieving higher trehalose yield based on substrate consumed. Interestingly, propionic acid yield was higher in fatty acid (0.51, 0.48, 0.46 and 0.46 g/g of substrate in Oleic acid, palmitic acid, stearic acid & tween 80) in comparison to without fatty acid (0.28 g/g of substrate). Lactic acid yield was nearby 0.1 g/g in all fatty acids and without fatty acid.

4.4.2.2. Effect of KCl on trehalose yields (based on biomass & substrate consumed)

Enhancement of trehalose production under osmotic stress in microbes was previously reported (Arguelles, 2000). In the previous section, it was also observed that in the presence of NaCl, trehalose accumulation increased in *P. shermanii* NCIM 5137. During neutralisation of crude glycerol, potassium chloride is formed. Hence, KCl may be the source of osmotic stress for microbe. Therefore, effects of different concentrations of KCl on trehalose yield were determined in a chemical defined media. The effects of 0.5% and 1% KCl on trehalose yield was evaluated by comparing yield obtained with non-stress (without KCl) media. Surprisingly, trehalose yield achieved with respect to substrate consumed was enhanced remarkably in the presence of KCl as compared to a media without KCl. In media supplemented with 1% KCl, maximum trehalose yields achieved with respect to biomass and substrate consumed were 459 mg/g of biomass and 87 mg/g of substrate which were quite similar to the values achieved with crude glycerol (fig 4.30). The final trehalose yield based on substrate consumed was ten times higher with media supplemented with 1% KCl as compared to media without KCl (fig 4.31). Besides it was also observed that final trehalose yield achieved with respect to substrate consumed was also increased with increase in osmotic pressure (KCl concentration) of media. Altogether, it can be concluded that higher osmotic stress in the crude glycerol media, resulted in subsequent enhancement of trehalose yield based on substrate consumed. Microbes accumulate compatible solutes or osmoprotectant during osmotic stress (due to presence of salts like NaCl or KCl) in their environment. One of well known compatible solute accumulated in microbes is trehalose (Argüelles, 2000).

Previously, enhanced trehalose yield under osmotic stress was reported in microbes including *P. freudenreichii*, *E.coli*, *Corynebacterium* and yeast. Over-expression of various enzymes in trehalose biosynthesis metabolic pathway (OtsAB and TreYZ) under osmotic stress was reported which led to enhanced trehalose yield. In other words, crude glycerol was more suitable for trehalose production as compared to pure glycerol due to the presence of KCl. However, obtained trehalose yield based on substrate consumed in the stationary phase with a chemically defined media in presence of 1% KCl was marginally less than the yield obtained with crude glycerol media. Thus it indicates that other unidentified minor impurities of crude glycerol probably may have a role in improving the trehalose yield based on substrate consumption. However, it should also be kept in mind that crude glycerol was used with complex media whereas chemically defined media contains pure components. The roles of components of complex media (yeast extract, peptone and tryptone) on trehalose production cannot be ruled out.

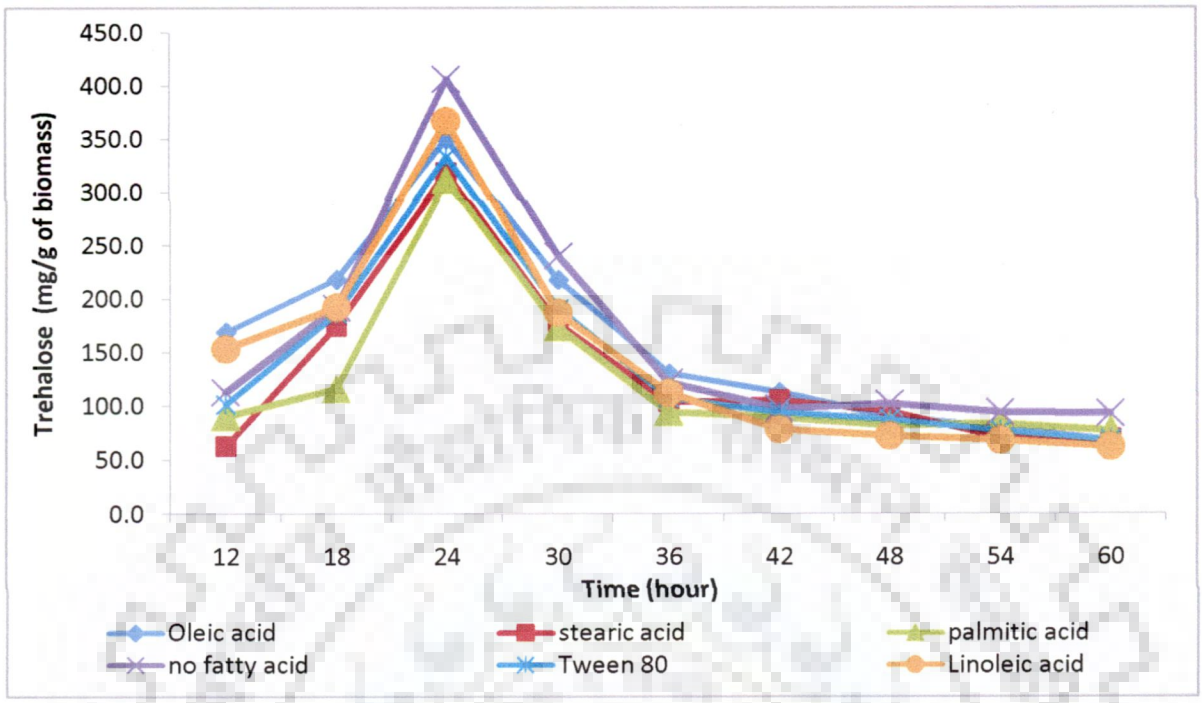


Fig 4.28 Effect of various fatty acids on trehalose yield with respect to biomass in chemically defined media (static flask study)

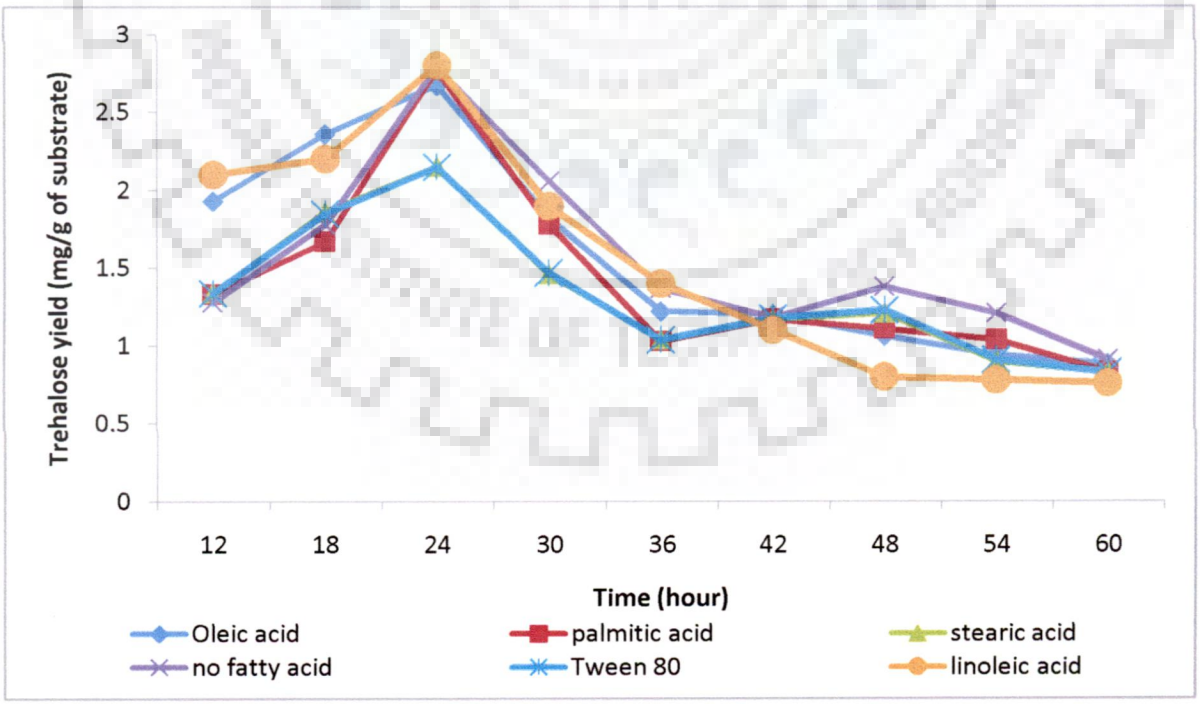


Fig 4.29 Effect of various fatty acids on trehalose yield with respect to substrate in chemically defined media (static flask study)

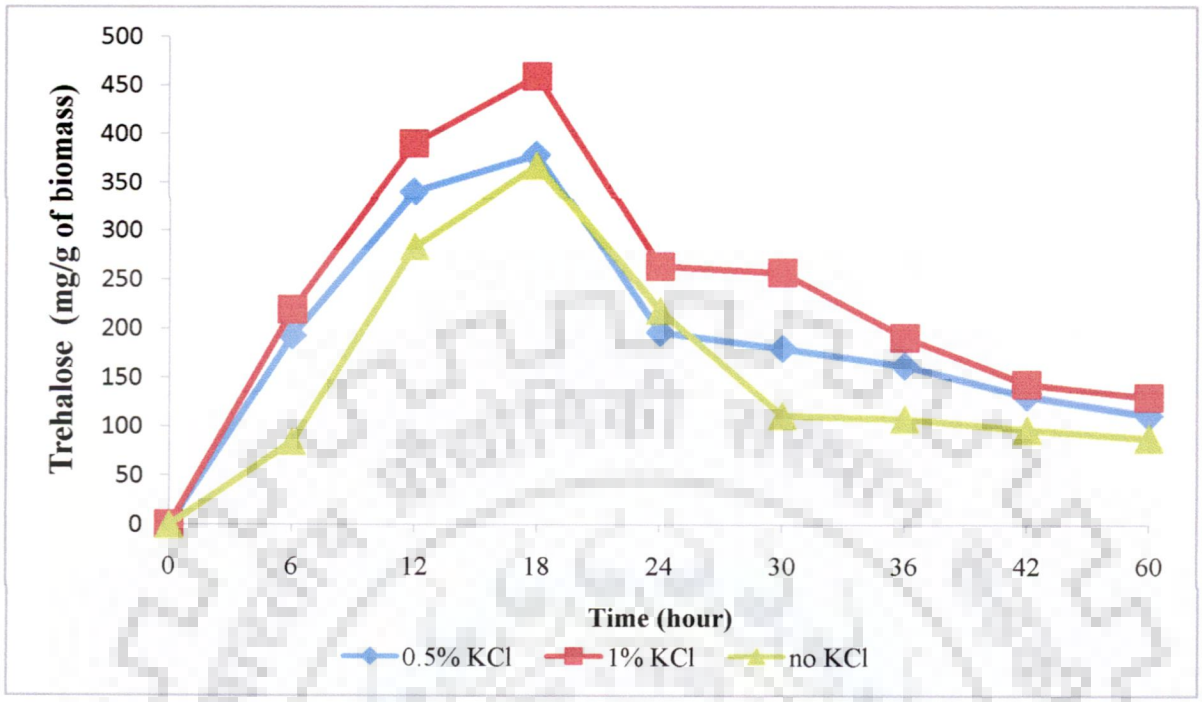


Fig 4.30 Effect of different concentrations of KCl on trehalose yield with respect to biomass in a chemically defined media (static flask study)

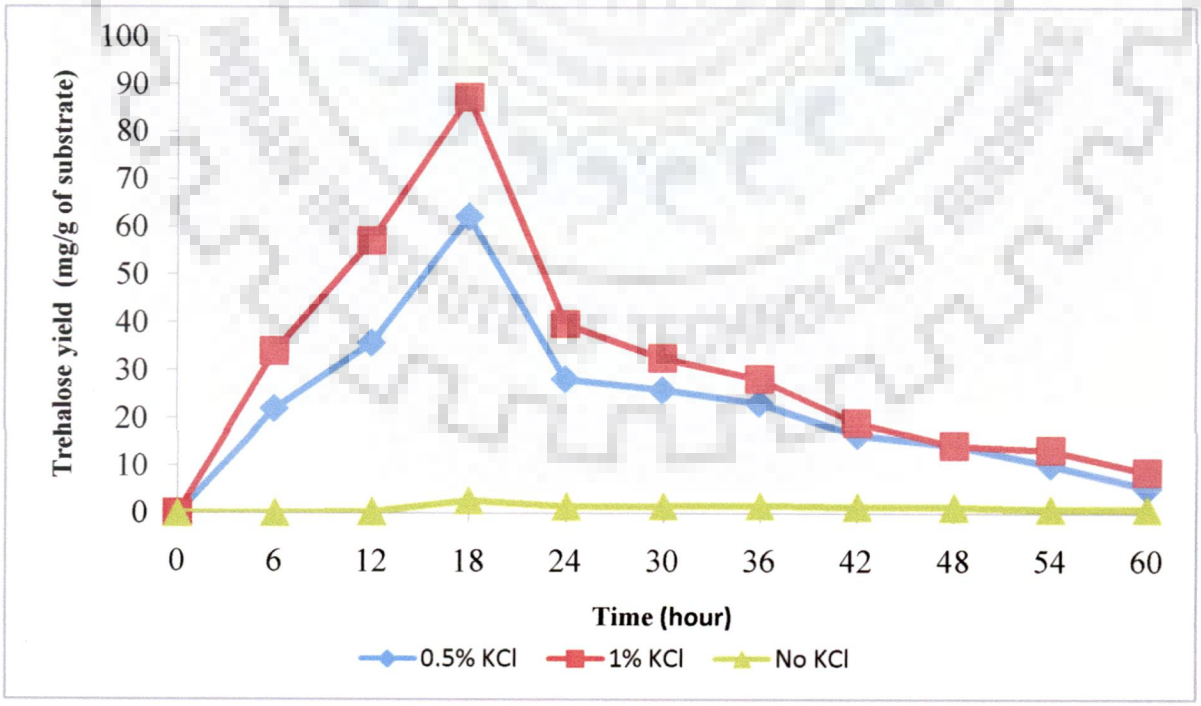


Fig 4.31 Effect of different concentrations of KCl on trehalose yield with respect to substrate consumed in a chemically defined media (static flask study)

4.4.3. Optimisation of crude glycerol concentration for complete substrate conversion

In the present study, complete substrate conversion was not achieved with 20 g/l of crude glycerol media in batch reactor. Therefore, it is not environmentally attractive to use 20 g/l of crude glycerol. Hence an effort was made to find optimum concentration of crude glycerol (10 and 5 g/l) which can ensure complete conversion of glycerol along with higher yields of trehalose and propionic acid. Interestingly, 90-95% substrate conversions were achieved with 10 and 5 g/l of crude glycerol in contrast to 65% conversion with 20 g/l crude glycerol (Fig 4.32). However, the final biomass achieved was approximately 3 g/l with 10 and 5 g/l of crude glycerol media and it was higher as compared to that obtained with 20 g/l of crude glycerol (2 g/l) (fig 4.32). Thus nearly complete substrate conversion and higher cell biomass were achieved with 10 and 5 g/l of crude glycerol media.

The comparison of final trehalose yields in three concentrations of crude glycerol of 20, 10 and 5 g/l are listed in table 4.7. In 10 g/l of crude glycerol, trehalose production with respect to biomass was approximately similar to that with 20 g/l (fig 4.33), but final trehalose concentration (mg/l) obtained with 10 g/l of crude glycerol media was 50% higher than that obtained with 20 g/l of crude glycerol media (table 4.7). Similarly, maximum and final trehalose yields obtained with respect to substrate consumed were 106 and 43 mg/g of substrate respectively with 10 g/l of crude glycerol (table 4.7, fig 4.34). Thus, in 10 g/l of crude glycerol media, the final trehalose yield based on substrate consumed was improved in comparison to 20 g/l of crude glycerol media. Although, trehalose yield with respect to biomass was lower with 5 g/l of crude glycerol but trehalose yield with respect to substrate consumed was similar to that obtained with 10 g/l crude glycerol (table 4.7). It can be concluded from table 4.7 that 10 g/l of crude glycerol is the optimum concentration for trehalose production as it improved the final trehalose concentration. Moreover, it was found that propionic acid yield was 0.63 g/g of substrate with 10 g/l crude glycerol (similar to that in 20 g/l) while with 5 g/l of crude glycerol it was 0.53 g/g of substrate, hence 10 g/l of crude glycerol media was also suitable for higher propionic acid yield (fig 4.34). Thus, 10 g/l of crude glycerol was most suitable for trehalose (0.04 g/g of substrate) and propionic acid (0.63 g/g of substrate) productions (fig 4.35). This study clearly demonstrates that crude glycerol can be efficiently utilized for the simultaneous productions of propionic acid and trehalose.

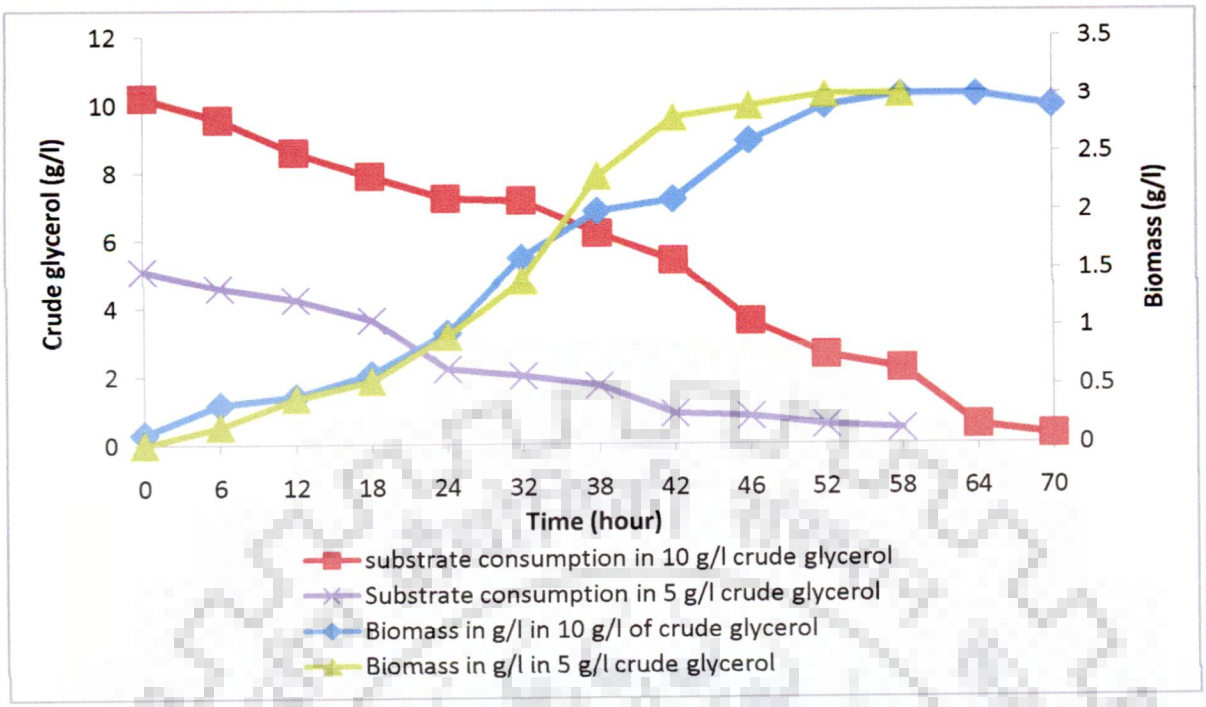


Fig 4.32 Comparison of residual substrate concentration and biomass growth profiles with 10 g/l and 5 g/l of crude glycerol.

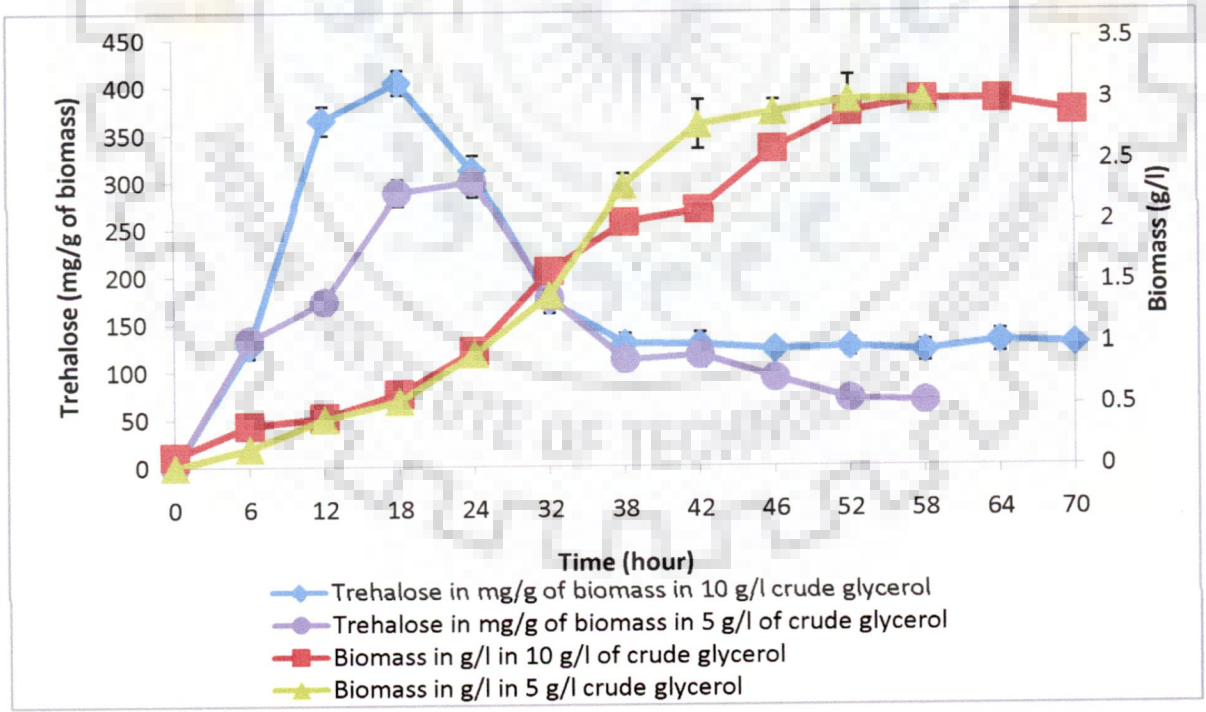


Fig 4.33 Comparison of trehalose yield with respect to biomass and biomass growth profiles with 10 g/l and 5 g/l of crude glycerol.

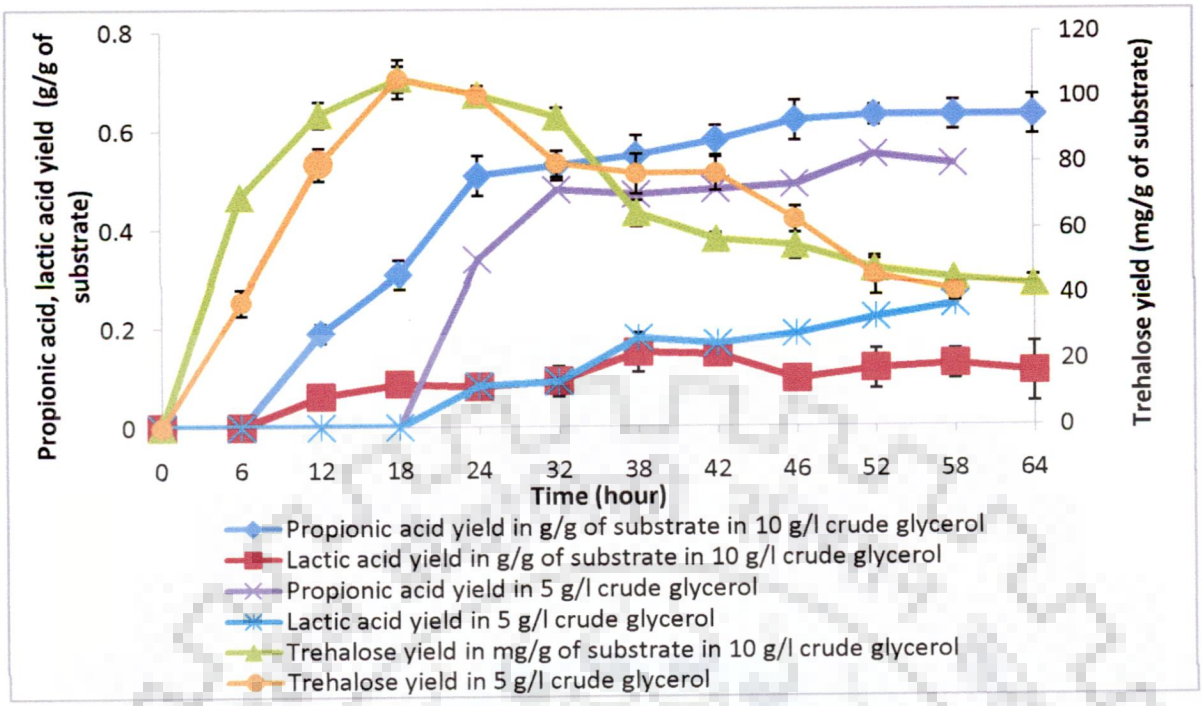


Fig 4.34 Comparison of yields of propionic acid, lactic acid and trehalose with 10 g/l and 5 g/l of crude glycerol in batch fermenter

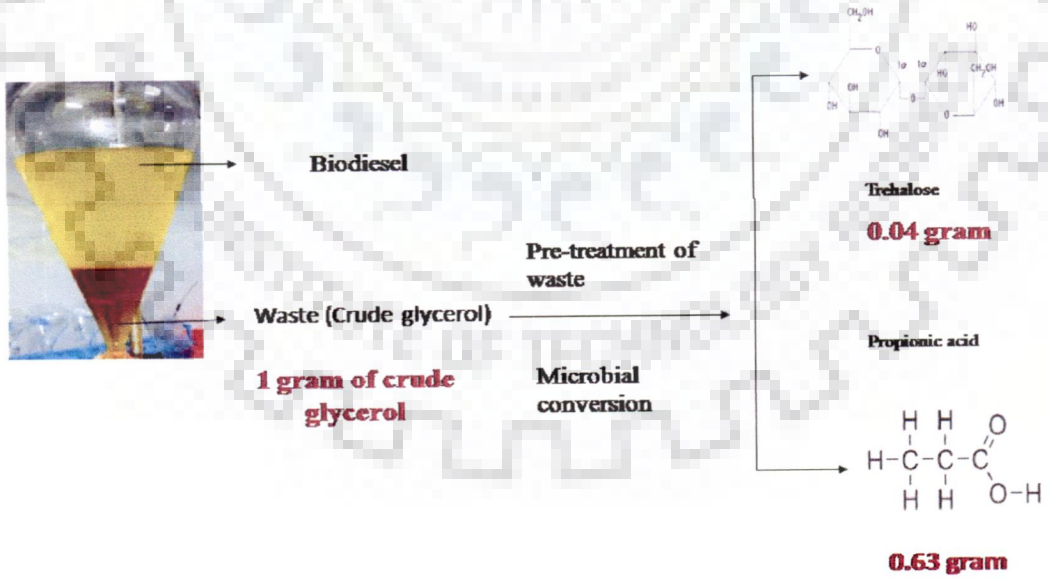


Fig 4.35 Production of trehalose and propionic acid from crude glycerol

Table 4.7 Comparison of final trehalose yields with respect to biomass (Y_{tx}) and substrate consumed (Y_{ts}), Propionic acid (Y_{pa}) and lactic acid (Y_{la}) yields obtained in different concentrations of crude glycerol.

Yield	Crude glycerol 20 g/l	Crude glycerol 10 g/l	Crude glycerol 5 g/l
Y_{tx} (mg/g of biomass)	128±3	131±8	68±4
Y_{ts} (mg/g of substrate)	21±2	43±2	41±2
Y_{pa} (g/g of substrate)	0.63±0.03	0.63±0.04	0.53±0
Y_{la} (g/g of substrate)	0.027±0	0.109±0	0.246±0
Trehalose (mg/l)	261±3	394±8	204±4

Trehalose is an important nutraceutical accumulated as a compatible solute in microbes. Commercial utilization of trehalose is hindered due to its high cost of production; hence search for economic carbon source is crucial. Despite the available knowledge on production of trehalose from glucose, sucrose, maltose and starch, report on trehalose production from glycerol has ever been proposed. Hence, to make trehalose production from glycerol feasible, we studied the fermentative production of trehalose from crude glycerol using *P. freudenreichii subsp. shermanii* NCIM 5137. Herein, the comparison of trehalose production in pure and crude glycerol media in batch reactor shows that maximum trehalose yield of approx 380 mg/g of biomass can be achieved and highest trehalose yield based on substrate consumed was improved 6.5 times in crude glycerol media as compared to that in pure glycerol media. The reasons for this superiority may be due to the presence of impurities in crude glycerol. Therefore, effects of various impurities present in crude glycerol were evaluated on trehalose yield based on substrate consumed and it was concluded that KCl was one of the major impurities responsible for achieving higher trehalose yields based on substrate consumed. Finally, 10 g/l of crude glycerol media was found to be optimum for higher trehalose and propionic acid yields. In summary, crude glycerol was demonstrated as a potential carbon source for economical trehalose production.

4.5. Study of trehalose metabolic pathway under osmotic and non-stress conditions

In previous section it was observed that trehalose production was enhanced under osmotic stress condition. Similar observations were also reported in *P. freudenreichii, E.coli, S. cerevisiae, Corynebacterium* (Cardoso et al., 2007; Strom et al., 1993, Arguelles, 2000, Wolf et al., 2003). In all these studies, efforts were made to identify specific genes responsible for higher trehalose accumulation under osmotic stress condition. Methodology followed for these studies included gene deletion mutant/gene over expression and biochemical characterisation. The common observation in all these studies is the prominent roles of OtsAB and UDP-glucose pyrophosphorylase in trehalose accumulation under osmotic stress condition. The role of trehalose synthase (TreS) in catabolic pathway of trehalose was already predicted in *P. freudenreichii and Corynebacterium* (Cardoso et al., 2007; Wolf et al., 2003). Interestingly, TreS role in converting glycogen into trehalose through maltose formation in Mycobacterium was also reported (Pan et al., 2008). It was also predicted that when trehalose concentration was low than glycogen is converted to trehalose. The role of TreYZ pathway was also predicted in *Corynebacterium* under osmotic stress (Wolf et al., 2003). However, TreYZ pathway was absent in *P. freudenreichii* as confirmed by genome study (Falentin et al., 2010). In case of *Corynebacterium* role of individual gene corresponding to fructose 1, 6 BiP, phosphoglucomutase (other than OtsAB, TreS and TreYZ) was evaluated for their influence on trehalose yield using gene deletion method. In the present study, instead of following the traditional method, simultaneous monitoring of group of enzyme activities and metabolites concentrations under osmotic and non-stress conditions were carried out. Although adopted methodology does not guarantee full proof conclusion about the role of individual enzyme, but it provides the overall system level information regarding metabolic regulation. Overall it can be concluded that our adopted methodology not only validated previous conclusions but it also provided new information regarding metabolic regulation involved in trehalose biosynthesis under osmotic stress. Thus the adopted methodology helps us in identifying critical metabolic steps involved in metabolic regulation. Further, these identified metabolic parameters can now be elucidated using the traditional method of gene deletion or gene over-expression methods. In the present study, initially a qualitative analysis was carried out followed by a multivariate data analysis method which ensures statistically correct interpretation of data. The objective of the folowed method involves identifications of metabolic parameters which are responsible for

enhanced trehalose accumulation in mid exponential growth phase as well as decrease in trehalose yield in late exponential phase.

Methodology adopted here includes comparison of relevant metabolic parameters under stress and non stress conditions. From the extent of over-expression or down regulation of an individual parameter a qualitative prediction about its role in overall regulation was made. Finally principle component analysis was carried out to identify critical metabolic parameters from loading plots. Similar methodology was adopted in case of β-glucan production in *Pediococcus parvulum* 2.6 with different carbohydrate sources (Velasco et al., 2007). In their studies, intracellular enzymes activities were monitored and finally it was predicted that α-phosphoglucomutase, UDP-glucose pyrophosphorylase were the bottleneck for biosynthesis polyscaaharides. Monitoring of intracellular enzyme activity was followed for studying EPS biosynthesis in *Lactobacillus helveticus* ATCC 15807 (Torino et al., 2005), *Streptococcus thermophilus* LT03 (Degeest & Vuyst, 2000). In all these studies, enzyme activities were either correlated with EPS production or were used to understand physiological factors that affect EPS biosynthesis. Therefore, in the present study a qualitative prediction was made about variation in trehalose accumulation by comparing the change of individual enzyme activity or metabolite concentration related to trehalose biosynthesis pathway in osmotic stress and non-stress conditions. The relevant enzymes activities and metabolites concentrations considered in the present study were OtsAB, Trehalase, trehalose synthase (TreS), UDP-glucose/ADP-glucose/GDP-glucose pyrophosphorylase (UDP-G pyro/ADP-G pyro/GDP-G pyro), phosphoglucomutase (PGM), UDP-glucose dehydrogenase (UDPGD), phosphoglucoisomerise(ISO), glucose-6-phosphate dehydrogenase(G6PD), glycogen, maltose, glucose-6-phosphate as shown in fig 4.36. Most prominent effects were difference in expression of NDP-glucose synthesizing enzymes and TreS. The activity of ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase increased by five, three and 1.5 times in osmotic stress condition as compared to non-stress condition respectively. Interestingly, activity of OtsA was highest with ADP-glucose followed by GDP-glucose and UDP-glucose under osmotic stress. Contradictory to *P. freudenreichii*, the activity of trehalose synthase (TreS) was increased with osmotic stress while no activity was detected in non-stress condition. It was also predicted that amylase activity is probably associated with TreS or independently expressed in osmotic stress condition.

4.5.1. Impact of osmotic stress on trehalose yield

Accumulation of trehalose as a protection strategy against osmotic stress is a widespread phenomenon in microbes. In previous section, it was already shown that there was increase in trehalose yield with respect to biomass with increase in KCl concentration (Fig 4.30). Similarly, there was significant increase in trehalose yield with respect to substrate consumed in osmotic stress condition as compared to non-stress condition (Fig 4.31). Thus osmotic shift in the media was counterbalanced by enhanced synthesis of trehalose and it can be observed that as the concentration of KCl was increased in the media, significant rise in trehalose yield was observed (especially trehalose yield with respect to substrate consumed). It is also interesting to observe that rate of substrate conversion was significantly less in osmotic stress condition as compared to non-stress condition (Fig 4.37). Once bacteria adapts to the osmotic environment of the media by increasing trehalose production, it either starts degrading trehalose or decrease the synthesis of trehalose during late exponential phase of growth. It indicates that there may exist some other mechanisms of adaptation in osmotic stress condition during late exponential phase of growth. Thus it can be propose that one of the early adaptation strategies of *P. freudenreichii* subspecies *shermanii* against osmotic stress includes adjustment of intracellular trehalose level in accordance of osmolarity of the given media. In the present work, study was restricted to trehalose biosynthesis pathway only.

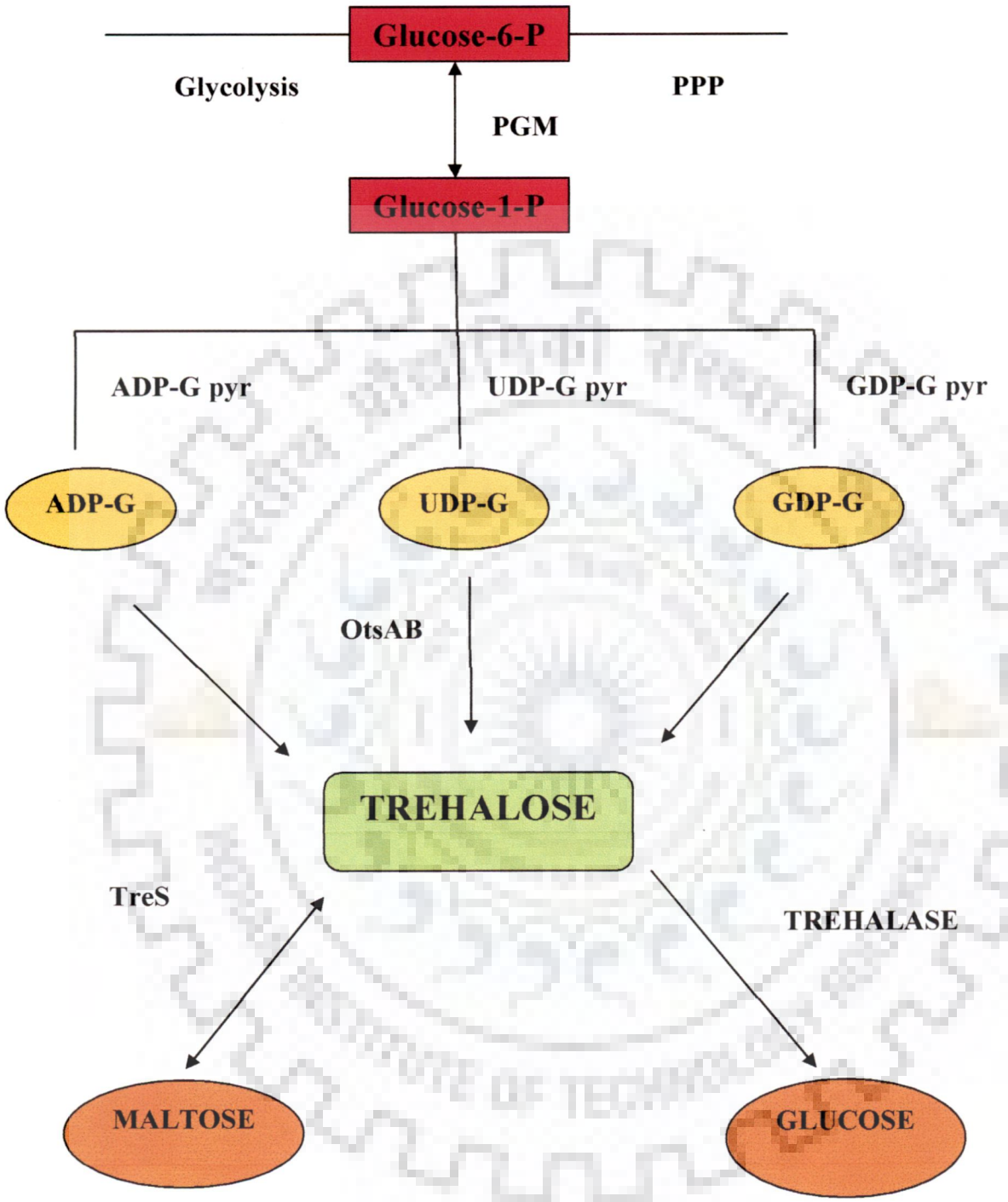


Fig 4.36 Trehalose biosynthesis pathway studied in *P. shermanii* NCIM 5137 in stress and non-stress conditions

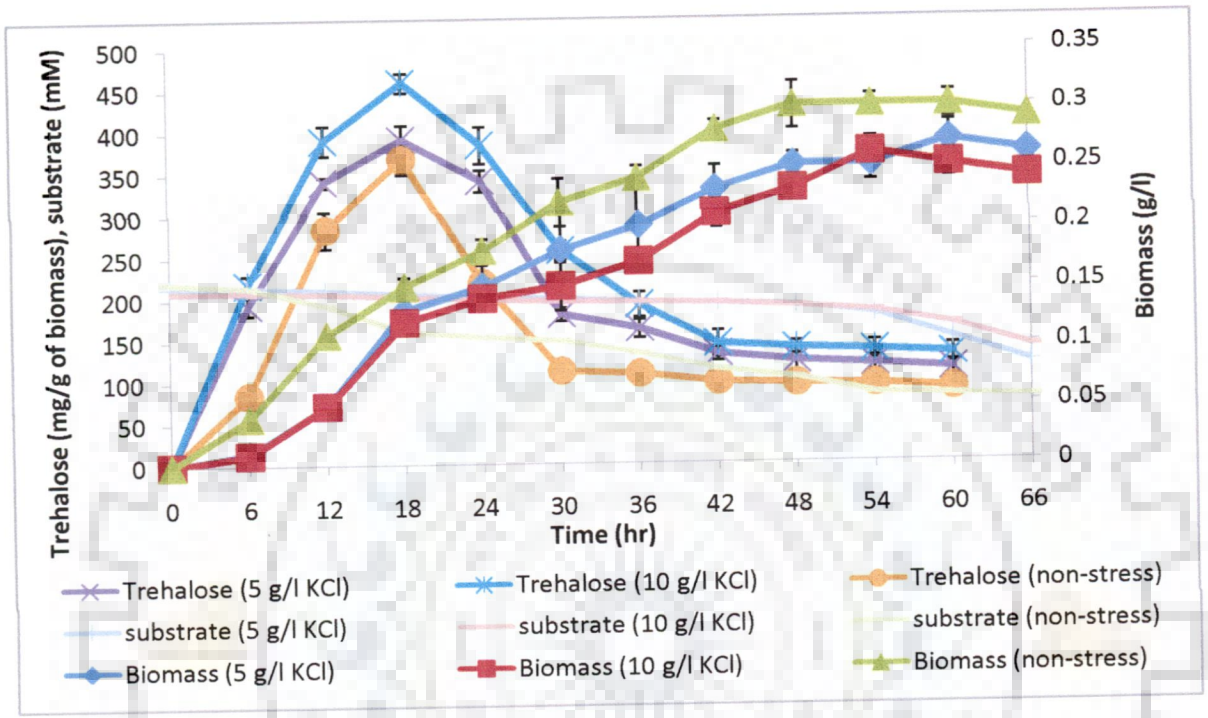


Fig 4.37 Trehalose yield, in mg/g of biomass, biomass growth and residual substrate concentration profiles in *P. shermanii* when grown in chemically defined media with glycerol as carbon source and osmotic stress in the form of 0.5% and 1% KCl

4.5.2. Role of NDP-glucose synthesising (NDP-glucose pyrophosphorylase) enzymes in response to osmotic stress

It is interesting to know how under osmotic stress condition, enhancement of trehalose production was achieved at the molecular level. In the present study, instead of using the traditional method of using deletion mutant or over-expressing recombinant, in-vitro relevant enzymes activities and metabolites concentrations under osmotic stress and non-stress growth conditions were monitored. From the comparison of individual enzyme and metabolite concentration in stress and non-stress conditions, a molecular level mechanism is proposed. In *P. freudenreichii*, importance of OtsAB pathway and catabolic nature of TreS pathway under osmotic stress were already proposed (Cardoso et al 2007). The enhancements of activities of OtsA and OtsB under osmotic stress are already reported (Cardoso et al 2007) and similar behaviour was also observed in the present study. In OtsAB pathway, enzyme OtsA uses substrates NDP-glucose and glucose-6-phosphate, NDP-glucose can be UDP- glucose, ADP-glucose and GDP-glucose. Most of earlier reported studies dealt with UDP-glucose but in *P. freudenereichii* it was reported that OtsA has activity with ADP-glucose only in crude extract while in pure form it has activity with all NDP-glucoses (Cardoso et al 2007). However, no study has ever reported the effects of osmotic stress on other nucleotide sugar synthesizing enzymes, mainly ADP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase. Hence activities of enzymes, ADP-glucose pyrophosphorylase, UDP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase synthesising ADP-glucose, UDP-glucose and GDP- glucose respectively were determined in non-stress and osmotic stress (5 g/l and 10 g/l KCl) media. For all enzyme activities, four stages of growth were taken, starting from the initial stage of trehalose synthesis (12 hr, fig 4.37) to subsequent three stages including highest trehalose accumulation stage. Although our main analysis is restricted to the maximum trehalose accumulation stage but other stages were also determined in the present study as these data were later used in multivariate analysis. One of the interesting observations about the activities of enzymes synthesising NDP-glucose was that the effect of osmotic stress was different on ADP-glucose pyrophosphorylase, UDP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase. From Fig 4.38 and 4.39 it can be observed that ADP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase were increased maximum up to five and three folds respectively in 10 g/l KCl media as compared to non-stress condition. In case of UDP-glucose pyrophosphorylase, maximum activity was

increased by 1.5 fold in osmotic stress condition (fig 4.40). It is also interesting to note that the effect of osmotic stress on UDP-glucose pyrophosphorylase is limited up-to 5 g/l KCl and thereafter no increase in activity was observed with further increase in extracellular concentration of KCl.

Thus it indicates that enhancement of trehalose accumulation needs higher supply of NDP-glucose and in the present case it seems increased demand for NDP-glucose is met by providing more ADP-glucose and GDP-glucose as compared to UDP-glucose. However, it is interesting to note that absolute activity of UDP-glucose pyrophosphorylase was always higher than GDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase. As activity of UDP-glucose pyrophosphorylase was also increased with osmotic stress, it can be predicted that UDP-glucose also serves as substrate for other metabolic reactions beside trehalose-6-phosphate synthesis. Two such metabolic reactions are UDP-glucose dehydrogenase and glycogen synthase. In the present case, it was observed that UDP-glucose dehydrogenase activity was decreased by five fold in 1% KCl media as compared to non-stress condition (table 4.8). Glycogen synthase reaction requires UDP-glucose or ADP-glucose as one of the substrate. In the present study, activity of glycogen synthase was determined using UDP-glucose as substrate. It was observed that there was no effect of osmotic stress on glycogen synthase (Table 4.8). Thus it can be concluded that excess demand of NDP-glucose was probably for enhanced trehalose synthesis and it was met mostly by ADP-glucose and GDP-glucose. Furthermore, diversion of UDP-glucose by UDP-glucose dehydrogenase was reduced during osmotic stress condition. Overall it can be predicted that during osmotic stress, cells adopts a strategy of diverting excess carbon flux towards trehalose synthesis by over-expressing ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase to maximum extent and downregulation of UDP-glucose dehydrogenase. In other words, when demand for excess NDP-glucose arises than cell machinery prefers ADP-glucose and GDP-glucose and at the same time reduces UDP-glucose consumption by down-regulating UDP-glucose dehydrogenase. As ADP-glucose was also used by glycogen synthase hence it is difficult to predict whether excess supply of ADP-glucose is solely used for trehalose synthesis. In the present study, an effort was made to evaluate the specificity of OtsA toward three NDP-glucoses as this can confirm the larger participation of ADP-glucose in trehalose synthesis.

Thus in the present study activity of OtsA was determined separately with ADP-glucose, GDP-glucose and UDP-glucose in stress and non-stress conditions. It was interesting to observe that activities of OtsA with different NDP-glucoses changes with

osmotic stress and OtsA had higher activity with ADP-glucose (product of ADP-glucose pyrophosphorylase) and GDP-glucose as compared to UDP-glucose in osmotic stress condition. This indirectly indicates that under in-vivo condition, OtsA has probably highest specificity for ADP-glucose as compared to GDP-glucose and UDP-glucose and this phenomenon has already been reported in literature (Cardoso et al 2007). In order to indirectly visualize the change in specificity of OtsA with three nucleotide sugars, ratio of activities of OtsA with ADP-glucose to UDP-Glucose and ratio of OtsA activities with GDP-glucose to UDP-Glucose were plotted in non-stress and osmotic stress (5 and 10 g/l KCl) conditions (Fig 4.41). From the figure 4.41 it can be observed that ratio of activities of OtsA with ADP-glucose to UDP-glucose and ratio of activities of OtsA with GDP-glucose to UDP-glucose were higher than one in osmotic stress while these ratios were less than one in non-stress condition. However, in media containing 10 g/l KCl, OtsA was more specific to ADP-glucose as compared to GDP-glucose which can be clearly observed in Fig 4.41 where ratio of OtsA activities with ADP glucose to GDP-glucose was more than one. Thus the activity of OtsA with ADP-glucose increases with concentration of KCl in the media. This clearly explain the convoluted relations of ADP-glucose pyrophosphorylase with trehalose synthesis during osmotic stress. However it is interesting to note that at late stage of growth, with 10 g/l of KCl, OtsA activity with ADP-glucose to OtsA activity with UDP-glucose decreased sharply as compared to media containing 5 g/l KCl. This behaviour needs further investigation with respect to other possible adaptation strategy used by cell against osmotic stress in late phase of growth. In the present study, specificity of OtsA was not represented by Km value as it needs to be carried out with purified enzyme. Purified enzyme was not employed in the present case as it was already reported that purified form has nearly equal specificity for ADP-glucose, GDP-glucose and marginally higher specificity with UDP-glucose in *P. freudenreichii*.

Further we investigated activities of enzymes related to glucose-6-phosphate, another substrate of trehalose synthesis by OtsA. Glucose-6-phosphate enters glycolysis (phosphoglucoisomerase) and pentose phosphate pathway (glucose-6-phosphate dehydrogenase). It also enters trehalose synthesis by inter-converting to glucose-1-phosphate by enzyme phosphoglucomutase which is further used to form NDP-glucose. Activities of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase didn't show any drastic differences in non-stress and osmotic stress but their activities were marginally lower in stress condition and this probably explains slower growth of cell in osmotic stress condition (table 4.8). On other hand, the activity of phosphoglucomutase was marginally

higher in osmotic stress condition as compared to non-stress condition. Altogether, from the results of activities of these three enzymes it can be inferred that effect of osmotic stress on these enzymes were marginal. From these enzymes activity profiles it can be proposed that cell machinery adopts a strategy of diverting more carbon towards trehalose biosynthesis by enhancing ADP-glucose pyrophosphorylase activity along with higher specificity of OtsA towards ADP-glucose and down-regulation of UDP-glucose dehydrogenase.

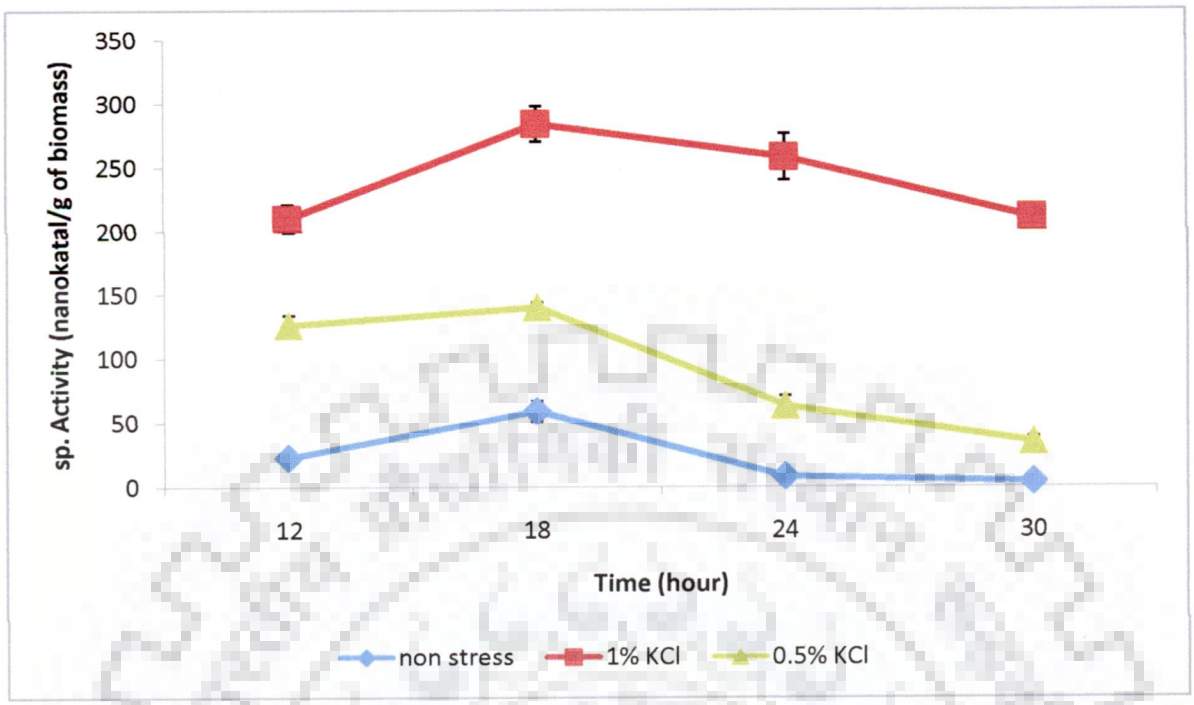


Fig 4.38 Specific activity of ADP-glucose pyrophosphorylase in non stress and osmotic stress (0.5% and 1% KCl) condition in a chemically defined media

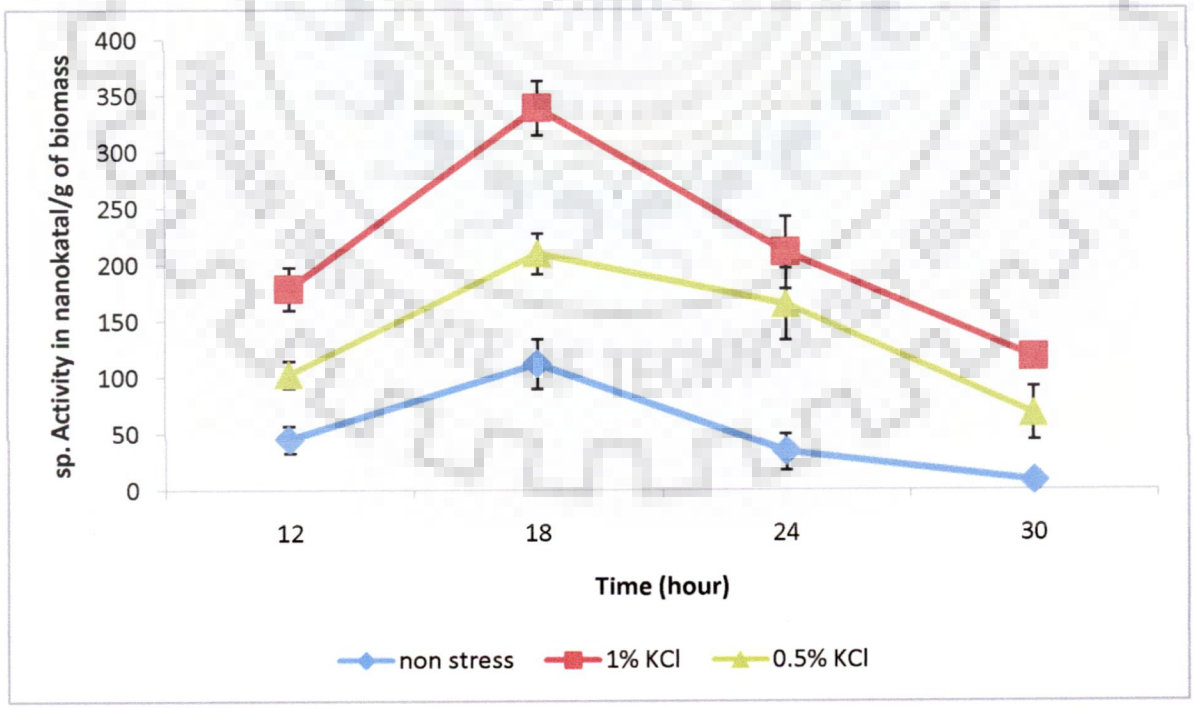


Fig 4.39 Specific activity of GDP-glucose pyrophosphorylase in non stress and osmotic stress (0.5% and 1% KCl) condition in a chemically defined media

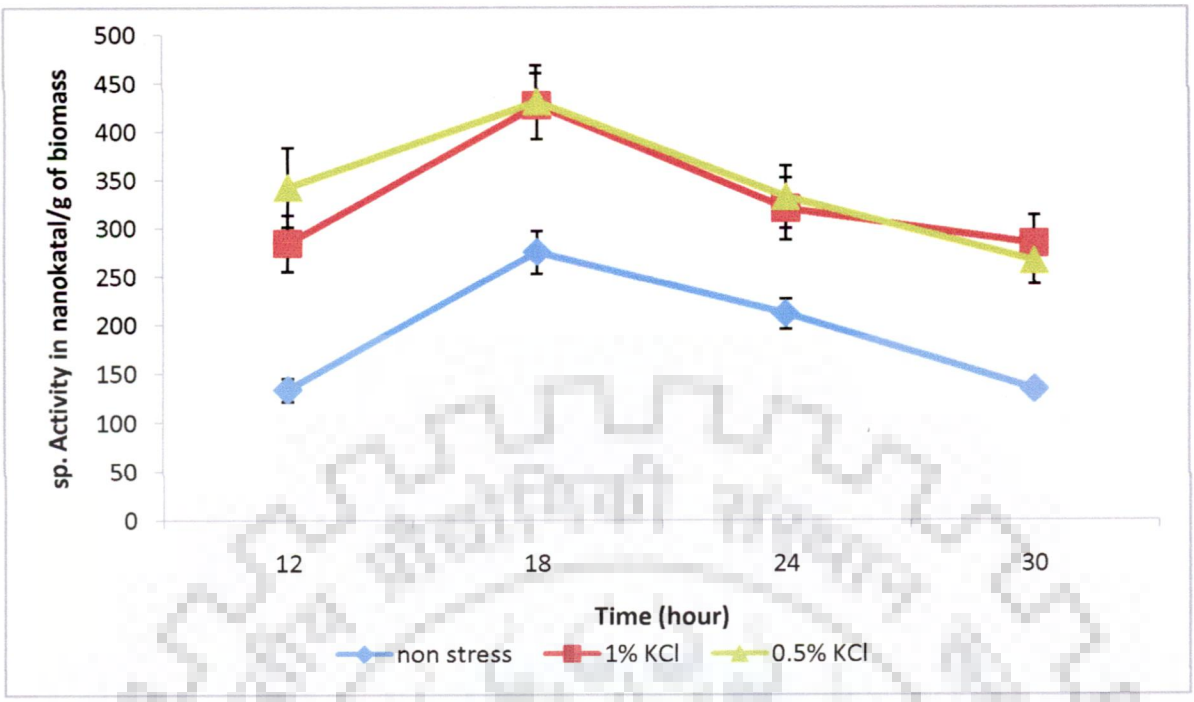


Fig 4.40 Specific activity of UDP-glucose pyrophosphorylase in non stress and osmotic stress (0.5% and 1% KCl) conditions with a chemically defined media

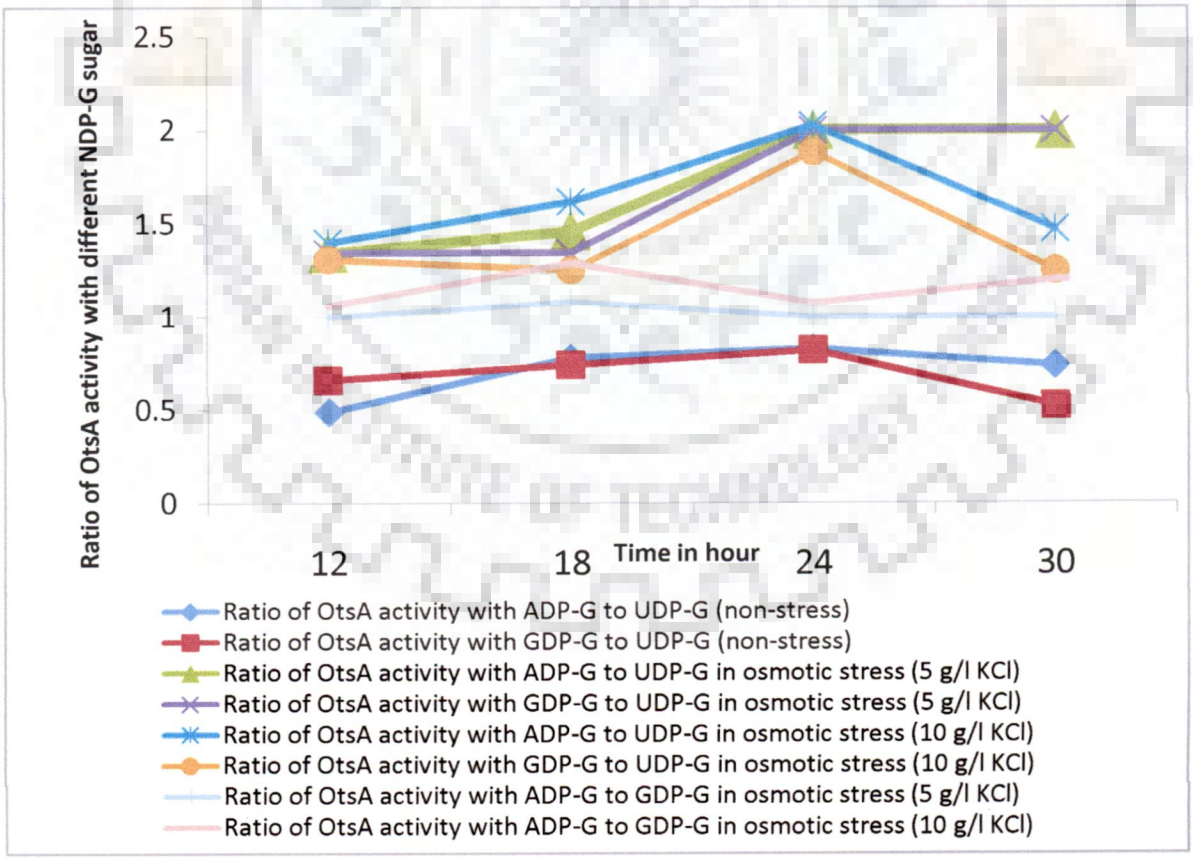


Fig 4.41 Ratio of OtsA activity with different NDP-G as substrate in non stress and stress conditions

Table 4.8: Specific enzyme activities in nanokatal/g of biomass and intracellular content of maltose (mg/g of biomass) and glycogen (glucose released in mg/g of biomass), OtsA (trehalose-6-phosphate synthetase), Pgm (phosphoglucomutase), Iso (isomerase), G6PD (glucose-6-phosphate dehydrogenase), UDPgD (UDP-glucose dehydrogenase), TreS (trehalose synthase), Tase (trehalase), Glycogen synthase (GS)

Hour	OtsA	Pgm	Iso	G6PD	UDPgD	TreS	Tase	Maltose	glycogen	GS
Non-stress										
12	4536±12	154±8	2493±24	2134±12	1088±8	0±0	12±0.6	8.5±1	53±6	82±4
18	6879±22	212±11	3456±21	3124±18	1678±4	0±0	23±2	10.1±0.8	123±4	113±3
24	3124±31	189±13	2889±11	1982±15	1231±14	0±0	12±1	3±0.5	88±8	95±6
30	2298±14	102±7	1768±8	1324±8	657±15	0±0	7±0.8	2.4±0.3	68±4	79±3
0.5% KCl										
12	5644±23	187±14	1142±9	884±4	334±19	4.23±0.8	83±4	0±0	24±4	79±5
18	7405±31	223±11	2564±15	2751±11	456±11	4.79±1	88±2	35±2	64±6	116±6
24	4385±28	198±8	1534±12	1534±8	232±8	3.2±0.5	71±3	30±4	23±3	78±5
30	3259±12	168±4	970±5	946±4	112±4	2.2±0.9	35±4	25±1	18±4	66±3
1% KCl										
`12	6553±23	190±16	1042±9	1042±8	284±12	5.4±1	94±6	0±0	18±4	76±2
18	8840±21	233±22	2116±8	2248±11	312±8	6.4±0.6	125±11	59±4	42±2	119±8
24	4450±14	230±11	1643±12	1643±14	172±4	3.4±0.7	76±8	32±2	16±1	102±6
30	4664±11	179±7	1164±8	1015±6	115±3	2.6±0.8	42±4	23±3	11±2	78±4

4.5.3. Role of enzyme trehalose synthase (TreS)

One interesting observation made during osmotic stress study was the presence of trehalose synthase (TreS) in osmotic stress condition and its absence in non-stress condition (Table 4.11). With the increase in KCl concentration in the media, there was a marginal increase in TreS activity. Similarly, trehalase activity increased (five fold) significantly with increase in KCl concentration of the media. However it is difficult to interpret whether this increase in trehalase activity was related to osmotic stress or trehalose content of cells. In case of yeast it was observed that with increase in trehalose content of cell, trehalase activity increases (Garre et al., 2009, Li et al 2011). Thus it can be proposed that trehalase enzyme gets induced by trehalose contents of cell. The role of trehalase in trehalose biosynthesis pathway is obviously catabolic in nature. As far as the role of TreS under osmotic stress is considered it is difficult to predict as there are contradictory reports on its role in *Corynebacterium* and *Mycobacterium.* In case of *Corynebacterium,* it was predicted that absence of trehalase resulted in a situation where TreS probably plays the role of catabolic enzyme which converts trehalose to glucose through maltose formation (Wolf et al., 2003). In case of *Mycobacterium*, it was postulated that at low concentration of trehalose it plays a role in the conversion of glycogen to trehalose through maltose formation (Pan et al., 2008). It was experimentally shown that TreS also contains amylase activity which is responsible for conversion of glycogen to maltose. Amylase activity and reversible inter-conversion of trehalose and maltose (referred as MTase activity by Pan et al., 2008) reported to be present in same protein. It was also reported that the presence of glycogen didn't affected the rate of trehalose to maltose conversion (Pan et al., 2008). In view of these contradictory observations, experiments were planned to elucidate the role of TreS under osmotic stress in *P. freundeichii sub sp. shermanii* NCIM 5137. In the previous section it was experimentally shown that under osmotic stress, organism adopts a strategy of diverting carbon flux towards trehalose synthesis by increasing the in-vivo activities of ADP-glucose pyrophosphorylase (maximum extent) and OtsA with ADP-glucose. Thus the question arises why under osmotic stress two catabolic routes are necessary for catabolism of trehalose? In first set of experiment, the presence of amylase activity of TreS was checked by incubating the cell extract with 0.5 g/l of glycogen and determining the maltose and trehalose contents of the reaction mixture. This study has been restricted to cell extract obtained from 18 hour grown cells (maximum trehalose accumulation stage) in media

containing 10 g/l KCl. From the result it can be seen that 55 mg/g of maltose was formed when incubated with glycogen whereas in control (only cell extract used) maltose content was 20 mg/g (table 4.12). Thus this study shows that amylase activity of TreS was present in cell extract of *P. freundenreichii subsp. shermanii* NCIM 5137. However it is difficult to confirm the association of amylase activity with TreS as we had used cell extract. To confirm whether the amylase activity was a part of TreS, non-stressed cell extract was used and incubated with 0.5 g/l of glycogen. In non-stress cell, where there was no TreS activity there was no formation of maltose in the reaction mixture (table 4.9). Thus it can be assumed that amylase activity obtained with stressed cell extract is either associated with TreS or is expressed independently under osmotic stress. However, it needs further investigation with purified TreS. In the present study, amylase activity was assumed as an independent enzyme which is expressed along with TreS during osmotic stress.

Pan et al., 2008 confirmed the association of amylase activity with TreS of M*ycobacteriumi sp.*. Interestingly there was also formation of trehalose when cell extract (obtained from osmotic stressed cells) was incubated with glycogen (table 4.9). The amount of trehalose formation in the reaction mixture was higher than the control reaction mixture where only cell extract (obtained from osmotic stressed cells) was incubated at identical conditions. Overall it can be postulated that glycogen can be converted to trehalose due to the presence of TreS and amylase activity. However before concluding on the role of TreS in the conversion of glycogen to trehalose, it was necessary to check the presence of TreYZ activity in the cell extract. TreYZ activity of cell extract was checked with soluble starch and no activity was found in cell free extract. Similar conclusions were also made from genome study of *P. freundreichii* (Falentin et al., 2010). Thus this study confirms that trehalose formation can be possible from glycogen through formation of maltose using TreS and amylase activity. Apparently, it seems that amylase activity (glycogen to maltose conversion) was higher than the maltose to trehalose conversion rate (Mtase activity according to Pan et al., 2008) when cell extract was incubated with glycogen. Reversible nature of TreS activity was also checked by using trehalose and maltose as substrate and determining maltose and trehalose formations respectively. From the result it can be seen that TreS activity in forward and reverse directions are almost similar (table 4.9). However, in vivo condition is significantly different from the in-vitro condition and thus there is a necessity to check the TreS activity in presence of glycogen as this storage carbohydrate is also present along with maltose and trehalose inside the cell (table 4.9).

The outcome of this study was surprising as 50% higher activities of TreS in both directions were obtained with glycogen and trehalose mixture or glycogen and maltose mixture as compared to activities obtained with only trehalose or maltose as substrate (table 4.9). This observation is contrary to the report on TreS of *Mycobacterium sp.* where presence of glycogen has no effect on TreS (specifically Mtase activity) (Pan et al., 2008). The question stills remains on the role of TreS and amylase activity in *P. freundreichii subsp. shermanii* NCIM 5137 under osmotic stress condition. Here we are proposing a model which explains the roles of glycogen and TreS along with amylase activity in trehalose biosynthesis. If Fig 4.42, interrelations between trehalose, maltose and glycogen formations under osmotic stress conditions are presented. Quantitative values of various rates in Fig 4.42 were taken from table 4.9. Maltose and trehalose formations rates from only glycogen with osmotically stressed cells were calculated from maltose and trehalose produced (table 4.9). The values of specific maltose and trehalose formations rate were 8.52 nkat/gm and 3.4 nkat/gm respectively from glycogen only. Thus if we compare the maltose formation rates from glycogen only and trehalose glycogen mixture at similar experimental conditions, it can be concluded that either there was no formation of maltose from trehalose or net formation rate of maltose from glycogen is around 3.04 nkat/gm (after considering maltose formation rate from trehalose only which was 5.52 nkat/gm). Thus from this proposed model it can be concluded that due to TreS, amylase activity and simultaneous presence of glycogen (0.5 g/l) net trehalose formation rate varies from 3-5.2 nkat/gm (fig 4.42). However it is difficult to accurately predict the net trehalose formation rate under in-vivo conditions from this experimental data. Besides, at high trehalose to maltose ratio this net estimated trehalose formation will decrease. Although this study, not able to predict the actual rate of trehalose formation but it indicates that in presence of high glycogen concentration, TreS and amylase activities, net formation rate of trehalose will be a positive value (anabolic nature). It can also be predicted that in absence of glycogen or low concentration of glycogen and high trehalose to maltose ratio, TreS has a catabolic role which has already been reported in literature. Here it is proposed that the equilibrium between trehalose and maltose is regulated by glycogen, TreS and amylase activities. In other words, catabolic and anabolic nature of TreS depends on intracellular glycogen concentration and trehalose to maltose ratio. It is worth to note that the present method of studying osmotic stress response is superior to the traditional method of using deletion mutant as deletion mutant cannot predict the simultaneous roles of TreS and glycogen in trehalose biosynthesis pathway.

Table 4.9 Trehalose and maltose formations using glycogen in non-stress and stress conditions, and activity of TreS in stress conditions using substrates glycogen + maltose/trehalose and only trehalose/maltose in osmotic stress (10 g/l KCl)

Substrate		
Osmotic stress	**Control (Cell extract + buffer)**	**Cell extract + substrate**
Glycogen (maltose formation)	20±0.6 mg/g	55±2 mg/g
Glycogen (trehalose formation)	14.2±1 mg/g	28.1±3 mg/g
Non-stress		
Glycogen (maltose formation)	7.98±0.2 mg/g	7.05±0.5 mg/g
Activity of TreS (osmotic stress	**Activity (nkat/g)**	
Glycogen + maltose (trehalose formation)	8.44±0.8	
Glycogen + trehalose (maltose formation)	8.36±0.7	
Maltose (trehalose formation)	5.48±0.5	
Trehalose (maltose formation)	5.52±0.3	

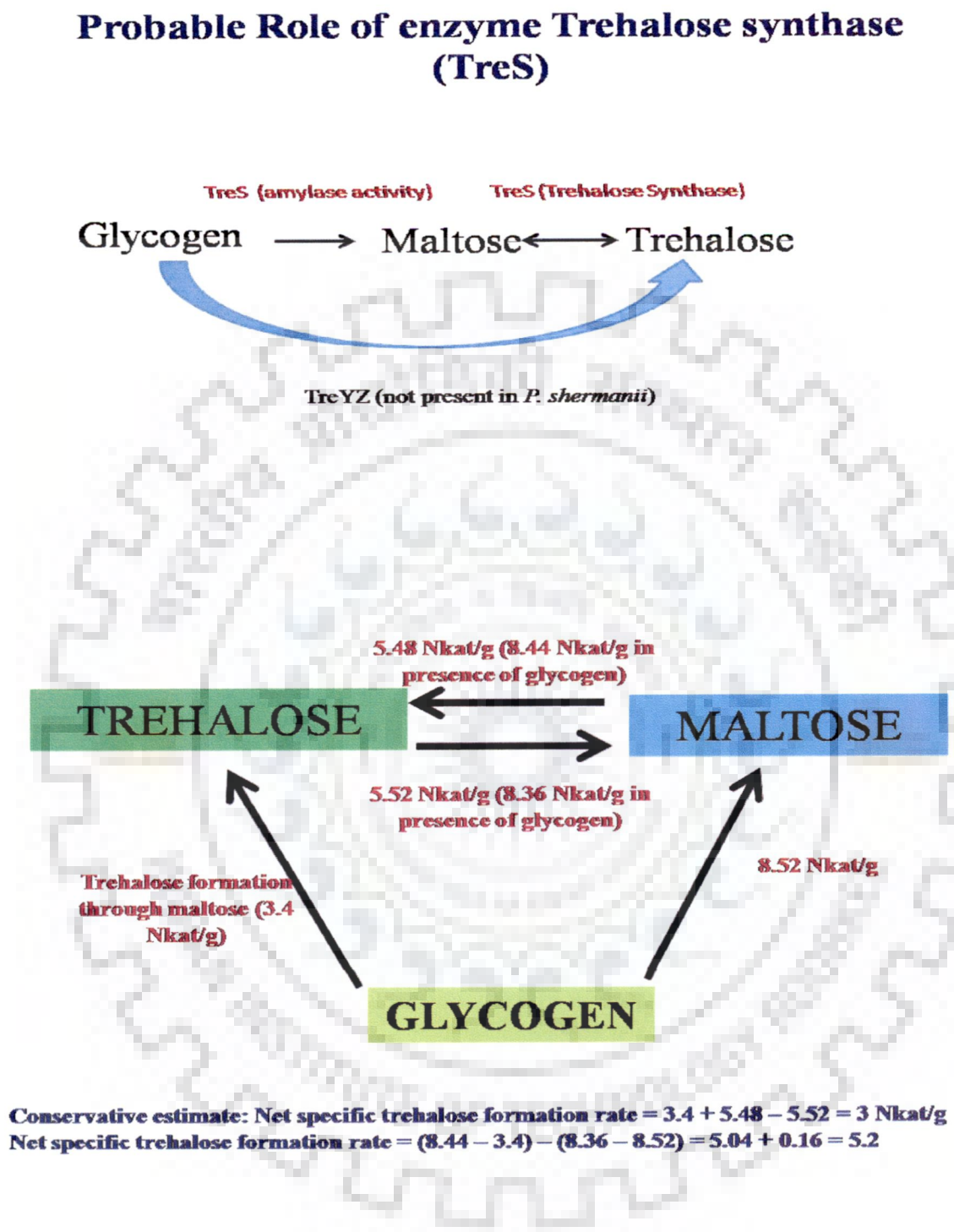


Fig 4.42 Probable interrelationship of trehalose, glycogen and maltose in non-stress and osmotic stress conditions

In summary, few remarkable new insights to trehalose biosynthesis under osmotic stress were revealed from this study. The probable role of TreS for synthesis of trehalose using glycogen as substrate under osmotic stress condition is predicted and ambiguity regarding the role of TreS needs to be further investigated in *Propionibacterium*. Another major observation was the difference in expressions of enzymes providing NDP-glucose under osmotic stress and higher expression of ADP-glucose pyrophosphorylase over other nucleotide sugar synthesizing enzymes, indicating regulatory role of ADP-glucose in osmotic stress condition. Thus from this study, it became apparent that glycogen, maltose and trehalose synthesis are inter related during osmotic stress conditions. Over expressions of ADP-glucose pyrophosphorylase and TreS were the strategy adopted by *P. shermanii* for minimizing the effect of osmotic stress. The decreasing trehalose content of cell in late exponential phase can be explained from higher trehalase activity and catabolic nature of TreS at low concentration of glycogen (observed during late exponential phase). However, decreasing activities of key enzymes like ADP-glucose pyrophosphorylase, GDP-pyrophosphorylase, OtSAB necessary for enhanced trehalose accumulation with growth in late exponential phase. Overall, a qualitative prediction about the prominent roles of ADP-glucose pathway and TreS during osmotic stress conditions was predicted from this study.

4.6. Analysis of trehalose metabolism by an approach of principle component analysis

The complex biological systems are difficult to decipher as they are characterised by a large number of parameters or variables which are either correlated linearly or non linearly with each other. Metabolic engineering strategies, typically via pathway over-expression and deletion mutant, continue to play a key role in optimizing the conversion efficiency of substrates into the desired products. However, metabolite production titre or yield remains difficult to predict based on reaction stoichiometry and mass balance only. In the present study, a large data of enzymes activities and metabolites concentrations from *P. shermanii* were collected and developed a multivariate statistics-based model to differentiate non-stress and osmotic stress phenotypes of microbial cells. This method provides empirical insights on parameters which differentiate osmotic stress phenotypes from non stress phenotypes without the need of any mechanism based model. Thus, it may provide guidelines for metabolic engineering design strategy for higher trehalose titre. In the previous section, qualitative analysis was carried out by comparing individual metabolic parameters under stress and non stress conditions. In the present study efforts were made to analyze enzymes activity and metabolites concentration profiles under osmotic stress and non stress conditions by using principle component analysis (PCA)

Thus the objective of this analysis was to quantitatively predict the level of influence of individual parameter on trehalose yield and for this purpose, a sophisticated data analysis method was required. PCA is a widely used unsupervised linear technique for dimensionality reduction. The central idea behind PCA is to transform the original dataset consisting of a larger number of interrelated variables to a new set of uncorrelated principle components (PCs), while retaining as much as possible the variation present in the original data set (Jolliffe, 2002). Therefore, PCA is used to decompose the overall data variation into a set of Principle components which can explain maximum percentage of variation. PCA has been used for various microbial physiology studies especially in *E. coli* (Guebel et al., 2008) and *Saccharomyces* (Westergaard et al., 2006)

The complete understanding of the global effects of osmotic stress on trehalose biosynthesis pathway are lacking in literature. In the present study, an attempt was made to address this gap in knowledge on the global effects of osmotic stress on trehalose biosynthesis pathway. In our previous section it was already reported that under osmotic stress, trehalose yield with respect to substrate consumed was significantly improved as

compared to non-stressed condition. The profiles of enzymes activities and metabolites concentrations related to trehalose biosynthesis pathway in osmotic stressed cells and non-stressed cells of *P. freundereichii subsp. shermanii* NCIM 5137 were reported in our previous section. In the present study, our objective was analysis of profiles of enzymes activities and metabolites concentrations in osmotically stressed cells and non stressed cells and devises a method to represent the data in such a way that it reflects the global effects of osmotic stress on trehalose biosynthesis pathway.

As the number of parameters monitored during stressed and non-stressed conditions were large (=13) (fig 4.38-40 & table 4.9) so there was a necessity to use data reduction technique like principal component analysis to derive new parameters which are physiologically significant. The usual methods of studying the effects of osmotic stress involves use of a mutant lacking a particular gene/gene sets or over expression of particular gene, does not give information on overall adaptation strategy used by the cell to protect it from stress. Beside it also lacks the quantitative prediction power. The methods adopted in the present study, enable us to assess the effects of osmotic stress on multiple enzymes or genes. Thus this method is more suitable for complete understanding of metabolic regulation associated with osmotic stress in *P. freundereichii subsp. shermanii* NCIM 5137. It was observed that one of the principal components can differentiate between osmotically stressed cells and non-stressed cells of *P. freundereichii subsp.* NCIM 5137. From the loading plot of principal components, it was also possible to identify the various enzymes which undergo over-expressions and enzymes that are down-regulated during osmotic stress conditions. This is the first attempt to analyze metabolic responses of a microbial system under osmotic stress condition using multivariate data analysis technique.

In recent times, principal component analysis was successfully used as a part of system biology. Usefulness of the multivariate data analysis in metabolomics was reported in various literatures (Guebel et al., 2009; Misra et al., 2002; Stephanopoulos et al., 2002). Principal component analysis is one such technique which focused on all systematic variations in the metabolic patterns or profiles. Based on the generated principal components and score and loading plots, prominent metabolic changes or phenotypes can be identified for wild and mutant. Thus in the present study, an effort was made to differentiate a stress and non-stressed cells using physiologically relevant principal components. Principal component analysis was carried out with in-vitro enzymes activities and metabolite concentrations obtained during culturing of *P. freundereichii subsp.*

shermanii NCIM 5137 in non-stress and stressed conditions in a chemically defined media. Significant variations in activities of enzymes and metabolite concentrations profiles were already reported in osmotically stressed cells of *P. freundereichii* subsp. *shermanii*. Total thirteen variables were selected and they were OtsAB, ADP-glucose pyrophosphorylase (ADP-G pyr), GDP-glucose pyrophosphorylase (GDP-G pyr), UDP-glucose pyrophosphorylase (UDP-G pyr), UDP-glucose dehydrogenase (UDP-GD), glucose-6-phosphate dehydrogenase (ppp), phosphoglucomutase (pgm), trehalase, Trehalose synthase (TreS), phosphoglucoisomerase(iso), glucose-6-phosphate (G6P), glycogen (glyc) and maltose (mal) concentrations. All these parameters were mean centered and normalized with their respective standard deviation. After principal component analysis, first two components were selected as it can explain 84.15% of the variance in data. The score plot of first two components was presented in fig. 4.43 and from this plot it was observed that non-stressed cells are distinctively in different zone as compared to stressed cells (0.5 % and 1% KCl). In the score plots, all non-stressed cells were on the right side of a new imaginary axis (Fig 4.43). Thus it can be inferred that this new axis can be used for differentiating osmotic stress phenotypes from non osmotic stress phenotypes in score plot. However, it is necessary to validate the developed principle components with new sets of independent experimental data. PCA modelling is a non supervised technique, so validation of PCA model will be considered successful if from the position of new set of data in score plot, actual state of osmotic condition of culture medium can be predicted.

To validate the PCA model, new dataset were projected on score plot using already developed principle components. In this new set of data, enzymes activities and metabolite concentrations of *P. freundereichii subsp. shermanii* NCIM 5137 grown in complex media containing glycerol, glucose and crude glycerol as carbon source were used (table 4.10 & 4.11). As the principal components contained standardized variable so first it was converted to normal variables using the following equation.

$$c1 = \frac{x1 - mean1}{(standard\ \text{deviation1})} \times loading\ factor1 + \frac{x2 - mean2}{(standard\ deviation2)} \times loading\ factor2 + \frac{x3 - mean3}{standard\ deviation3} \times loading\ factor3 + \frac{x4 - mean}{standard\ deviation} \times loading\ factor + upto \dots\dots\dots\dots x13$$

Where x1, x2, x3 are metabolic parameters and c1 = score value of first principle component.

From the score plot (Fig 4.44) it can be seen that cells grown in media containing either glycerol or glucose are on the right side of the new imaginary axis indicating non-stress phenotypes. Datasets used for projection were from normal culture conditions where there was no osmotic stress in the media. Thus, it can be concluded that principal components are able to predict phenotypes of new sets of data which were not used for deriving principal components. In previous section, on trehalose production with crude glycerol derived from biodiesel waste it was observed that trehalose yield obtained with respect to substrate consumed was highest and it was predicted that due to the presence of impurities like KCl and other unknown impurities, cells accumulated higher amount of trehalose. In the present study we would like to see whether enzymes activities and metabolites concentrations profiles obtained with crude glycerol media can be projected properly on the score plot based on already derived principal components. From the score plot of these new sets of data, it can be seen that cells were in osmotically sensitive phenotype zone and thus this observation was matching with our previous prediction (Fig 4.44). So it can be concluded that developed model correctly predicts osmotic state of any new sets of experimental data.

Careful observations of score plot indicate that all these data can be differentiated based on the value of first principal component. Lower the score (higher the negative value) of first principal component (except one point) higher is the effect of osmotic stress on cell. Thus here it is proposed a new scale for osmotic stress phenotype which can be used for differentiating osmotic stress phenotype from non-osmotic stress phenotype of *P. freundereichii subsp. shermanii* NCIM 5137. It is also interesting to note that score values of first principal component of crude glycerol grown cells were in the extreme left (negative value of first principal component) which indicates according to new scale, higher osmotic stress was present on the cell. In case of cells grown in crude glycerol probably stress arises not only from KCl but also from some other impurities which are difficult to analyze due to complex nature of crude glycerol.

Second Principal component can be used to differentiate highest trehalose accumulation stage from other stages where trehalose yields were lower than the highest trehalose accumulation stage. It can be seen that in osmotic stress and non stress conditions highest trehalose accumulation stage (data labels with bold red outline) have negative score value for second principal component. However, if three highest (0%, 0.5% and 1%) trehalose yield stages are compared than it can be concluded that first principal component

should have lowest score value (more negative value) for first principal component and second principal component should have minimum negative score value. The highest trehalose accumulation stage of crude glycerol (biodiesel) grown cells has score values of -15.97 and -1.24 whereas glycerol (in complex media) grown cell has score values of 1.37 and -2.49 for first and second principal component respectively. The corresponding trehalose yields with respect to substrate consumed are 104 and 2.1 mg/g of substrate consumed for crude glycerol and glycerol with complex media respectively.

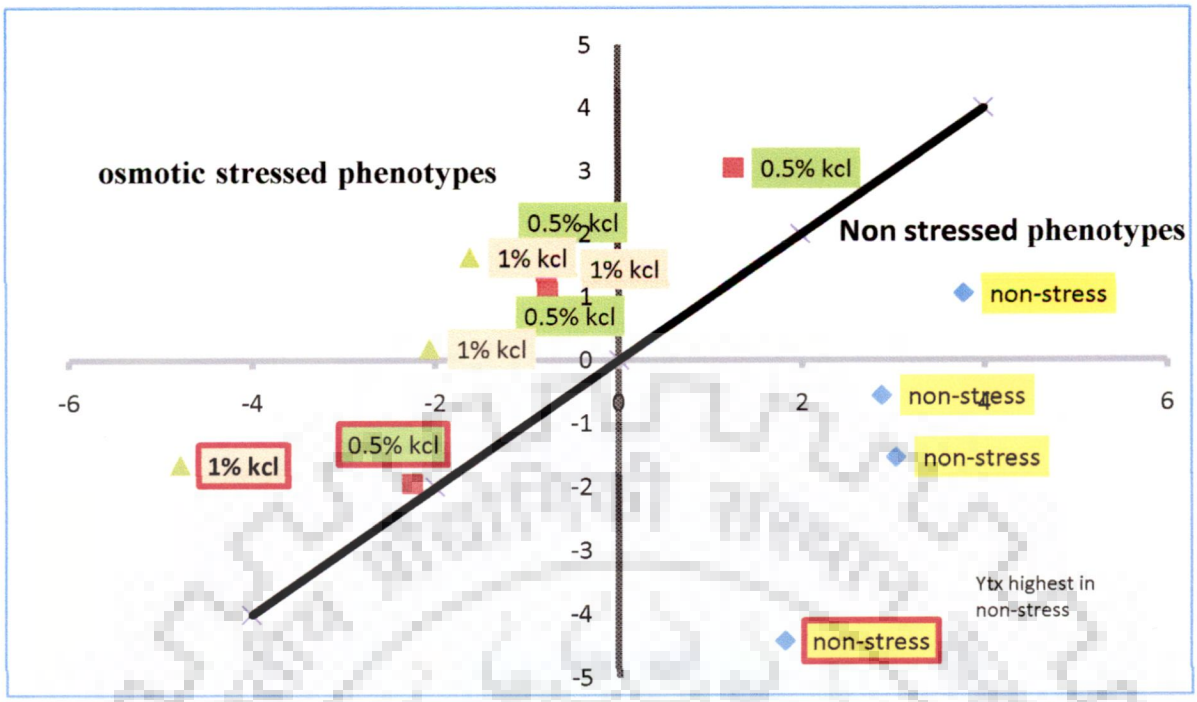


Fig 4.43 Score plot of first principal component and second principal component differentiating non-stress and stress phenotypes. Data labels with bold red outline are highest trehalose yield stage in respective non stress (yellow color), 0.5 % KCl (green color) and 1% KCl (pink color).

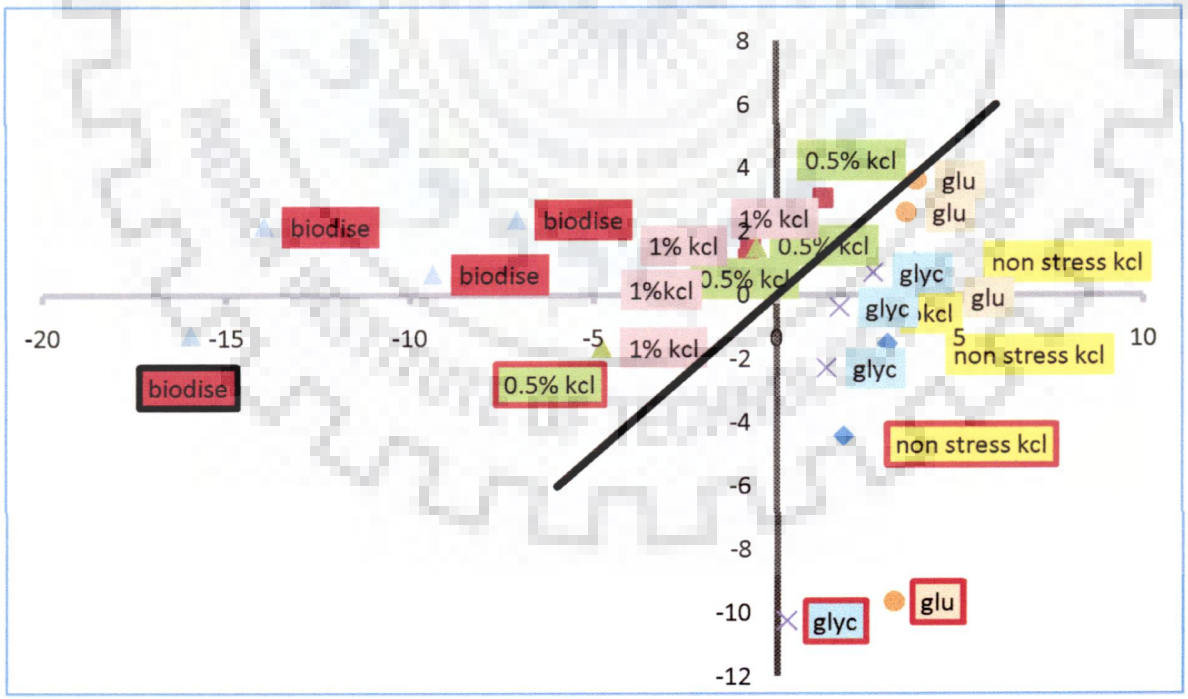


Fig 4.44 Projection of independent experimental data on score plot, differentiating microbes grown in non-stress (glucose (glu) and glycerol (glyc)) and osmotic stress (biodiesel waste, KCl as osmotic stress source) conditions.

After introduction of new scale for assessing the extent of osmotic stress it is worthwhile to explore the global effects of osmotic stress on various enzymes of trehalose biosynthesis pathway through loading plot of principal components (table 4.12, fig 4.45). In the loading plot thirteen different parameters can be differentiated based on their response against osmotic stress. From score plot, it was concluded that under osmotic stress condition, score value of first principal component should have more negative value (fig 4.43). Thus in loading plot, parameters having negative loading factor value of first principal component will be over-expressed whereas parameters having positive loading factor value of first principal component will be down regulated. From the loading plot it can be seen that among the thirteen parameters, eight parameters have x value less than -0.25 and three parameters had values higher than 0.1 (table 4.12, fig 4.45). Two parameters have values higher than -0.007. Thus it can be predicted that under osmotic stress condition, trehalase, trehalose synthase, ADP-glucose pyrophosphorylase, UDP-glucose pyrophosphorylase, phosphoglucomutase, GDP-glucose pyrophosphorylase and OtsA will be over-expressed and phosphoglucoisomerase, UDP-glucose dehydrogenase will be down-regulated. Similarly maltose contents of cell will increase with simultaneous decrease in glycogen contents under osmotic stress condition. Now if highest trehalose yield is considered than then it should have lower score value (higher negative value) of first principal component (which is same as osmotic stress phenotype) and minimum negative score value of second principal component. Thus for achieving higher trehalose yield, activities of trehalase, trehalose synthase, ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase, UDP-glucose pyrophosphorylase, phosphoglucomutase, OtsA should be overexpressed (all metabolic parameters have high negative loading factors for first principal component) whereas UDP-glucose dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and isomerise activities and glycogen concentration should decrease (as high loading factors in first principal component). However, extent of down regulation will be maximum with UDP-G-dehydrogenase and glycogen. If extent of over expressions of ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase are considered than it should be highest with ADP-glucose pyrophosphorylase followed by GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase (as these parameters have nearly equal loading factors for first principal component but loading factor for second principal component of ADP-glucose pyrophosphorylase has positive value whereas loading factors for second principal component of GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase have

negative value) (table 4.12). Similarly, it can also be predicted that OtsA and phosphoglucomutase should have similar level of over-expression but it should be less than ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase (as loading factor for second principal component of phoshphoglucomutase and OtsA have higher negative value). By same analogy it can be predicted that trehalase and TreS should also have over-expressions to a maximum extent as they have positive loading factor values for second principle component and lowest loading factor value for first principal component. However the role of trehalase is ambiguous as it has a role in catabolic pathway of trehalase. Thus these quantitative predictions can be made from principal components. The advantages of principal component analysis were quantitative prediction and visualization of global effects of osmotic stress in *P. shermanii* NCIM 5137.

Table 4.10: Trehalose, enzyme activities and metabolite concentration in carbon source crude glycerol, Trehalose in mg/g of biomass (mg/gb), enzyme activities in nanokatal/g of biomass (nkat/gb) and metabolite concentration in ng/gb of biomass (Nm/gb)

Trehalose mg/gb	OtsA nkat/gb	PGM nkat/gb	UDPGy nkat/gb	ADPGy nkat/gb	GDPGy nkat/gb	UDPGD nkat/gb	Iso nkat/gb	PPP nkat/gb	Trease nkat/gb	TreS nkat/gb	G6P nkat/gb	maltose mg/gb	Glycogen mg/gb
386±4	6045±11	211±5	383±4	608±4	674±4	44±1	973±6	1661±16	555±6	30±1	$1.8x10^5$± 5	0±0	0±0
404±6	8108±9	294±5	396±3	635±6	615±8	132±2	1290±11	2579±12	568±7	31±2	$2.5x10^5$± 8	77±1	65±2
281±8	2986±12	238±6	240±7	433±3	490±11	96±1	1046±13	1839±12	365±4	22±1	$2.5x10^5$± 5	44±6	34±3
261±3	2198±4	88.2±5	242±8	423±1	469±12	46±1	728±4	1719±8	342±8	19±4	$2.2x10^5$± 5	30±4	23±2

Where OtsA-trehalose-6-phosphate synthetase, PGM-phosphoglucomutase, UDPGy-UDP-glucose pyrophosphorylase, ADPGy-ADP-glucose pyrophosphorylase, GDPGy-GDP-glucose pyrophosphorylase, UDPGD-UDP-glucose dehydrogenase, Iso-phosphoglucoisomerase, G6PD-glucose-6-phosphate dehydrogenase, trease- trehalase, TreS-Trehalose synthase, G6P-glucose-6-phosphate concentration, UDPG-UDP-glucose concentration

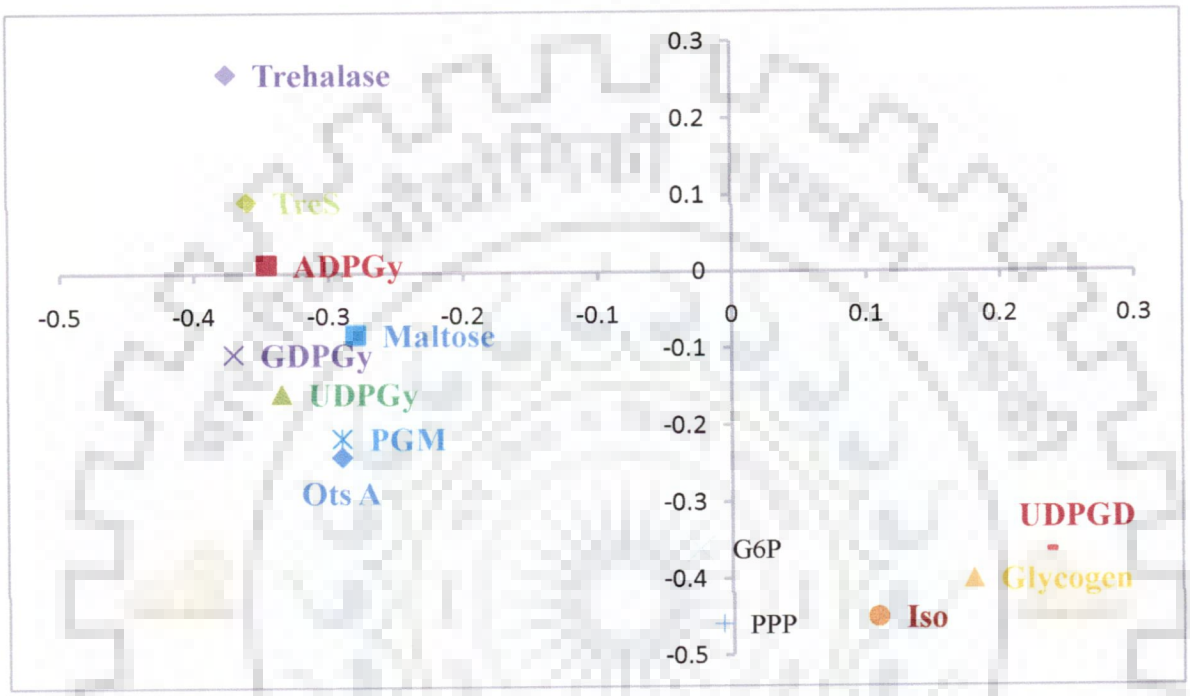


Fig 4.45 Loading plot of principal component analysis

Table 4.11: Intracellular trehalose content, enzyme activities and metabolite concentration in carbon source-glucose and glycerol in *P. shermanii* NCIM 5137, PGM-phosphoglucomutase, UDPGy-UDP-glucose pyrophosphorylase, ADP-Gy-ADP-glucose pyrophosphorylase, GDPGy-GDP-glucose pyrophosphorylase, UDPGD-UDP-glucose dehydrogenase, BIP-fructose biphosphotase, Iso-Isomerase, G6PD-glucose-6-phosphotase dehydrogenase, Trase-Trehalase, TreS-trehalose synthase, G6P, UDPg, PA, LA concentration of glucose-6-phosphate UDP-glucose, propionic acid, lactic acid, trehalose in mg/g of biomass, enzyme activities in nanokatal/g of biomass (nkat/g) and metabolites in mg/g of biomass

Trehalose Mg/g biomass)	Ots A nkat/g biomass	PGM nkat/g biomass	UDPGy nkat/g biomass	ADPGy nkat/g biomass	GDPGy nkat/g biomass	UDPGd nkat/g biomass	Iso nkat/g biomass	G6PD nkat/g biomass	Trase nkat/g biomass	TreS nkat/g biomass	G6P Nmol/g biomass	Maltose Mg/g	Glycogen Mg/g
Glucose													
87±2	404±7	61.8±4	41±2	5.1±3	109±6	1938±8	4188±11	4283± 12	78±2	1.7±0.05	$8x10^5$±1.6	22±4	28.5±3
88±4	255±6	42.2±3	29±1	4.0±0.4	60±2	1399±9	1593±12	1895±9	46.7±1	1.2±0.3	$3.4x10^5$±2	34±2	19.37±2
68±8	176±4	41.1±3	27±3	0.6±0.01	11±4	1099±4	1380±8	1271±8	33.7±2	0.7±0.02	$1.6x10^5$±4	59±3	13.2±2
63±3	123±3	30.9±2	20±2	0	6.7±0.2	848±3	1108±9	1407±4	36.7±1	0.4±0.01	$1.1x10^5$±4	44±2	12.19±1
Glycerol													
381±11	7293±9	302±8	238±4	71±1	172±1	1948±11	1193±4	2478±9	41±3	0	$7.6x10^5$±4	5±0.5	139±3
270±8	3419±11	264±4	195±3	27±1	55±3	1066±12	1131±5	926±11	18±3	0	$4.2x10^5$±3	8±0.8	33±2
219±9	2351±8	234±2	159±2	4±1	7.9±0.1	272±4	957±3	403±4	18±3	0	$3.8x10^5$±4	12±1	30±5
130±4	2313±4	160±1	107±5	1.5±0.01	7.8±0.2	423±2	471±4	225±9	11.7±4	0	$3.6x10^5$±5	7±0.4	28±1

Table 4.12 Loading factors of PC 1 and PC 2

Enzyme/metabolite	Loading factor (PC 1)	Loading Factor (PC 2)
OtsA	-0.29	-0.24
ADP-G pyr	-0.34	0.01
UDP-G pyr	-0.33	-0.15
GDP-G pyr	-0.37	-0.105
PGM	-0.29	-0.21
Iso	0.108	-0.45
PPP	-0.007	-0.45
UDPGD	0.235	-0.36
TreS	-0.36	0.094
Trehalase	-0.37	0.26
Maltose	-0.28	-0.08
Glycogen	0.18	-0.4
Glucose-6-P	-0.02	-0.36

However, it is necessary to validate our predictions based on developed principal components using independent experimental data sets. In the present study, all thirteen parameters were compared between crude glycerol grown cells (18 hr) and pure glycerol grown cells (18 hr) in complex medium. The ratio of individual parameters in crude glycerol to pure glycerol medium was plotted and from Fig 4.46 it can be seen that maximum over expressions were obtained with trehalase, ADP-glucose pyrophosphorylase, maltose concentration and in case of trehalose synthase, the ratio tends to infinity (not shown in Fig 4.46). The down regulation of UDP-glucose dehydrogenase and decrease in glycogen content was also observed in crude glycerol medium as compared to pure glycerol medium. In case of isomerase, there was marginal over expression in crude glycerol media as compared to pure glycerol media and this observation is contrary to the prediction obtained from loading plot. In case of OtsA and phosphoglucomutase, extents of over-expressions in crude glycerol media as compared to

pure glycerol media were nearly similar and this is in accordance to the predictions obtained with loading plot.

The discrepancy in observations and predictions needs further investigation and one probable reason may be that other than osmotic stress some other type of stress is also working in crude glycerol media. Thus this study first time analyzed globally the effects of osmotic stress on various enzymes and metabolites of trehalose biosynthesis pathway. The osmotic stress response of cells can be predicted from loading plot and thus developed principal component analysis model can differentiate independent experimental data into stress phenotype and non-stress phenotype. This is first such report on application of principal component analysis to analyze osmotic stress response of microbial cell. Although trehalose biosynthesis requires few metabolites and enzyme reactions but it seems to have a more complex metabolic regulation. The bacterial cell adapts to changing environments by changing the level of various biochemical metabolites and enzymes activities. Understanding of this complex phenomenon is not very easy without statistical or multivariate approaches. Hence in the present study we had shown application of one such approach known as principle component analysis. We collected maximum number of relevant enzymes activities and concentration of metabolites under osmotic stress and non-stress conditions. Predictions about the influences of these variables on trehalose bio-synthesis were attempted by principle component analysis. This approach was suitable as it gave influence level of individual variable and first time it was revealed that expression of nucleotide sugar synthesising enzymes had varying influences on trehalose synthesis and role of ADP-glucose pyrophosphorylase was prominent. In this study, study of influences of various relevant enzyme activities and metabolite concentration on trehalose biosynthesis were elucidated which is rarely reported in literature and concluded that regulation of trehalose synthesis is complex and not limited to few enzymes. Using the principal component analysis it was possible to quantitatively predict the effect of over-expression of individual enzyme on trehalose biosynthesis. Overall, it can be concluded that influence level of various enzymes of trehalose biosynthesis pathway on trehalose yield are equally predicted by qualitative analysis (section 4.5) and principal component analysis. Quantitative prediction as well as overall prediction about whole trehalose biosynthesis pathway was made.

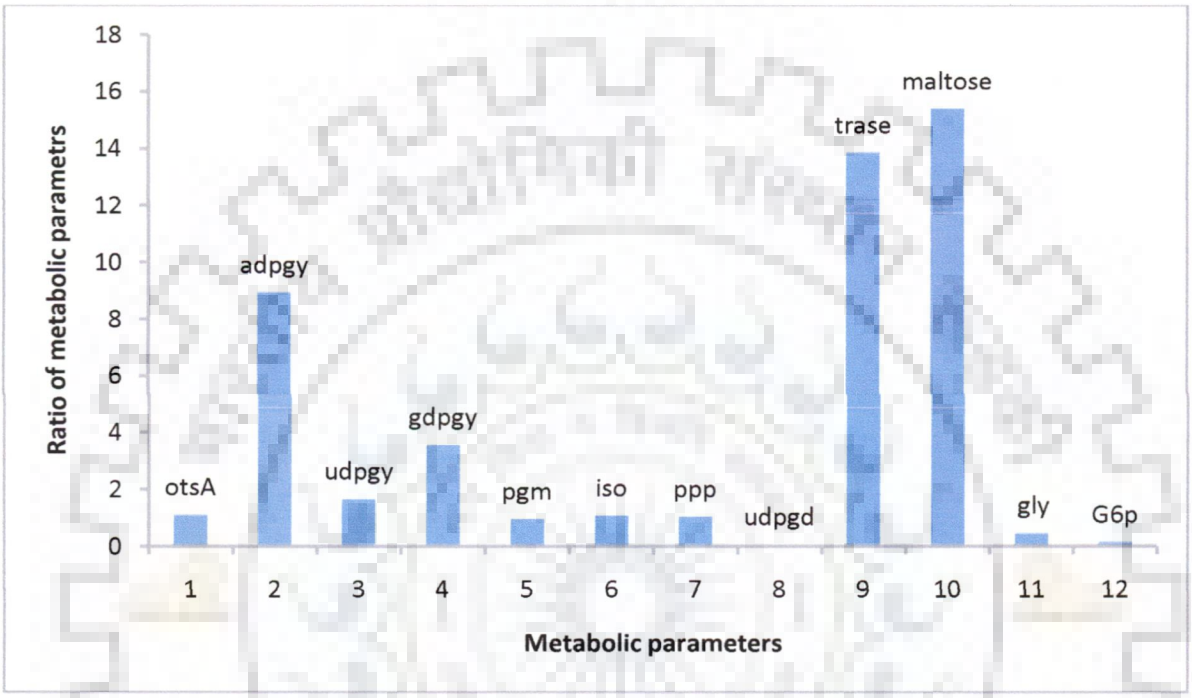


Fig. 4.46 Ratio of metabolic parameter in crude glycerol to pure glycerol at highest trehalose accumulation stage of growth

4.7. Use of osmotically sensitive mutant of *Propionibacterium freudenreichii* subspp *shermanii* for the simultaneous production of organic acids and trehalose from biodiesel waste based crude glycerol

The consequence of osmotic stress is higher trehalose accumulation in *Propionibacterium shermanii*; hence we tried to isolate an osmotically sensitive mutant by chemical mutagenesis with the objective of achieving higher trehalose yield at minimal osmotic stress condition. Amongst the 1000 colonies of EMS treated cells; four mutants were sensitive to 3% of NaCl (not able to grow at and above 3% NaCl concentration). Another peculiar behaviour was that these mutants were able to grow at 120 µg/ml of nisin whereas parent strain was not able to grow above 10 µg/ml of nisin concentration. These mutants were also sensitive to 0.05% of SDS (Sodium dodecyl sulphate). These osmotic sensitive and nisin resistant mutants (*Propionibacterium shermanii* Nis^r) were sensitive to other antibiotics like ampicillin and penicillin (parent strain were also sensitive to these antibiotics). Amongst these four mutants one mutant was found to be highest trehalose producer. In mutant, in non-stress condition trehalose yields both with respect to biomass and substrate in carbon source glycerol and crude glycerol were higher in comparison to parent strain. In crude glycerol, yield of trehalose was much higher than parent strain (600 mg/g of biomass highest while final 400 mg/g of biomass). Amongst all the enzymes expression of ADP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase were significantly higher as compared to parent strain and their role for enhanced trehalose production was proposed in mutant. Relation between enhanced trehalose accumulation and nisin resistance with osmotic sensitivity in any microbe was first time reported here.

4.7.1. Mutagenesis and trehalose yield in mutant in contrast to parent strain

The common defence strategy of microbes against environmental stresses includes accumulation of organic compatible solutes like trehalose, glutamate, proline e.t.c. In particular, it is reported that there is enhanced trehalose accumulation under osmotic stress in *P. freudenreichii* (Cardoso et al., 2007, Ruhal et al., 2011). In the present study, it was also observed that trehalose accumulation enhanced under osmotic stress condition in *P. shermanii* NCIM 5137. Based on these observations, it was envisaged that any bacterial cell unable to survive in higher osmolality, probably will have the natural tendency of accumulating higher trehalose even in non stress condition. To this end, the advantage of chemical mutagenesis was taken to isolate a bacterial mutant cell with poor viability in osmotic stress and exploring its potential for higher trehalose production. Previously, this

type of strategy involving mutant development by chemical mutagenesis for higher trehalose yield was successfully applied in yeast. In fact, trehalase mutant (obtained after chemical mutagenesis) had shown strong improvement in trehalose accumulation (Chi et al 2003).. Similarly a thermo sensitive mutant of yeast accumulating trehalose with higher yield was also reported recently (Wang D-S et al., 2011).

The screening for isolation of mutant bacterial cell with osmotic sensitivity was done after treating cells of *P. shermanii* NCIM 5137 with chemical mutagen EMS (ethyl methane sulphonate) as described in material and methods. Each mutagen treated single cell colonies were replica plated on agar plates with 0%, 1%, 2%, 3%, 4% and 8% NaCl. From these colonies, desired mutants were selected based on their inability to grow at lowest concentration of NaCl and corresponding colonies grown in non-stress (0% NaCl) condition were chosen for further study. In other words, the colonies which grow on non-stress plate while not on lowest concentration of NaCl plates were selected. About thousand single cell colonies were screened for osmotic sensitivity using the above methodology. It was observed that all colonies except four were able to grow at 1%, 2%, 3% and 4% NaCl containing agar plates. These four colonies were not able to grow at 3% NaCl agar plates or agar plates with 4% and 8% NaCl.

Thus, finally four mutants which were sensitive to 3% NaCl (unable to grow in media with 3% NaCl) were selected and designated as osmotically sensitive mutant. Parent strain was able to grow upto 4% NaCl plate. Four osmotic sensitive mutants were obtained but these mutants were further screened for their ability to produce trehalose. Additionally, these mutants were non-viable at and above 0.05% SDS whereas parent strain was able to grow at this SDS concentration. Therefore, these mutants were osmotically sensitive and for surviving in osmotic stress condition it should have the potential to accumulate osmolytes like trehalose. The four mutants were screened for trehalose production ability in media with carbon source glycerol as listed in table 4.13. Glycerol was chosen as carbon source because target was trehalose production from crude glycerol. In table 4.13, trehalose yields obtained with respect to biomass and substrate consumed at stationary phase are shown and for comparison with parent strain data was also presented. Thus it can be concluded that mutant 1 is better suited for trehalose production. Except mutant 3, all other mutants showed higher trehalose production capacity.

Interestingly, during the evaluation of physiological changes in mutant cell it was found that mutant cells became more nisin resistance as compared to parent strain. It seems

that nisin binding was lost in these mutants as they were resistant up to the concentration of 120 $\mu g.ml^{-1}$ of nisin as compared to parent strain which was unable to grow above 10 $\mu g.ml^{-1}$ of nisin (table 4.14). In fact, all these mutants and parent strains were sensitive to other antibiotics like penicillin and ampicillin. This improvement of trehalose yield in a nisin resistant and osmotic sensitive mutant is first time reported here. On the basis of trehalose yield in table 4.13, mutant 1 was chosen for further study of trehalose production. As it was observed that parent strain accumulates higher trehalose in carbon source glycerol, mutant was also studied for trehalose production with carbon source glycerol in static flask condition. In mutant, the highest trehalose yield with respect to biomass was 331 mg/g of biomass and final trehalose yield in stationary phase was 228 mg/g of biomass (fig 4.47) while in parent strain final trehalose yield was 88 mg/g of biomass which was 2.5 times lower than mutant strain (fig 4.47). It was also observed that biomass growth was slow in mutant as compared to parent. In view of these results it was concluded that this approach of developing mutant sensitive to particular stress has proven effective for enhanced production of trehalose in non-stress condition. Therefore this strategy showed remarkable improvement of final trehalose yield in mutant in comparison to parent strain due to lower degradation rate of trehalose with growth. The trehalose accumulations in mutant with other carbon sources like glucose (with osmotic stress), glucose and sucrose are shown in fig 4.48, 4.49 and 4.50 and trehalose yield with respect to biomass in parent strain are also listed in table 4.15 which illustrate that glycerol is the best carbon source for trehalose production. When comparison was made of trehalose yield with respect to substrate consumed, it was observed that yield was 2.7 fold higher in glycerol media as compared to sucrose media. Thus our selection of glycerol as carbon source instead of sucrose seems to be correct.

Table 4.13: Comparison of trehalose yield based on biomass (mg/g of biomass, Y_{tx}) and substrate consumed (mg/g of substrate consumed, Y_{ts}) of isolated osmotic sensitive mutants in contrast to parent strain in media with glycerol as carbon source and static flask culturing.

Yield	**Y_{tx}**	**Y_{ts}**
Mutant 1	233±2	11.8±1
Mutant 2	191±4	8.8±0.5
Mutant 3	66±2	6.2±1
Mutant 4	184±6	8.62±0.4
Parent	88±4	0.72±0.03

Table 4.14: Nisin resistance in osmotic sensitive mutants at different concentrations of nisin ($\mu g.ml^{-1}$) in comparison to wild strain

Mutant no.	**0.001**	**0.01**	**0.5**	**1**	**10**	**40**	**80**	**120**
Mutant 1	++++++	++++++	++++++	++++++	++++++	++++++	++++++	++++++
Mutant 2	++++++	++++++	++++++	++++++	++++++	++++++	++++++	++++++
Mutant 3	+++	+++	+++	+++	+++	+++	+++	+++
Mutant 4	+++	+++	+++	+++	+++	+++	+++	+++
Parent	+	+	+	+	-	-	-	-

++++++	very good growth	+	poor growth
+++	good growth	-	no growth

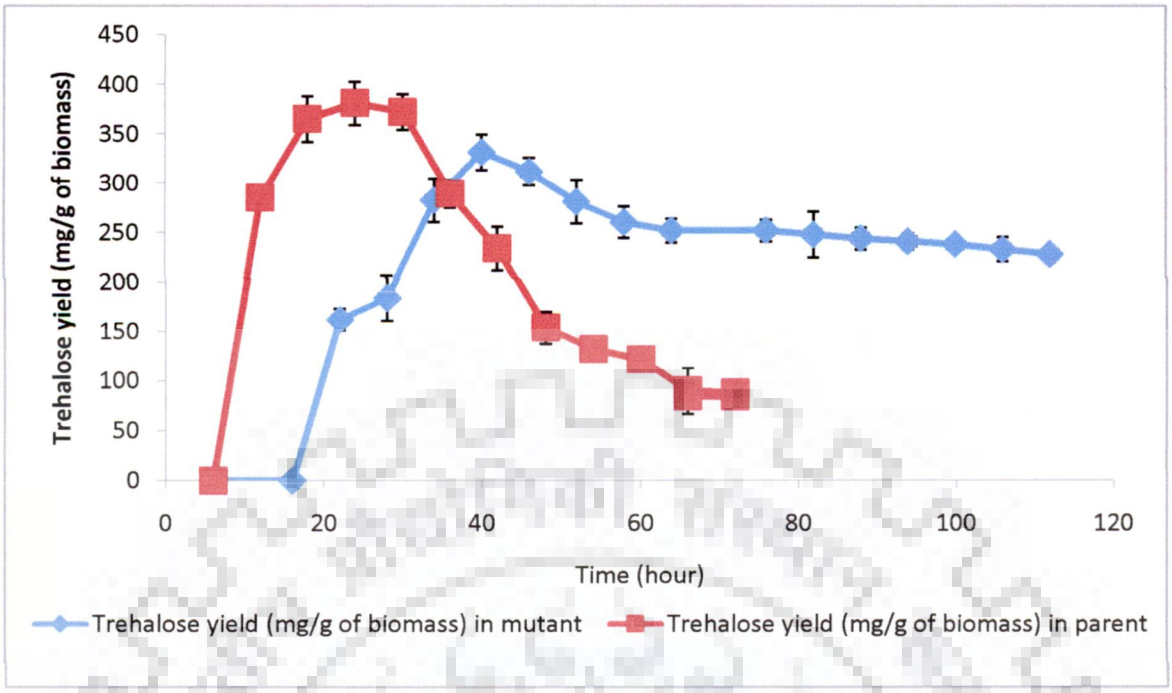


Fig 4.47 Trehalose yield in parent and mutant strain in medium with pure glycerol as carbon source

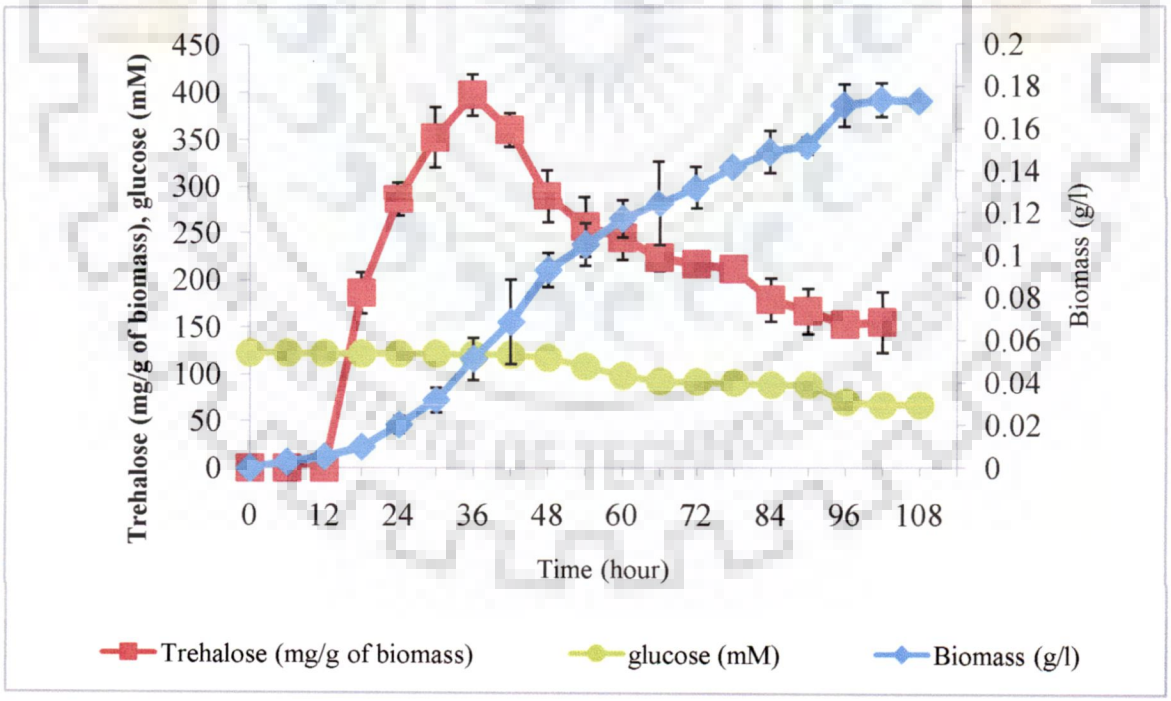


Fig 4.48 Trehalose yield, substrate concentration and biomass growth profiles in mutant under osmotic stress with carbon source glucose

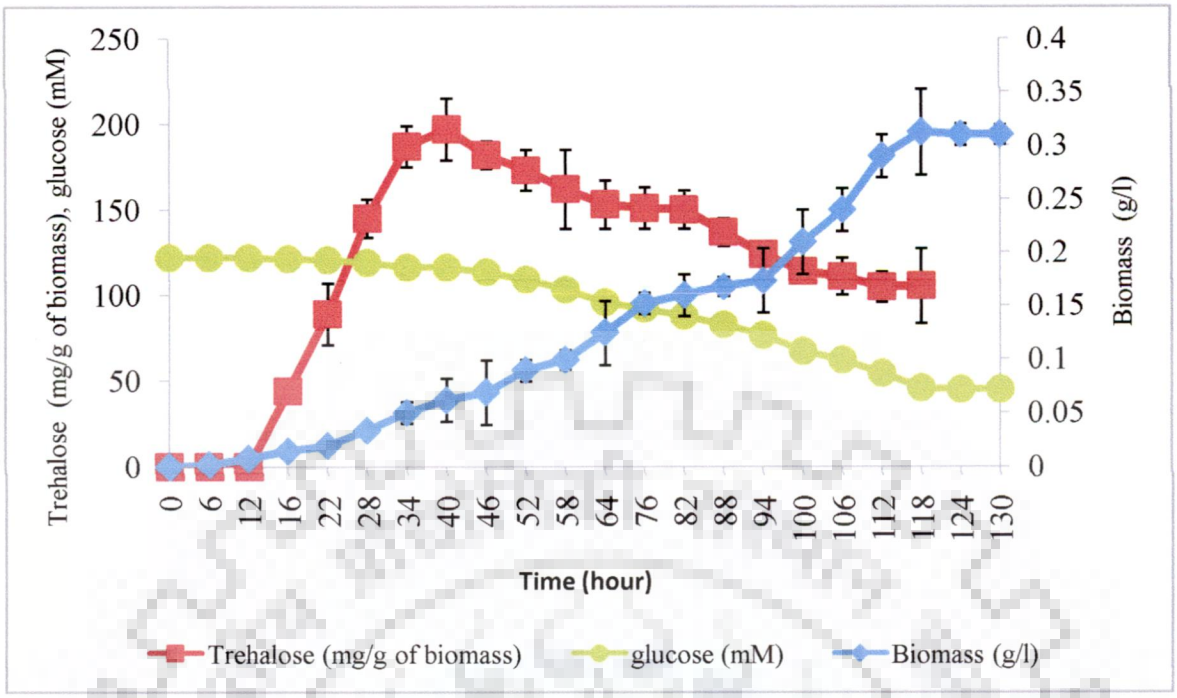


Fig 4.49 Trehalose yield, substrate concentration and biomass growth profiles in mutant under non-stress condition with glucose as carbon source

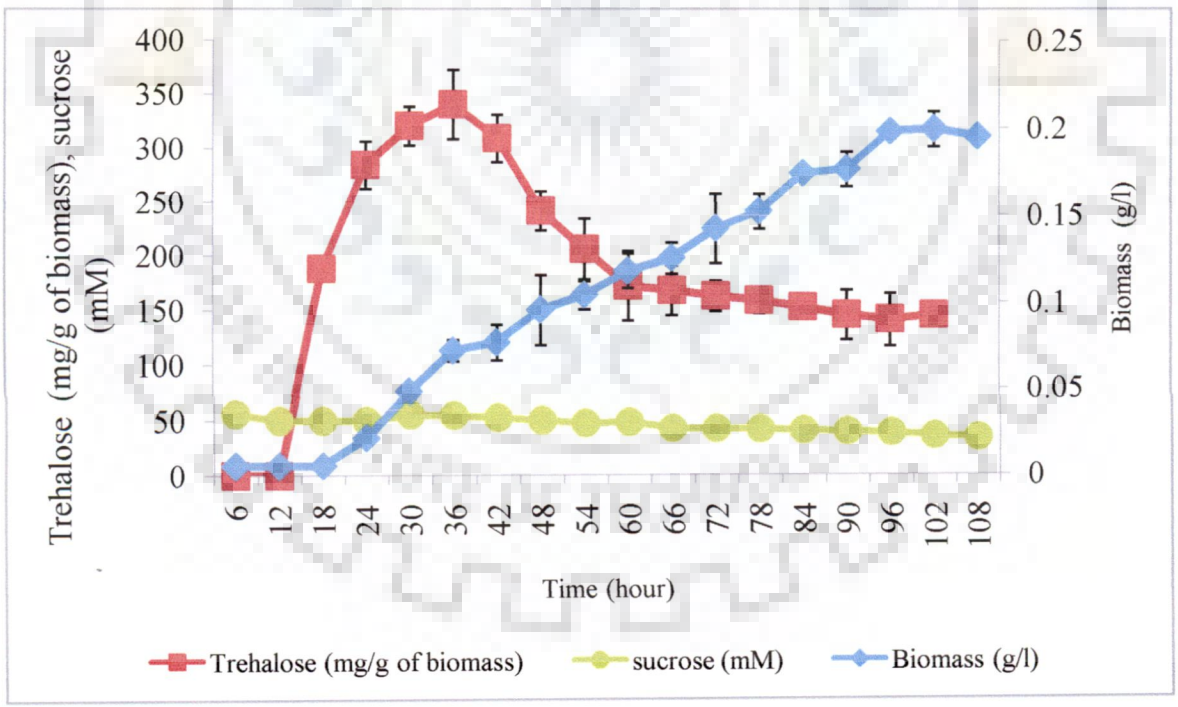


Fig 4.50 Trehalose yield, substrate concentration and biomass growth profiles in mutant with sucrose as carbon source

Table 4.15 Comparison of trehalose yields in osmotic stress and with various carbon sources, where Ytx is trehalose yield in mg/g of biomass and final refer to trehalose at the stationary phase. Yts is trehalose yield (final) with respect to substrate consumed

Osmotic stress/C-source	**mutant** $Y_{tx\ highest}$	**mutant** $Y_{tx\ final}$	**Mutant** $Y_{ts\ final}$	**Parent** $Y_{tx\ highest}$	**Parent** $Y_{tx\ final}$
Osmotic stress	396	154	2.6	122	44
Glucose	196	105	2.39	93	15
Sucrose	341	148	4.4	172	85
Glycerol	331	233	11.8	381	89

From these trehalose production profiles with these carbon sources it can be concluded that mutant 1 seems to be a better trehalose producer. Thus strategy of developing an osmotically sensitive mutant for higher trehalose accumulation is successful in the present study. This is probably the first report on developing an osmotically sensitive mutant for higher trehalose production.

4.7.2. Production of trehalose with crude glycerol

Production of chemicals from microbes is always advantageous as microbes can use waste materials to synthesize many chemicals while conventional chemical synthesis methods depends more on petroleum based substrates. Easy scalability of microbial process also makes them most suitable for production of molecules like trehalose and they are better than conventional methods which requires large amount of natural resources. Since highest trehalose yield achieved was with substrate glycerol, hence trehalose production from an alternative cheap source of glycerol known as crude glycerol obtained from biodiesel waste was studied for trehalose production with a mutant 1. In a similar study, crude glycerol was found more suitable for higher trehalose production with parent strain (Ruhal et al., 2011).

During static flask studies with crude glycerol as carbon source highest trehalose yield with respect to biomass was 685 mg. (g of biomass)$^{-1}$ and final yield was 412 mg. (g of biomass)$^{-1}$, which according to our knowledge is the highest yield ever reported (fig 4.51). In a recent study with mutant strain of *Saccharomycopsis* trehalose yield of 28% with respect to biomass (in the present study 41%) was reported using cassava starch as carbon source (Wang D-S et al., 2011). Similarly in *Corynebacterium* recombinant strain was able to produce trehalose with yield of 31% of biomass with glucose as carbon source (Carpinelli et al 2006). Trehalose yields with respect to biomass and substrate were improved almost two-three folds in mutant (412 mg/g of biomass, 0.08 g/g of substrate) in comparison to parent strain (final 128 mg/g of biomass and 0.04 g/g of substrate) (fig 4.52).

The yields of microbial metabolites during fermentation are function of cultivation conditions and hence a study on trehalose production in bioreactor was carried out to avoid the lowering of pH during fermentation as observed in static flask study. Thus, study of trehalose production with selected mutant was done under controlled conditions of pH and dissolved oxygen in batch reactor. In batch reactor with pure glycerol as substrate, highest trehalose yield achieved with respect to biomass was 376 mg.(g of biomass)$^{-1}$ while final trehalose yield was 138 mg. (g of biomass)$^{-1}$. On other hand highest yield of trehalose with respect to substrate consumed was 80 mg. (g of substrate consumed)$^{-1}$ and final yield was 27 mg. (g of substrate consumed)$^{-1}$ (Fig 4.53 & 4.54). In crude glycerol media highest trehalose was 530 mg. (g of biomass)$^{-1}$ while final yield was 391 mg. (g of biomass)$^{-1}$ and trehalose yield with respect to substrate consumed was 142 mg. (g of substrate consumed)$^{-}$

[1]while final yield was 90 mg. (g of substrate consumed)$^{-1}$ (Fig 4.52 & 4.53). In crude glycerol, final trehalose yield achieved with respect to biomass was three fold higher while with respect to substrate it was three fold higher as compared to pure glycerol media. While complete substrate consumption was not achieved and propionic and lactic acid yields were 0.46 g. (g of substrate consumed)$^{-1}$ and 0.3 g. (g of substrate consumed) respectively (fig 4.52). In comparison to parent strain, propionic acid yield was lower in mutant strain but lactic acid yield was higher under similar experimental condition. Thus this study is advantageous as one gram of crude glycerol results in 0.46 gram of propionic acid, 0.3 gram of lactic acid and 0.09 g of trehalose.

In the present study, trehalose yield achieved with respect to biomass was higher than that obtained with yeast strain (Wang et al., 2011) using starch as substrate. On other hand in *Corynebacterium* trehalose yield obtained with respect to substrate was higher than that obtained in present study. Advantage of present study was trehalose production from crude glycerol whereas in *Corynebacterium* glucose was used. Beside two major organic acids were also produced along with trehalose using the developed method.

4.7.3. Physiology of mutant in relation to enzyme activities

Trehalose accumulation was enhanced in the mutant and it was higher with crude glycerol. As crude glycerol contain KCl as impurities so it can be predicted that due to osmotic stress trehalose production was highest in the mutant. The metabolic parameters which are directly or indirectly involved with trehalose biosynthesis were investigated as they might have influence on the improved efficiency of trehalose production from mutant cell of *P. shermanii*. Three pathways of trehalose biosynthesis are known as OtsAB, TreYZ and TreS pathway. In *P. freudenreichii*, OtsAB pathway is followed (Cardoso et al., 2007). In the present study no activity of TreS and TreYZ were observed in mutant. Hence OtsAB pathway was considered for the study. OtsA is trehalose-6-phosphate synthetase which synthesizes trehalose-6-phosphate from substrates NDP-glucose (UDP-glucose/GDP-glucose/ADP-glucose) and glucose-6-phosphate. Hence enzyme activities involved in synthesis and utilization of NDP-glucose and glucose-6-phosphate were considered for study with mutant. NDP-glucose synthesis occurres through ADP-glucose/UDP-glucose/GDP-glucose pyrophosphorylase while glucose-6-phosphate is involved in glycolysis and pentose phosphate pathway hence enzyme activities of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were determined.

Similarly, activities of phosphoglucomutase, UDP-glucose dehydrogenase, trehalase were also determined along with maltose and glycogen concentrations.

It was noticed that on comparison of enzymes activities at highest trehalose accumulation stage in mutant and parent strain with carbon source glycerol, activities of some enzymes were higher in mutant together with higher trehalose yield. The activities which are different in mutant and parent were compared in carbon source glycerol. The activity of OtsA was 1.2 fold higher in mutant as compared to parent strain (table 4.16). Interestingly, expression level of enzymes synthesising NDP-glucose were different from each other (table 4.16). Amongst them activity of ADP-glucose pyrophosphorylase was around 18 fold higher in mutant, similarly activities of GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase were 6.5 fold and 1.3 fold higher in mutant in comparison to parent strain (table 4.16). On the other hand, activity of trehalose degrading enzyme trehalase was 20 fold higher in mutant strain which may be presence of high trehalose content as was noted in yeast. No TreYZ and TreS activities were found (table 4.16). In mutant strain, there was no accumulation of glycogen and maltose when grown with glycerol. Thus one of remarkable difference with parent strain was absence of glycogen and maltose.

Other intracellular enzyme activities of glucose-6-phosphate dehydrogenase (PPP), phosphoglucomutase did not show any remarkable difference while activity of UDP-glucose dehydrogenase was lower in mutant which signify that mutant has set up its priority for higher trehalose synthesis and hence decrease the UDP-glucose degradation by UDP-glucose dehydrogenase. In case of phosphoglucoisomerase, activity almost doubled in mutant strain as compared to parent strain. From the enzymes activities it was clear that over-expressions of ADP-glucose/GDP-G pyrophosphorylase together with OtsA was effective for higher trehalose yield. It seems that by diverting flux through ADP-glucose and GDP-glucose is more favourable for higher trehalose yield. Similar observation was also made during study of osmotic stress condition in parent strain. Thus a bacterium probably uses ADP-glucose as regulatory molecule for higher trehalose yield. Figure 4.54 shows the difference in enzyme activities in mutant and parent strain. In previous reports in yeast higher activities of OtsA, UDP-G pyrophosphorylase and phosphoglucomutase under osmotic stress were reported (Voit, 2003), similarly in *Corynebacterium* over-expression of OtsA together with UDP-G pyrophosphorylase has resulted in higher yield of trehalose (Carpinnelli et al., 2006). Hence in the present study the preferential use of alternate

pathway through ADP-G or GDP-G instead of UDP-G under osmotic stress condition is first time reported.

Many remarkable insights were obtained with parent strain was again validated from the study of osmotic sensitive mutant. Although the true mechanism of protective effect of trehalose was not clearly understood but it was sure that this compound can protect against osmotic stress. In another words, *P. shermanii* adopts the defence strategy against the osmotic stress through enhanced trehalose accumulation. In fact, it was supported during our study with parent strain that effect of osmotic stress results in enhanced trehalose. Additionally, trehalose yield was improved tenfold in mutant strain in comparison to parent strain and hence efficient conversion of crude glycerol from biodiesel waste into trehalose was achieved. While at the physiological level, our proposed importance of ADP-glucose pyrophosphorylase and GDP-glucose pyrophosphorylase on trehalose accumulation was supported in mutant as activities of these enzymes were much higher as compared to parent strain. The decrease in trehalose content of mutant cell in late exponential phase was less than the parent cell under similar experimental condition (Fig 4.47). Although, the trehalase activity responsible for catabolism of trehalose was 20 fold higher in mutant cell as compared to parent strain, still the rate of decrease of trehalose content was low in mutant cell. This decrease in trehalose content with growth can be explained by comparing the level of individual enzyme activity at late exponential phase and maximum trehalose accumulation stage. In case of ADP-glucose pyrophosphorylase, the decrease in activity was four fold in mutant cell whereas in parent cell decrease was 50 fold. Similarly, for OtsA, decrease in enzyme activity was twofold in mutant cell and it was 3.5 fold in parent cell. Similar, conclusion can also be made for GDP-glucose pyrophosphorylase. Thus it can be concluded that due to low rate of decrease of key enzymes activities with growth, rate of decrease of trehalose content of cell in late exponential phase was arrested.

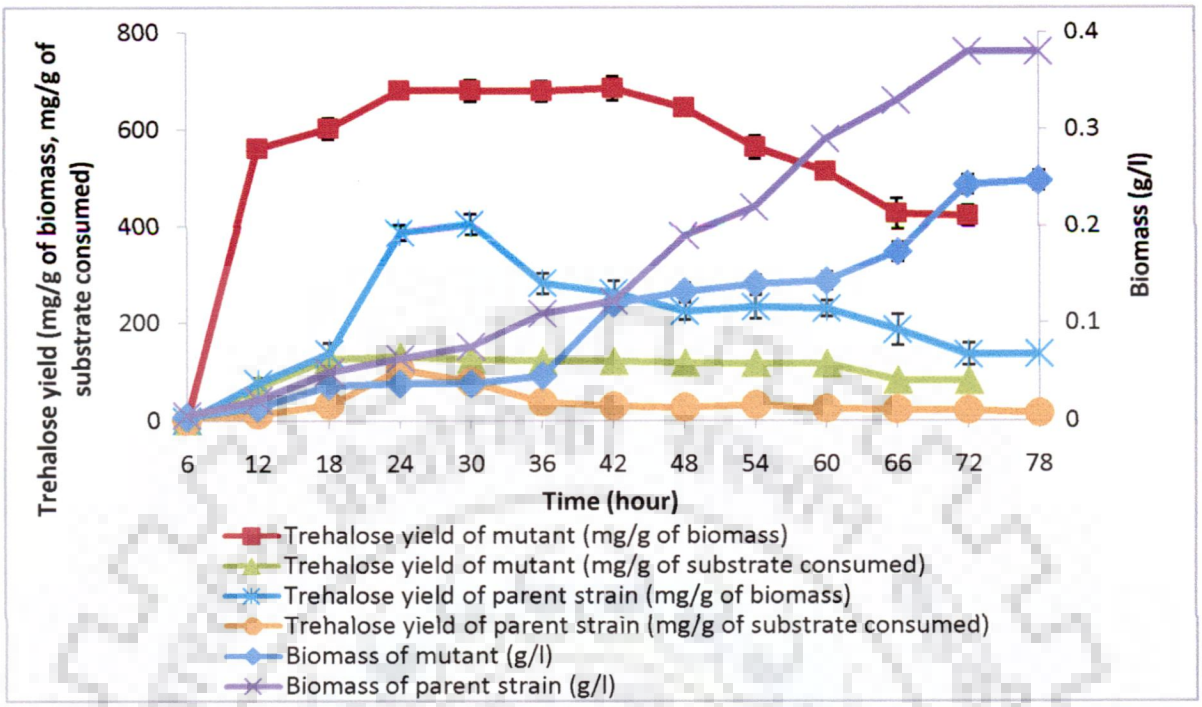


Fig 4.51 Comparison of trehalose yield in static flask conditions in mutant and parent strain using crude glycerol as carbon source

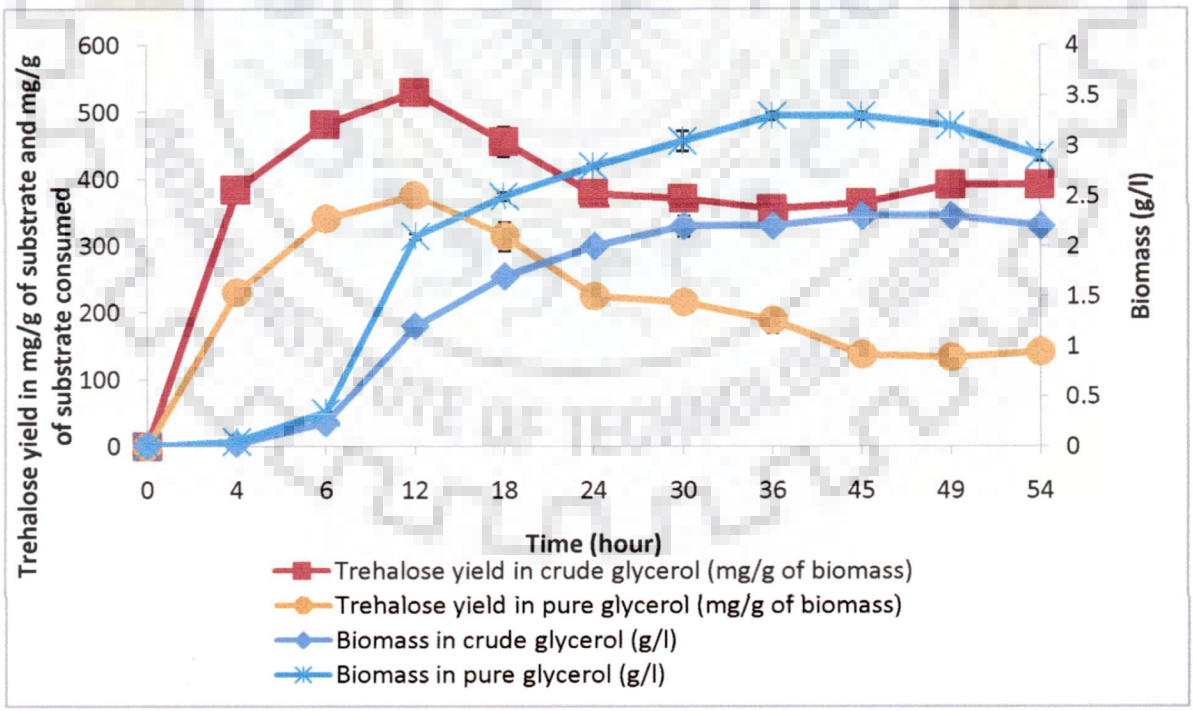


Fig 4.52 Batch reactor fermentation of mutant in media with pure and crude glycerol as carbon source

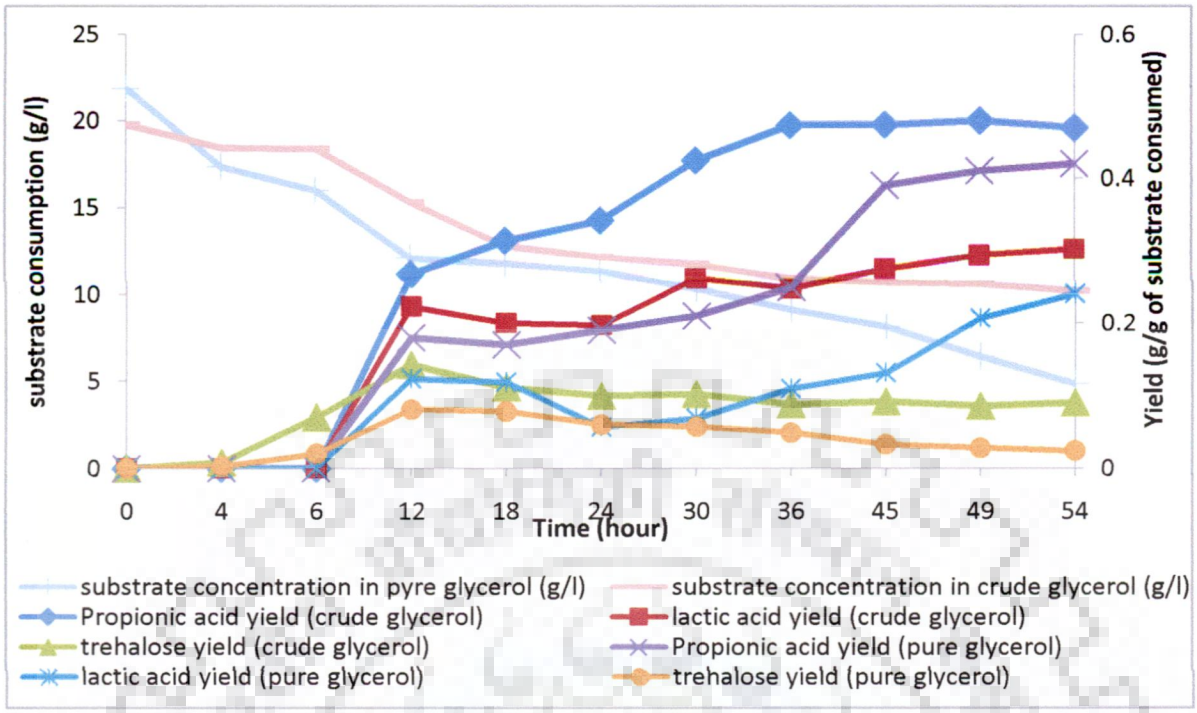


Fig 4.53 Trehalose, propionic acid and lactic acid yields in medium with pure and crude glycerol as carbon source in batch reactor

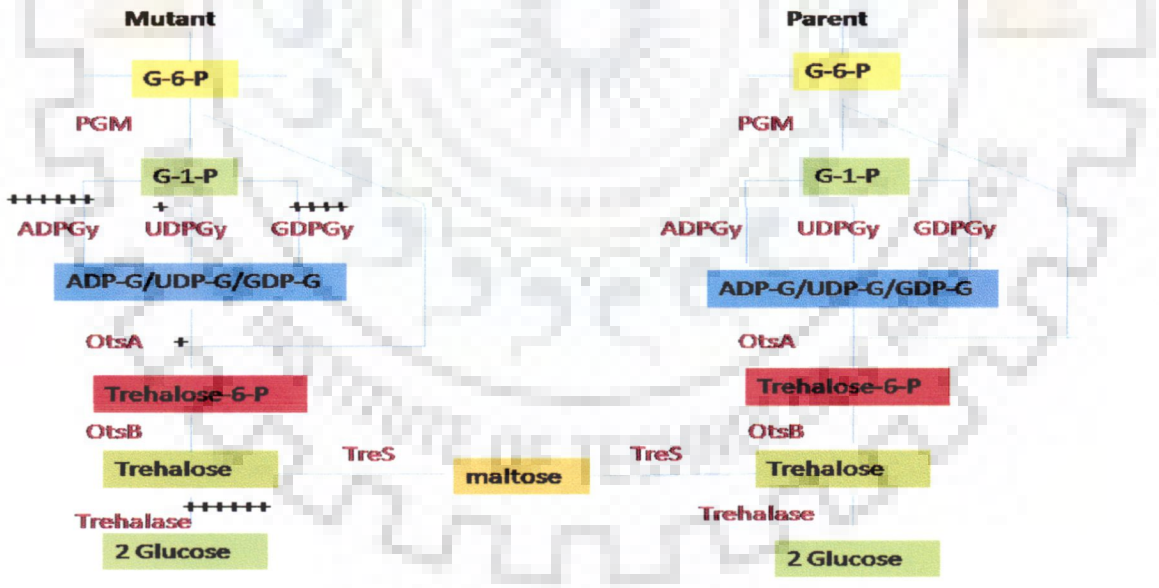


Fig 4.54 Comparison of changes in enzyme activities in parent and mutant strain in relation to trehalose biosynthesis. Degree of increase of enzyme activity is shown by + sign, where PGM (phosphoglucomutase), ADP-G/UDP-G/GDP-G pyr (ADP-G/UDP-G/GDP-G pyrophosphorylase), G-6-P is glucose-6-phosphate, glucose-1-phosphate (G-1-P)

Table 4.16: Specific enzyme activities in nanokatal/g of biomass, OtsA (trehalose-6-phosphate synthetase), Pgm (phosphoglucomutase), Iso (isomerase), G6PD (glucose-6-phosphate dehydrogenase), UDPgD (UDP-glucose dehydrogenase), F16diP (fructose 1, 6 diphosphtase), TreS (trehalose synthase), Tase (trehalase), ADP-G/GDP-G/UDP-G pyrophosphorylase (ADP-G/GDP-G/UDP-G pyr), trehalose yield Ytx (mg/g of biomass and Yts (mg/g of substrate consumed), glycogen (Glyc) and maltose (Mal) (mg/g of biomass). Key enzymes which have different enzyme activities in mutant and parent strains are shown by bold figures

Hour	OtsA	Pgm	Iso	G6PD	UDPgD	TreS	Tase	ADP-G pyr	GDP-G pyr	UDP-G pyr	Ytx	Yts	Glyc	Mal
Mutant														
24	3116±12	102±8	1113.9±24	1531±12	139.7±8	0±0	555±6	751.8±1	598.7±6	125.3±11	132±8	48±3	0	0
30	**8816**±22	200±11	2497±21	3124±18	**337**±4	0±0	**939**±21	**1337.8**±0.8	**1122**±4	328.8±21	331±14	50±3	0	0
36	6803±31	174±13	1353±11	1982±15	314±14	0±0	582±18	877.6±0.5	828±8	264.5±22	311±11	42±2	0	0
42	4981±14	109±7	769±8	1324±8	192±15	0±0	124±8	352.7±0.3	312±4	216.4±18	257±9	14.4±3	0	0
Parent														
12	**7293**±9	302±8	1193±4	2478±9	**1948**±11	0±0	**41**±3	**71**±1	**172**±1	238±4	381±11	2.1±0.3	139±3	5±0.5
18	3419±11	264±4	1131±5	926±11	1066±12	0±0	18±3	27±1	55±3	195±3	270±8	2.8±0.2	33±2	8±0.8
24	2351±8	234±2	957±3	403±4	272±4	0±0	18±3	4±1	7.9±0.1	159±2	219±9	1.18±0.1	30±5	12±1
30	2313±4	160±1	471±4	225±9	423±2	0±0	11.7±4	1.5±0.01	7.8±0.2	107±5	130±4	0.62±0.04	28±1	7±0.4

4.8. Enhancement of trehalose titre by aeration in osmotic sensitive mutant of *Propionibacterium freudenreichii subspp shermanii* with carbon source crude glycerol from biodiesel waste

During trehalose production with mutant strain it was observed that complete substrate conversion was not achieved even under pH controlled condition. However, attempt to improve conversion by decreasing crude glycerol concentration was not successful in static flask culturing condition. In static flask condition, a final trehalose yield with respect to substrate consumed was decreased from 81 mg/g to 15.08mg/g when substrate concentration varied from 20 to 5 g/l of crude glycerol. Similarly, final trehalose yield with respect with respect to biomass was also decreased by 3 fold when crude glycerol concentration was varied from 20 to 5 g/l. It was reported in literature that under aerobic conditions with *P. freudenreichii* biomass growth was higher (Quesada-Chanto et al 1998). Therefore, in the present study the effort was made to study the effect of aeration with mutant. Although, it was already observed that under oxidative stress trehalose yield decreased with parent strain. So here the objective was to increase the biomass concentration with minimal decrease in trehalose yield. In the present study, effect of aeration was investigated on biomass, trehalose accumulation and organic acid production in osmotic sensitive mutant of *P. freudenreichii subspp shermanii* and was compared with parent strain. The effect of aeration was studied in shake flask cultures at 200 rpm while in bioreactor dissolved oxygen was controlled at 30% of air saturation. In both cases crude glycerol (from biodiesel waste) was used as carbon source. Under aeration condition, trehalose titre, trehalose yield and biomass reached 1.3 g/l, 241 mg/g of biomass and 5.4 g/l with crude glycerol as carbon source with osmotic sensitive mutant. The economic production of high titre of trehalose (absolute trehalose) from biodiesel waste (crude glycerol) is highlighted in the present study. Propionic acid bacteria are known to be anaerobic but it was reported that it is not very sensitive to aerobic conditions (Wystke et al., 1972, Quesada-Chanto et al., 1998). It was also reported that *P. freudenreichii* can grow under aerobic conditions which led to increase in biomass and changes in the organic acid formation profiles (Quesada-Chanto et al 1998). This made us to envisage an experiment where osmotic sensitive mutant and parent strain of *P. freudenreichii subsp shermanii* NCIM 5137 were cultured under aeration condition (shake flask with 200 rpm) with pure and crude glycerol as carbon source. Trehalose content and biomass growth profiles were measured at regular intervals of time. Final yields of organic acids like

propionic acid and lactic acid were estimated at stationary phase. During shake flask study at 200 rpm, final biomass of 5.1 g/l was achieved in parent strain and 5.9 g/l in mutant with pure glycerol media (Fig 4.55). Similarly, 5.7 g/l and 5.4 g/l biomass was achieved with crude glycerol media in parent and mutant strain respectively (Fig 4.56). Therefore, biomass achieved in aeration condition was approx 10 fold higher in comparison to static flask condition. Interestingly, trehalose titre was 1.4 g/l in mutant, 361 mg/l in parent strain with crude glycerol and 158 mg/l, 678 mg/l in parent and mutant strain with pure glycerol medium respectively. Unfortunately, trehalose yield with respect to biomass was lower in crude and pure glycerol under aeration as compared to static flask condition. There was marginal decrease in trehalose yield with respect to substrate consumed (table 4.17). One of the prominent advantages was 98% substrate conversion of crude glycerol under aerobic condition. Since, highest trehalose titre was obtained in crude glycerol with mutant hence similar experiment was conducted in batch reactor with mutant in crude glycerol under controlled conditions of pH and dissolved oxygen. Final trehalose titre of 1.56 g/l was obtained (Fig. 4.57). Recently, 1.7 g/l of trehalose titre was reported in pure glycerol with recombinant *E. coli* (OtsAB overexpression) and in presence of trehalase inhibitor (validamycin) (extracellular) (Li et al., 2011). In the present work, 1.56 g/l of trehalose titre was obtained with crude glycerol (biodiesel waste, an alternate cheap source of glycerol) with osmotic sensitive mutant of *P. shermanii* NCIM 5137 without addition of trehalase inhibitor (not economical as very costly). Although, it should be noted that trehalose yield was lower in aerobic condition as compared to static flask condition (table 4.7 & 4.17), but final trehalose titre was increased by two fold as compared to static flask condition (anaerobic). The lactic acid and propionic acid yields were 0.53 and 0.21 g/g respectively in aerated condition with mutant. The productivity achieved with mutant in crude glycerol media under controlled conditions (batch fermentor) was 31mg/l/h and this value is comparable with other reported value (47 mg/l/h) obtained with recombinant *E.coli*_ along with trehalase inhibitor(Li et al. 2011). In comparison to productivity obtained with recombinant *Corneybacterium sp.* this productivity is significantly low but improvement can be achieved by high-cell density fermentation. In case of S.*fibuligera A11*, productivity of 122 mg/l/h was achieved with a biomass concentration 22.8 g/l (Wang et al. 2011). Thus future directions should be directed towards improving productivity by increasing biomass concentration in aerobic fermentation.

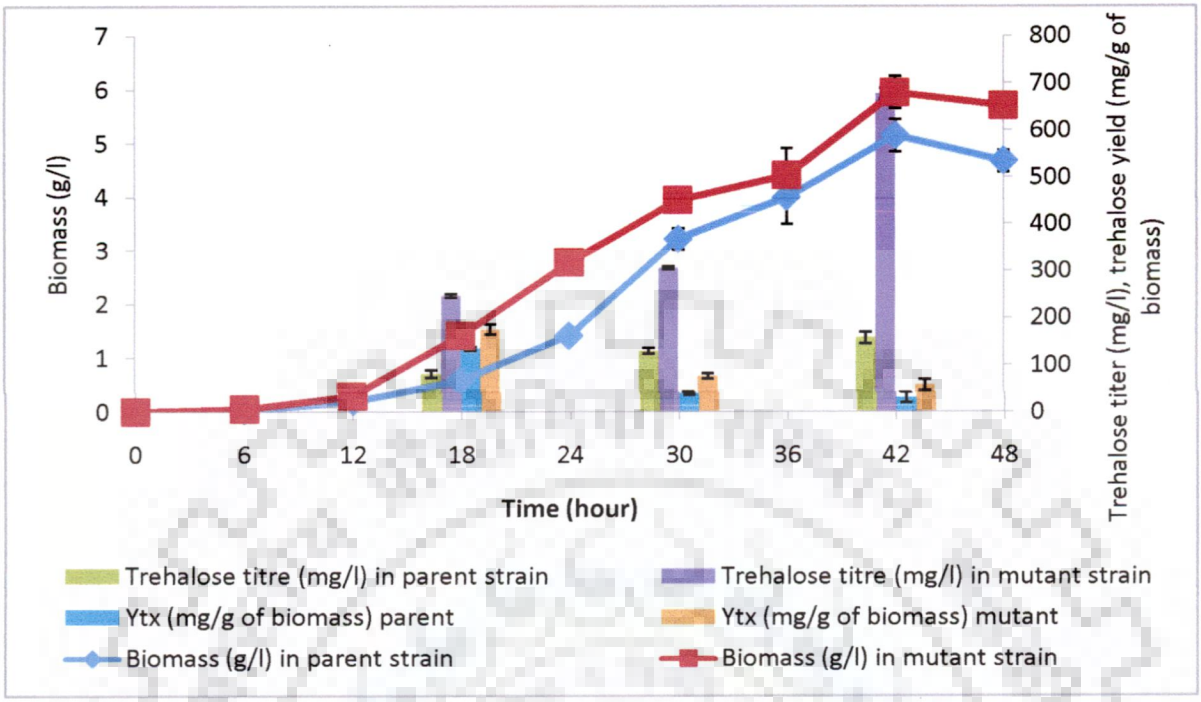


Fig 4.55 Comparison of trehalose titre, trehalose yield, biomass growth with pure glycerol media in mutant and parent strain in shake flask

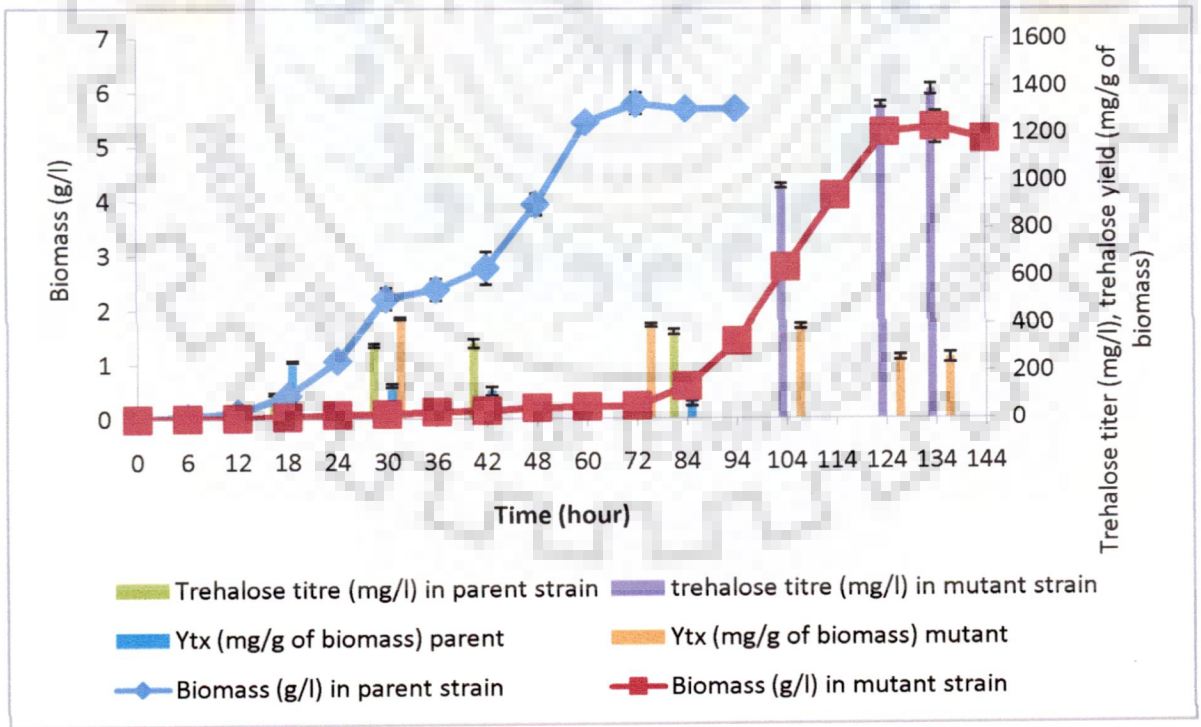


Fig 4.56 Comparison of trehalose titre, trehalose yield and biomass growth with crude glycerol media in mutant and parent strain in shake flask

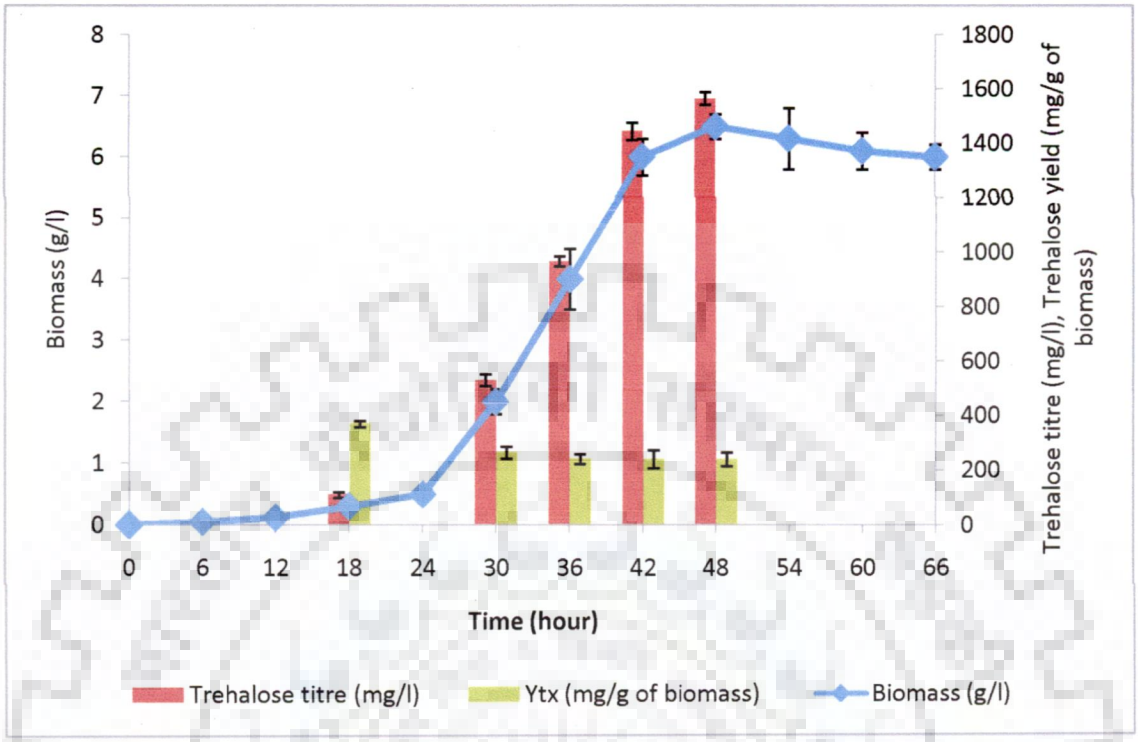


Fig 4.57 Trehalose titre, yield and biomass growth with crude glycerol media in mutant in batch reactor

Table 4.17 Comparison of final trehalose, biomass and organic acids in parent and mutant strain in aeration conditions

Parameters	**Parent Pure glycerol**	**Parent Crude glycerol**	**Mutant Pure glycerol**	**Mutant Crude glycerol**
Biomass (g/l)	5.1±0.5	5.7±0.8	5.9±0.6	5.4±0.7
Trehalose titer (mg/l)	158±6	361±11	678±14	1303±22
Trehalose yield (Ytx) (mg/g of biomass)	31±0.6	63.5±0.9	115±11	241±11
Trehalose yield (Yts) (mg/g of substrate consumed)	7.9±0.8	36.4±0.6	32.6±1	69.4±3
Propionic acid yield (g/g of substrate consumed)	0.31±0.01	0.56±0.03	0.38±0.03	0.53±0.02
Lactic acid yield (g/g of substrate consumed)	0.34±0.03	0.22±0.01	0.28±0.02	0.21±0.01
Substrate consumption (%)	100%	87.8%	100%	94%

Chapter 5

Conclusions

Chapter 5

CONCLUSIONS

The objective of the present study was to evaluate the physiology of trehalose biosynthesis pathway under osmotic stress and non-stress conditions. The physiological study was carried out with the objective of identifying key metabolic parameters which have significant influences during osmotic stress condition and these parameters ultimately leads to enhance trehalose accumulation in *P. shermanii* NCIM 5137. The final objective was to develop a mutant of *P. shermanii* NCIM 5137 which incorporates or validates some of the findings of physiological study and results in developing an efficient process for higher trehalose production. In recent times, dairy *Propionibacterium* got significant attention in various industrial processes due to their various applications and just entry to post-genomic era. However, restricted proper host-vector system and limited availability of various molecular tools for dairy *Propionibacterium* makes our effort of understanding the physiology of trehalose biosynthesis more important. Dairy *Propionibacterium* is a safe food microbe which comes under the category of GRAS with potential probiotic properties and hence it is not surprising that the potential of this bacterium as a cell factory for the production of flavours vitamins and nutraceutical had been explored. Trehalose is non-reducing disaccharide sugar ubiquitously distributed in nature and is well known for its stress protecting role in microbe. Trehalose acts as compatible solute and is known as nutraceutical. The study of its physiology for trehalose biosynthesis can be targeted for two objectives, one for development of recombinant strains with higher trehalose yields and hence enhanced stress tolerance which is a prerequisite for various bio-production processes. Second it will be useful for production of this nutraceutical economically. In general, production of particular metabolite using bacterial cell are achieved by genetic engineering which extends substrate range of existing pathway or improve production efficiency by introducing novel pathway from some other microorganism (heterologous). For all these metabolic engineering strategies, analyses of cellular metabolism are required because it involves several hit and trials. On the other hand, it is almost impossible to engineer all the genes of central metabolism together and then study its effect on metabolite production. Hence understanding of these biological complexity through the approaches of system biology using mathematical and statistical algorithm reduce the chances of error and can better predict the yield of targeted metabolite. Keeping this view in mind, an attempt was made to use multivariate approach to understand the complex

metabolic synthesis of trehalose and its regulation under stress and non-stress conditions. Furthermore, some novel insights to trehalose biosynthesis pathway were highlighted in the present study.

In the present study trehalose biosynthesis was studied in *P. shermanii* NCIM 5137 which is a dairy *Propionibacterium*. During our preliminary study with *P. shermanii* it was concluded that trehalose accumulation was enhanced in response to osmotic stress and also by changing the carbon source. This led us to determine the effect of economical carbon source on trehalose accumulation. It was found that carbon source starch, sucrose and glycerol were accumulating higher trehalose in comparison to glucose. Interestingly, glycerol was most suitable carbon source for trehalose accumulation. Additionally, crude glycerol an alternate cheap source of glycerol was studied for its suitability for trehalose production. In fact it was observed that crude glycerol was superior carbon source as compared to pure glycerol. Thus an effort was made to identify the parameters which are responsible for superior performance with crude glycerol. The effects of various impurities (fatty acid, KCl) present in crude glycerol on trehalose yields were evaluated with a chemically defined media. It was finally concluded that KCl was responsible for achieving higher trehalose yield with respect to substrate consumed in crude glycerol media. The effect of KCl was further evaluated at molecular level by monitoring enzymes activities and metabolites concentrations associated with trehalose biosynthesis pathway under osmotic stress and non-stress conditions. The profiles of enzymes activities and metabolites concentrations were initially analyzed to qualitatively predict the influence level of individual metabolic parameters on trehalose biosynthesis. An attempt was made to take the advantage of multivariate data analysis techniques for analysis of trehalose biosynthesis physiology under stress and non-stress conditions. The profiles of enzymes activities and metabolite concentrations related to trehalose biosynthesis pathway were taken as metabolic variables in principal component analysis. The principal component analysis was able to differentiate the osmotically stressed cells from non-stressed cells. Further the osmotic state of new independent experimental data was correctly predicted by the developed principal component model. Over all physiological changes associated with osmotic stress phenotypes were clearly visible from the loading plot of principal component analysis. Besides quantitative predictions about extent over-expressions of various enzymes necessary for higher trehalose accumulation was made and validated with independent experimental data. From the physiological study it was predicted that over-

expressions of ADP-glucose pyrophosphorylase along with OtsA and phosphoglucomutase and downregulation of UDP glucose dehydrogenase will lead to higher trehalose yield in *P. shermanii* NCIM 5137. The role of trehalose synthase was not very clear from physiological study but it seems glycogen of content of cell may have a role in regulating anabolism and catabolism of trehalose by TreS. Principal component analysis also indicates that trehalase should be over expressed under osmotic stress condition but knowing the catabolic role of trehalase it is surprising. The prominent role of ADP-glucose pyrophosphorylase and OtsA in higher trehalose accumulation with osmotically sensitive mutant also validates conclusions obtained with parent strain.

Final trehalose yields achieved with respect to biomass and substrate consumed in mutant strain was significantly higher than the parent strain under similar experimental conditions. One of the probable reasons is due to lower rate of decrease of ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase, OtsA activities with growth in the mutant as compared to parent strain. The major outcome of present study includes achieving 110 times higher trehalose yield with respect to substrate consumed in mutant with crude glycerol as compared to parent strain with pure glycerol. Similarly trehalose yield with respect to biomass was also improved by five times in mutant with crude glycerol as compared to parent strain with pure glycerol.

Thus in summary, in the present work the improvement of trehalose production with subsequent gathering of knowledge on physiology of trehalose biosynthesis was demonstrated. Trehalose is accumulated under osmotic stress hence accumulation of trehalose was exploited for understanding trehalose biosynthesis and applied for trehalose production. In summary, improvement in trehalose accumulation and new insights on physiology of trehalose synthesis was achieved and were presented.

The conclusions and main results can be presented as point wise in two parts with respect to trehalose production and metabolism

Trehalose production

- *Propionibacterium shermanii* NCIM 5137 was most suitable strain for study of trehalose biosynthesis amongst the major strains of dairy *Propionibacterium* procured from different culture collection centres of India.

- There was no effect of nitrogen source (inorganic/organic) while aeration and pH reduced trehalose accumulation but change in carbon source has beneficial effect on trehalose accumulation.
- Amongst the environment stress conditions, osmotic stress was most effective as it enhanced trehalose accumulation in comparison to non-stress condition which indicate prominent role of trehalose in osmotic stress tolerance.
- In all cheap carbon source studied (sucrose, maltose, soluble starch and glycerol) trehalose accumulation was higher in comparison to substrate glucose. Interestingly, highest trehalose accumulation was in carbon source glycerol and this led us to explore alternate cheap source of glycerol like biodiesel waste for trehalose production.
- Crude glycerol from biodiesel waste was explored for production of trehalose and was compared with pure glycerol media with *P. shermanii* NCIM 5137. Interestingly, trehalose yield was higher in crude glycerol media with 10 g/l of concentration (131 mg/g of biomass, 43 mg/g of substrate) in comparison to pure glycerol media (52 mg/g of biomass, 8 mg/g of substrate). This process was advantageous as it also led to production of relatively pure propionic acid with yield of 0.63 g/g of substrate.
- It was also concluded that amongst the impurities found in biodiesel waste, fatty acids seems to have no beneficial effects on trehalose yield based on substrate consumed while the presence of KCl in the crude glycerol was probably responsible for achieving higher trehalose yield based on substrate consumed.
- As our study concluded that osmotic stress has important role in enhancement of trehalose, therefore it led us to explore this phenomenon in the development of osmotically sensitive mutant with higher trehalose yield. This mutant was nisin resistant and it was able to produce higher trehalose yield (391 mg/g of biomass, 90 mg/g of substrate).
- Further, it was observed from literature that under aerobic conditions *Propionibacterium freudenreichii* enhances biomass concentration; this led us to envisage that increasing biomass will make increase in intracellular absolute trehalose concentration. It was found true, when under aerobic conditions trehalose titre of 1.5 g/l was obtained with crude glycerol from biodiesel waste with osmotic sensitive mutant of *P. shermanii* NCIM 5137, which was competitive with the reported process in *E. coli* where 1.7 g/l of trehalose titre was obtained with

recombinant strain from pure glycerol and by addition of validamycin in fermentation medium.

Trehalose metabolism

One of our objectives was to understand metabolism of trehalose together with prospect of its production from *P. shermanii* NCIM. The important conclusions with respect to metabolism were

- In our cluster analysis study with regard to accumulation of trehalose, maltose and glycogen in different carbon source, it was observed that accumulation of trehalose was more similar to glycogen accumulation.
- During study with production of trehalose from crude glycerol, it was concluded that presence of KCl was responsible for higher trehalose yield, this led us to study in detail effect of osmotic stress by subsequent increase of KCl concentration (0.5% and 1%) in chemically defined media and measuring concentration of metabolites and enzymes activities related to trehalose biosynthesis. It was concluded that NDP-glucose pyrophosphorylase behave different under osmotic stress. ADP-glucose pyrophosphorylase increases fivefold (in comparison to 3 and 1.5 fold with GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase respectively) under osmotic stress. Additionally, specificity of OtsA increases for ADP-glucose under osmotic stress as compared to GDP-glucose and UDP-glucose.
- Similarly, another important observation was regarding role of TreS, especially with respect to glycogen conversion into trehalose under osmotic stress. Amylase activity was observed in osmotic stress condition but it was not possible to conclude the association of amylase activity with TreS. It was also postulated that intracellular glycogen concentration plays a role in trehalose maltose inter-conversion equilibrium. In other words, catabolic and anabolic role of TreS depends on glycogen concentration and trehalose to maltose ration.
- Further, a Principal component analysis was applied for studying the physiology in non-stress and osmotic stress. Principal components were able to differentiate phenotypes of osmotic stress from non stress phenotypes
- It was also concluded that for achieving higher trehalose yield, activities of trehalase, trehalose synthase, ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase, UDP-glucose pyrophosphorylase, phosphoglucomutase, OtsA

should be over-expressed (all metabolic parameters have high negative loading factors for first principal component) whereas UDP-glucose dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and isomerase activities and glycogen concentration should decrease (as high loading factors for first principal component).

- The outcomes of the physiological study carried out with parent strain under osmotic stress and non-stress conditions were again validated by study with mutant.

Future Prospects

1. Improvement of trehalose titre by increasing the biomass concentration either by manipulating substrate concentration or by aeration. Fed-batch fermentation can also be employed for achieving higher trehalose titre along with higher biomass concentration.
2. Improvement of trehalose yield by site directed mutagenesis. Specifically targeting ADP-glucose pyrophosphorylase, OtsA and phosphoglucomutase. Similarly, development of trehalose deletion mutant can further improve the trehalose yield.
3. Study of purified TreS for better understanding of role TreS for glycogen conversion into trehalose under osmotic stress.
4. Purification of OtsA and characterization of its specificity with various NDP-glucoses and identifying any regulatory molecule in the cell extract which is responsible for higher specificity of OtsA with ADP-glucose.

Chapter 6
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Paper in international journals

- **Ruhal R.,** Aggarwal S., Choudhury B. 2011. Suitability of crude glycerol obtained from bio diesel waste for the productions of trehalose and propionic acid. Green Chemistry. 13, 3492-3498. (**Impact factor 5.47**).

- **Rohit Ruhal** and Bijan Choudhury. 2011. Use of osmotically sensitive mutant of *Propionibacterium freudenreichii* subspp *shermanii* for the simulataneous productions of organic acids and trehalose from biodiesel waste based crude glycerol. Bioresource Technology. (**Under revision**). (**Impact factor 4.4**)

- **Rohit Ruhal** and Bijan Choudhury. Analysis of influence of UDP-G synthesising enzymes on trehalose synthesis in *Propionibacterium shermanii* by multivariate analysis (**Manuscript under preparation).**

- **Rohit Ruhal** and Bijan Choudhury. Prominent role of TreS and ADP-glucose pyrophosphorylase under osmotic stress in *Propionibacterium.* (**Manuscript under preparation).**

- **Rohit Ruhal**, Vineet shrivastava and Bijan Choudhury. A principle component approach for determining influence of various enzyme activities on trehalose biosynthesis in *Propionibacterium shermanii* (**Manuscript under preparation**).

Book Chapter

- **Rohit Ruhal** and Bijan Choudhury. Membrane separation in food process design. Book chapter of Blackwell series entitled Food process and design. (**In press**).

Conferences/Symposium

- Abstract published in 49th international conference AMI conference organized in DU, Delhi, India. (November 18-20, 2008) entitled: "Production of trehalose *in Propionibacterium shermanii* in stress conditions."

- Abstract submitted in international conference ISBT 2011 (April 2011). Trehalose accumulation in *Propionibacterium shermanii* in different cheap carbon sources. International conference conducted by Research journal of Biotechnology.

- Participation in national conference "National workshop of biotechnology" 2010 as instructor to PG students from all over India for analytical tools including HPLC, GC and FPLC