EVALUATION OF SALT STRESS ADAPTATION ON GROWTH AND FERMENTATION CHARACTERISTICS OF YEAST IN INDUSTRIAL MEDIA

M.TECH DISSERTATION REPORT

Submitted in partial fulfilment of the requirement for the award of the degree of

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DECLARATION

I, Abantika Chowdhury declare that my Dissertation report titled ***"Evaluation of Salt Stress Adaptation on Growth and Fermentation Characteristics of Yeast in Industrial Media"*** submitted in the partial fulfilment of the requirement for the award of the degree of **Master of Technology** in **Industrial Biotechnology** to Delhi Technological University is a record of original research work. No part of this report has been submitted for award of any other degree. All the given information and work are true to my sense and knowledge.

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CERTIFICATE

This is to certify that the Dissertation report entitled ***"Evaluation of Salt Stress Adaptation on Growth and Fermentation Characteristics of Yeast in Industrial Media"*** submitted by **Ms. Abantika Chowdhury,** Roll No. **2K15/IBT/01** in the partial fulfilment of the requirement for the award of the degree of **Master of Technology** in **Industrial Biotechnology** to Delhi Technological University, is an authentic record of candidate's own research work carried out under my knowledge at International Centre for Genetic Engineering and Biotechnology (ICGEB, New Delhi) under guidance of Dr. Shireesh Srivastava. The data enclosed in the report is original and has not been submitted elsewhere for honouring of any other degree to the best of my knowledge and belief.

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This is to certify that the Dissertation report entitled ***"Evaluation of Salt Stress Adaptation on Growth and Fermentation Characteristics of Yeast in Industrial Media"*** is the bonafide work carried out by **Ms. Abantika Chowdhury**. She has successfully completed the research work during August 2016 –July 2017 in the partial fulfilment of the requirement for the award of the degree of **Master of Technology** in **Industrial Biotechnology**, Delhi Technological University, Delhi. It is an authentic record of candidate's own work carried out by her under my guidance at Centre for Advanced Bioenergy Research, International Centre for Genetic Engineering and Biotechnology (ICGEB, New Delhi). The information and data enclosed in the report is original and has not been submitted elsewhere for honouring of any other degree to the best of my knowledge and belief.

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‘If I have seen further it is by standing on the shoulder of giants’

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Abantika Chowdhury

CONTENTS

LIST OF ABBREVIATIONS

CSL	Corn Steep Liquor
C2S0	Media containing 2% CSL and no salt
C2S3	Media containing 2% CSL and 3% salt
Cons.	Consumption
CYR	Adenylate Cyclase gene
Eth	Ethanol
Glu	Glucose
Glyc.	Glycerol
HOG	High Osmolarity Glycerol
HPLC	High Performance Liquid Chromatography
NaCl	Sodium Chloride salt
O.D.	Optical Density or Absorbance
Prod.	Production
Sp. Eth Prod.	Specific Ethanol Production (Ethanol produced per O.D/cell)
Sp. Glu Cons.	Specific Glucose Consumption (Glucose consumed per O.D/cell)
YPD	Yeast Extract Peptone Dextrose

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1.

ABSTRACT

The progressive transition to green economy and sustainable energy is a driver for the development of biofuels, a renewable fuel. My work focuses on improving the ethanol production of yeast cells using salt stress in industrial media. *Saccharomyces cerevisiae*, the most widely used commercial yeast for industrial ethanol production is used for the experimentation. The yeast strain used is *S. cerevisiae* CEN.PK 122 (diploid), which is an auxotrophic strain proven to exhibit double ethanol yield on biomass in high glucose concentration as compared to prototrophic strains due to selective transition of respiratory metabolism to fermentative metabolism. To further improve fermentation capacity of the yeast, salt stress is employed. The yeast cells are gradually adapted to high salt stress (3% NaCl) for three generations and tested for fermentative capacity in industrial grade corn steep liquor media using different seeding cell densities. It is found that once adapted to 3% salt stress, now salt adapted yeast cells are capable of improved fermentation and ethanol production on release of salt stress attributed to their enhanced growth as compared to unadapted yeast cells (control). Salt adapted yeast shows 1.5 times ethanol production than control. This enhanced fermentation by adapted cells occurs at higher cell density suggests molecular interaction and signalling among cells induced by salt stress to sense the environment and shift metabolic pathways for better adaptability and fermentation. There is metabolic modification of yeast using salt stress. Additionally, adaptation to one type of stress leads to protective response to other stress factors making salt stress adapted yeast ideal for use in industrial fermentation.

The research paves way to solve the huge ethanol demand as biofuel by higher production in industrial media. Fermentation process accounts about 20% of the total production cost of bio-refineries that converts biomass to biofuel. Improved fermentation efficiency will decrease the price of ethanol biofuel, thus exploring native and overseas markets. This technology will revitalize Indian economy by decreased dependency on imported fuel and generating cheaper bioethanol fuel.

2.

INTRODUCTION

Yeasts are an important group of lower eukaryotic microorganisms used in several industrial biotechnology processes since ancient times for brewing, baking and bread making. In modern times, it serves as a commercial workhouse for production of fuels, chemicals and pharmaceutical products. Among all yeast, *Saccharomyces cerevisiae* is most abundantly used in industrial bioprocesses since it is a model organism of eukaryotic systems with established data on physiology, genetics, genes along with robustness under process condition and ease of genetic & metabolic engineering. Yeast ferment sugars (primarily glucose in case of wild type *Saccharomyces species*) producing ethanol along with generation of ATP by anaerobic respiration. *Saccharomyces species* can grow in a range of conditions (pH, Temperature, osmotic pressure/salt stress) and capable of fermenting ethanol in anaerobic or partial anaerobic condition. *S. cerevisiae* is capable of dealing with stress condition while culturing (Bai *et al*. 2008) making it an apt choice for salt stress adaptation.

In our experimentation, *S. cerevisiae* CEN.PK 122 (diploid) is used for fermentation of 10% glucose with or without salt stress. High concentration of glucose (10%) is selected since yeast majorly utilizes glucose as carbohydrate source and our strain *S. cerevisiae* CEN.PK 122 is an industrial strain proven to exhibit high gravity fermentation to convert highly concentrated sugar in media for large production of ethanol. High Gravity fermentation has various advantages as it decreases the water requirement and energy costs for the process due to enhanced overall productivity and higher ethanol in fermentation product saves energy during distillation (Wang *et al.* 2007). Moreover, this strain is an auxotrophic and characterised for double ethanol yield on biomass in high glucose concentration as compared to prototrophic strains due to selective transition of respiratory metabolism to fermentative metabolism. To further improve fermentation capacity of the yeast, salt stress is employed.

In our experimentation, *S. cerevisiae* CEN.PK 122 (diploid) is used to **test the Maintenance Energy Hypothesis**. The Maintenance Energy hypothesis suggests that salt adapted yeast cells are capable of

fermenting more sugar (glucose) to ethanol along with more ATP generation in order to maintain active cell metabolism and sustain life. If the maintenance energy hypothesis is proved right, it confirms enhanced ethanol fermentation capacity of the salt adapted yeast cells in osmotic (salt) stress. Higher specific productivity or ethanol production per cell of *S. cerevisiae* leads to higher yield of ethanol with same media concentration. Therefore, the production process becomes more economical and paves way for increased bioethanol production and utilization as a biofuel, which is a cleaner and renewable fuel. The higher ethanol yield on biomass (higher specific productivity in case of salt adapted yeast) would be advantageous in immobilized cell systems, as reduced yeast biomass could greatly reduce the mass transfer limitations through the immobilization matrix. Hence, salt adapted yeast can be used for industrial scale ethanol production that requires immobilization of cells.

Our experimentation is divided into 3 phase :

1. Serial Adaptation of yeast cells on minimal 2% CSL media supplemented with 3% salt stress (NaCl).
2. Comparative analysis of growth & fermentation characteristics of salt adapted yeast cells in presence and absence of salt stress in industrial media to investigate improvement in Ethanol Production.
3. Observation of cellular characteristics like colour and size of adapted and unadapted (control) yeast cultured in presence and absence of salt stress for concluding remarks for the difference.

In first stage, the yeast strain is gradually adapted to high Salt (NaCl) stress cultured in industrial grade media (Corn Steep Liquor with high glucose concentration). The adapted cells are selected by the fact, non-adapted yeast cells die and are reduced in number while the adapting cells survive and transforms into Salt tolerant yeast and eventually adapted to salt stress when cultured in high salt supplemented media. The media was kept minimal (2% CSL) so that the cells adapt in Salt stress and not merely grow. If there is higher concentration of CSL, yeast cells will be able to grow even in high salt stress because of higher nutrients supplemented from media to cells to sustain life. Here, yeast cell may not

adapt to the condition and loose its tolerance property as soon as the stress condition is removed. Therefore, it requires an optimization of CSL concentration in culture media (estimated to be 2% CSL) such that the cells do not die and cell growth is not a mere result of extra nutrients in the culture media but yeast cells get adapted to high NaCl concentration. The yeast strain respond to salt stress by adjusting its metabolic activities and become adapted over time. In other words, **the yeast cells are metabolically engineered/modified by osmotic stress and tested for the desired quality of enhanced ethanol specific productivity.** The tolerant yeast cells are maintained on 2% CSL + 2% glucose + 3% NaCl to allow better survival of yeast cells and low metabolic activity while storage and also on YPD plate containing salt. Serial Adaptation 1 (Sr1), Serial Adaptation 2 (Sr2) and Serial Adaptation 3 (Sr3) are 3 generations of salt adapted yeast obtained by sequential adaptation of previous generation. The cells are maintained on CSL plate (+3% NaCl) shows improved growth and fermentation capacity on next culturing, whereas shows same growth when maintained on YPD plate (3%NaCl). Therefore, plating on CSL(+3% salt) plate is an important part of serial adaptation process.

In second stage, growth and fermentation profiles of salt adapted yeast growing in presence & absence of salt (3% NaCl) stress and unadapted (control) cells growing in absence of salt stress are compared with different seeding cell densities (low to high). Growth, ethanol productivity, yield and glucose consumption of adapted vs control provide proof for Maintenance Energy Hypothesis. Emphasis on 3% Salt tolerance is due the fact sea water is approximately 3.5% saline and thus, can be directly added to the fermentation broth replacing water. This will reduce the cost of adding salt to the media in industrial fermentation. Another approach is testing our hypothesis using marine yeasts for fermentation profile in salt condition. But since, most ethanol industries are optimized for fermentation using *Saccharomyces cerevisiae* and optimization of culture conditions for marine yeasts will be costly. So, studies on *S. cerevisiae* are carried out in our experiments for effective fermentation.

The push toward sustainable energy has gained substantial momentum in recent years. This raises the demand for a renewable biofuel. Ethanol biofuel is promising since, it can be obtained by degradation of bio-waste and subsequent fermentation. This serves dual purpose of waste management and sustainable energy supply since bio-waste is most abundant and renewable. The adverse climatic conditions & increasing pollution from continuous use of fossil derived fuels are the driving factors for the development of cleaner fuel like bioethanol. Fossil Fuels are one time gift that lifted us from subsistence agriculture and eventually should lead us to a future based on Renewable Resources. The market for renewable resources is $30 billion and expected to reach $1 trillion with major share of biofuel (Silicon Valley Bank 2012). So, there is huge scope for development of bioethanol as biofuel. **The main challenge is production cost of ethanol which can be lowered by technological innovation, selection of robust strains and enhanced & faster fermentation process as targeted in our research.**

3.

REVIEW OF LITERATURE

3.1. *Saccharomyces cerevisiae* in Fermentation

Saccharomyces cerevisiae, a common species of yeast is the most extensively studied model of eukaryotes and is a commercially valuable organism for ethanol production. It can grow in a range of condition, so slight variation of condition like pH, temperature, osmotic stress, anaerobic/ partial anaerobic conditions would not affect cell growth and fermentation of ethanol. Also, *S. cerevisiae* can grow in presence low concentration of inorganic salts as in industrial media; making it the most favourable choice for industrial fermentation process because of its versatility. Yeast ferment sugars (primarily glucose in case of wild type *Saccharomyces species*) producing ethanol along with generation of ATP by anaerobic respiration. *S. cerevisiae* is capable of dealing with stress condition while culturing (Bai *et al*. 2008) making it the right choice for salt stress adaptation.

Two forms of fungal yeast cells can survive and grow: haploid and diploid. The haploid (asexual form of yeast) cells undergo a simple lifecycle of mitosis and growth and under conditions of high stress generally die. The diploid cells (sexual form of yeast) undergo a simple lifecycle of mitosis and growth under optimal conditions and double their population. Average doubling time of *Saccharomyces cerevisiae* is 100 minutes (with maximum of 120 minutes). However, growth rates vary enormously both between strains and between environments. Mean replicative lifespan is about 26 cell divisions. Yeast under extreme stress conditions can form spores which is rare. For *S. cerevisiae*, optimal pH is 5.0 and optimal Temperature is 30°C for growth and metabolic activity.

3.2. Why does yeast ferment?

All strains of *S. cerevisiae* can grow both aerobically and anaerobically on glucose, maltose, galactose and fructose but glucose and fructose are known to be two best fermenting sugars. The ability of yeasts to use different sugars can differ depending on whether they are grown aerobically or anaerobically.

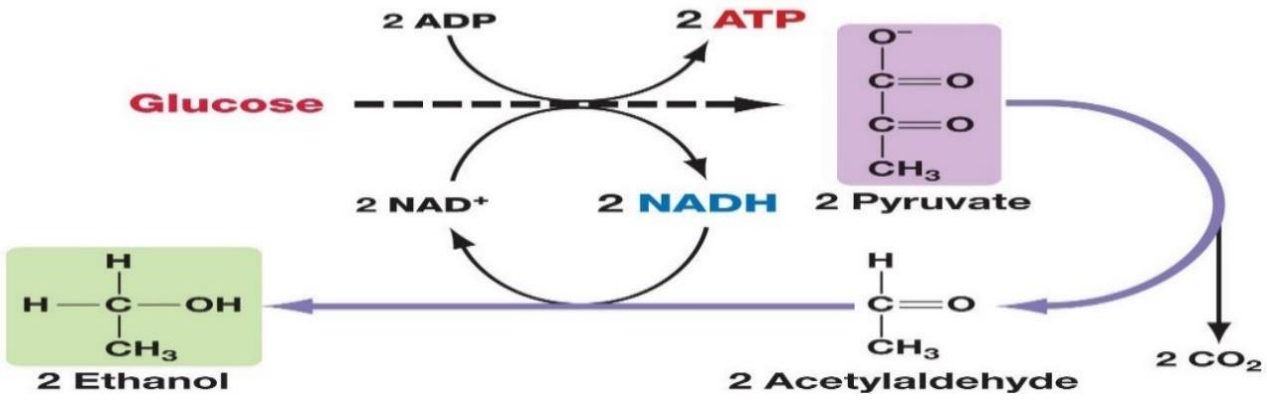


Figure 1: Alcoholic Fermentation of glucose to generate ethanol & energy molecule (net 2ATP/glucose molecule).

In limited oxygen or anaerobic condition, fermentative pathway of yeast is activated as an additional route for the uptake of glucose and not as an alternative to aerobic respiration. Aerobic respiration produces more energy than anaerobic respiration (fermentation), yet yeast cells choose to ferment. The possible answer is the requirement of additional ATP (energy molecules) for transfer of pyruvate in mitochondria and synthesis of numerous enzymes involved TCA cycle for aerobic respiration. A considerably smaller number of enzymes are required for cell growth as against high mitochondrial respiration cost. Therefore, the yeast cell would minimize the number of active reactions in attempting to optimize resource management and preferentially ferment sugars to meet the energy requirement (Simeonidis *et al.* 2010). In case of stress condition, there is an immediate requirement of ATP by yeast

cells to sustain life, fairly reasoning enhanced fermentation to generate more ATP and ethanol in case of osmotic or salt stress.

3.3. *Saccharomyces cerevisiae* CEN.PK 122

Saccharomyces cerevisiae CEN.PK 122 is a diploid auxotrophic strain of yeast. Auxotrophic strains are incapable of synthesizing a particular organic compound required for its growth and are created by genetic mutations from wild type prototrophic strains (capable of synthesizing all compounds required for growth). Auxotrophic strains are able to grow in media supplemented with the deficient compound. Hence they are used in screening of genetic recombination experiments.

The isogenic family of CEN.PK strains was developed by crossing of different laboratory strains of *S. cerevisiae* in the 1990's by a consortium of German yeast researchers. CEN.PK possesses a mutation in CYR1 gene (A5627T corresponding to a K1876M substitution near the end of the catalytic domain) of Adenylate Cyclase which eliminates glucose-induced and acidification-induced cAMP signalling and delays glucose-induced loss of stress resistance (Vanhalewyn *et al.* 1999; Dumortier *et al*. 2000). Hence, CEN.PK strains respond and adapt to stresses better than wild strains. These strains combine good accessibility to classical and molecular genetics techniques with excellent growth characteristics under controlled, industrially relevant (stress) conditions (van Dijken *et al.* 2000) and are used in systems biology studies (Canelas *et al.* 2010), metabolic engineering studies in fermentation of pentose sugars for production of ethanol (Kuyper *et al.* 2005) and as host for the production of heterologous proteins.

Nowadays, a lot of studies is carried for the improved production of ethanol by fermentation in order to convert biomass waste into liquid second generation biofuel (McKendry 2002; Caspeta *et al.* 2013).

To this purpose, new yeast strains capable to produce ethanol in different cultivation conditions are investigated (Nielsen *et al.* 2013).

Saccharomyces cerevisiae CEN.PK 122 (auxotrophic diploid yeast strain) is used in our experimentation because –

(i) Industrially, diploid or multi-ploid yeasts are used because they are more stable in industrial condition as compared to haploids strains.

(ii) Diploids are more adaptive to surrounding/ environmental conditions than haploids i.e., diploids are more prone to become stress tolerant.

(iii) *S. cerevisiae* CEN.PK -auxotrophic yeast strains are proven to exhibit double ethanol yield in high glucose concentration as compared to prototrophic strains due to selective transition of gluconeogenic & respiratory metabolism to fermentative metabolism (Landi *et al.* 2011; Paciello *et al.* 2014). The greater the number of autotrophies the faster is the metabolic shift from respiratory to fermentative metabolism for ethanol production.

High concentration of glucose (10%) is selected since yeast majorly utilizes glucose as carbohydrate source and our strain *S. cerevisiae* CEN.PK 122 is an industrial strain proven to exhibit high gravity fermentation to convert highly concentrated sugar in media for large production of ethanol (Pereira *et al.* 2010). High Gravity fermentation has various advantages as it decreases the water requirement and energy costs for the process due to enhanced overall productivity and higher ethanol in fermentation product saves energy during distillation (Wang *et al.* 2007).

3.4. Industrial Media for Fermentation

Corn Steep Liquor (CSL) is a viscous concentrate of corn solubles and by-product of corn wet-milling. CSL is an important component of industrial grade fermentation media. It is cheaper than commonly used laboratory grade fermentation media (Yeast extract-Peptone-Dextrose media) while its performance is comparable to lab grade nutrient rich media (YPD) with similar growth of yeast and fermentation of ethanol. Besides its low cost, CSL medium consists of all components required for commercial scale processes thereby, making it industrially relevant.

Constituents of Industrial fermentation broth :

CSL – excellent source of organic nutrients (amino acid, vitamins, minerals, organic Nitrogen)

Urea – inorganic N - source

Inorganic Minerals – Magnesium Sulphate, Copper Sulphate

Glucose (10%) – C-source. High glucose (10%) concentration is used for culturing because yeast majorly utilizes glucose as carbohydrate source and the industrial grade media has approximately same level of glucose for high ethanol production. Our strain is optimized for high gravity fermentations (in high glucose concentrations).

Salt (3% NaCl) – for enhanced ethanol productivity of yeast cells in osmotic stress (salt stress). Substantial number of cells are able to grow and adapt in short interval at around 3% Salt concentration. Sea water is approximately 3.5% saline and can be directly added to the fermentation broth replacing water. This will reduce the cost of adding salt to the media in industrial fermentation.

Industrial production of ethanol is carried in continuous reactor which requires process optimization and media component optimization for maximum production of ethanol. Under optimum operating condition the maximum of 50% ethanol yield is achieved in CSTR where rate of formation of ethanol follows Michael-Menten equation (constants: Vmax and Km).

3.5. Effect of High Salt Stress on Growth and Metabolism of *Saccharomyces cerevisiae*

One mechanism that explains *S. cerevisiae* ability to cope with salt (NaCl) stress relies in its ability to regulate ion concentrations. Yeast cells will increase their uptake of salt cations (Na^+) in the presence of high salt concentrations (Li *et al.* 2012) until the intracellular concentration of salts equal that of the environment, thereafter yeast cells will reduce cell salinity by introducing compatible solutes. The production of glycerol help protect the cells from NaCl stress by dissipating the driving force for Na+ uptake through the plasma membrane. *S. cerevisiae* increases its intracellular concentration of potassium ions in order to maintain equal electrical charge without the presence of toxic concentrations of sodium ions inside the cell. V-ATPase have the greatest effect in the stress response to salinity in *S. cerevisiae* (Hamilton *et al.* 2002). There is an increase in this enzyme immediately after addition of small concentrations of NaCl and a continued increase in higher salt concentrations. This was due to the creation of a proton motive force that helps the Na+/ H+ antiporter move Na+ cations into vacuoles to reduce salt concentration in the cytoplasm and maintain a constant cytoplasmic salt concentration to avoid disrupting homeostasis.

There are metabolic surprises during adaptation of yeast cell to saline condition (Blomberg 2000; Mager and Siderius 2002). Key features of the cellular response in yeast to saline conditions are as follows :

(i) **The initial response of the cell is efflux of water, which leads to cell shrinkage and growth arrest.** As an immediate consequence of the exposure of yeast to high osmolarity in the surrounding medium, cells rapidly loose intracellular water which leads to loss of turgor and hence shrinkage of cells. Water is recruited from the vacuole into the cytoplasm to partially compensate for the sudden increase in macro-molecular concentration. In addition, depending on the severity of the osmotic

stress, cytoskeleton collapses leading to depolarization of actin patches. These immediate effects are caused by the physico-mechanical forces operating under hyperosmotic conditions. Basic cell processes such as transcription is disrupted and cellular division is arrested at this stage.

(ii) **The second phase is main osmoregulatory strategy of the cell** is exclusion of the salt via ion pumps followed by regain lost water to restore turgor or cell volume by increased production and accumulation of glycerol (major osmo-protectant in salt stress) and trehalose sugar (stabilise cell membranes). Thus, dehydration effect is retarded by the production of trehalose & glycerol in yeast.

(iii) **Third phase involves activation of Hog (High Osmolarity Glycerol) signaling pathways** that lead to rapid & high production of glycerol to restore cell function participating in osmoregulation and protection of enzymatic activities under salt stress. The HOG pathway triggers MAPK (mitogen-activated protein Kinase) pathway (Lodder *et al.* 1999). This results in the rapid phosphorylation and nuclear translocation of MAP kinase Hog1p. Nuclear Hog1p mediates regulation, both repression and activation of gene expression of the sodium pump-encoding ENA1 gene to efflux Na^+ ions. Hog1p also regulates transcription of the *GPD1* gene encoding glycerol 3-phosphate dehydrogenase (Albertyn *etal.* 1994) to prevent glycerol efflux in order to keep increasing glycerol synthesis & intracellular glycerol accumulation.

(iv) Other signalling pathways involves Wsc1 Proteins that are putative receptors in stress response in yeast *Saccharomyces cerevisiae* and are upstream regulators of the stress-activated MAP kinase cascade leading to heat shock response and for maintenance of cell wall integrity (Lodder *et al.* 1999). Cardiolipin (CL), an anionic phospholipid located on mitochondrial membrane mediates cross talk between mitochondria and vacuole during salt stress (Chen *et al.* 2008). Reactive Oxygen

Intermediates (ROI), earlier considered toxic products of aerobic metabolism are now believed to be produced as signalling molecules to control process of stress response (Mittler 2002).

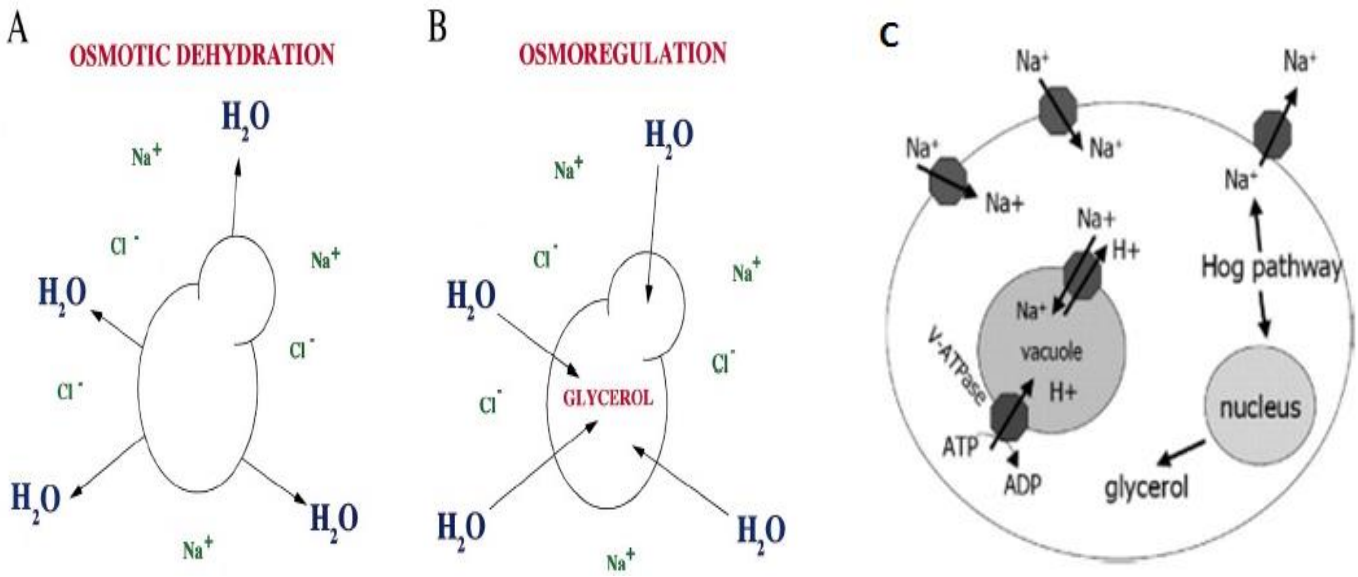


Figure 2: Cellular Response of *Saccharomyces cerevisiae* to Hyper-osmolarity (high salt stress).
{A. Osmotic Dehydration – efflux of water lead to cell shrinkage and arrested growth
B. Osmoregulation & Turgor restoration -recovery of water & glycerol production
C. Activation of V-ATPase & Hog pathway to efflux salt ion & maintain cellular homeostasis by glycerol accumulation}

Yeast cells relocate their energy from basic functionalities such as reproduction to the activation of these pathways that consume most of the energy available in stress condition. This increased expenditure in the stress response translates to slower reproduction and hence, reduced growth rates. But yeast cells are slowly able to adapt to salt stress with no decrease in cell concentration suggesting cells were able to prevent cell death at the cost of reduced reproduction and respond to stress with more energy expenditure. Generally in stress condition, there is an immediate requirement of ATP to sustain life, fairly reasoning enhanced fermentation to generate more ATP and ethanol. Thus, in stress condition there is an immediate requirement of ATP to sustain life obtained by enhanced fermentation generating more ATP and ethanol.

Yeast being a eukaryote, bears striking resemblances with higher plants and thus, if mechanism involved in salt tolerance of yeasts are known, it may be possible to extrapolate the same to higher plants (Osakabe *et al.* 2012) for sensing the environment and responding to abiotic stresses including salt stress. The progressive salinization of soil, estimated at around 20% of irrigated land (Ghassemi *et al.* 1995) has made the genetic improvement of salt tolerance an urgent priority for the future of agriculture. It may then be well within the ambit of the science of the day to develop salt resistant plants by recombinant DNA technology, especially commercial and crop plants which do face a severe salt stress, particularly in tropical and temperate regions. Presently, few plants have been made salt tolerant including transgenic tomato using yeast HAL1 gene (Gisbert *et al.* 2000).

Transcriptional and Proteomic Response of *S. cerevisiae* to Salt Stress

S. cerevisiae contains roughly 200 proteins, which respond to salt stress (Blomberg 1995), but only 18 to 30 proteins increased their expression considerably when cells were shocked with high salt stress (Norbeck and Blomberg 1996).

Upon osmotic shock yeast cells synthesize and accumulate glycerol, a osmolyte that may stabilize soluble enzymes and restore the cell turgor pressure required for growth. Therefore, some of the proteins showing increased synthesis rates upon osmotic shock are enzymes involved in glycerol metabolism. Along with upregulation of enzymes like GPD-1p for glycerol production (Albertyn *et al.* 1994), induction of salt tolerance & adaptation require de-novo protein synthesis and upregulation of Heat Shock Proteins (HSP-107).

Gene	Gene product
Up-regulated	
CTT1	Catalase T
DAK1	Dihydroxyacetone kinase
ENO1	Enolase I
GCY1	Putative glycerol dehydrogenase
GPD1	Glycerol-3-phosphate dehydrogenase
GPP1/2	Glycerol-3-phosphatase I and II
HSP104	Heat shock protein
TDH1	Glyceraldehyde-3-phosphate dehydrogenase I
Down-regulated	
ADH1	Alcohol dehydrogenase I
ALD6	Aldehyde dehydrogenase
ENO2	Enolase II
GDH1	Glutamate dehydrogenase (NADP)
HXK2	Hexokinase pII
MET6	Methionine synthase
PDC1	Pyruvate decarboxylase
TDH3	Glyceraldehyde-3-phosphate dehydrogenase III

Figure 3: Gene and Proteins up-regulated and down-regulated by hyperosmotic stress (Norbeck and Blomberg 1996).

3.6. Ethanol Fermentation in NaCl stress and Maintenance energy

Maintenance Energy is the amount of energy required by the cells for maintaining their life processes & growth. It also includes the energy spent while maintaining chemical gradients of ions / metabolites across cellular membrane. There are 2 Maintenance Energy terms:

(i) Constant maintenance energy term (m) – independent of the specific growth rate

(ii) Varied maintenance energy term (m') – dependent on specific growth rate, m' decreases linearly with increase in specific growth rate and becomes zero at the maximum specific growth rate.

In order to maintain life in high salt concentration, the cell need to spent more maintenance energy (ATP molecule). This extra energy demand is supplied through high rate of ATP generating process i.e., fermentation in yeast. Thus, enhancing fermentation of pyruvate to Ethanol and ATP in cytoplasm.

Therefore, theoretically: ↑Salt Stress → ↑Ethanol yield

For yeast cells to ferment and convert sugars into ethanol, the yeast cells first need to adapt to stress (high NaCl concentration). The NaCl tolerant/ adapted yeast cells capable of fermenting more ethanol are selected and maintained on high salt containing solid media (agar plates). The adapted cells are selected by the fact, non-adapted yeast cells die and are reduced in number while the adapting cells survive and transforms into Salt tolerant yeast with continual culturing in high salt supplemented media. Theoretically, yeast is capable of enhanced fermentation and ethanol production by salt stress adaptation according to maintenance energy hypothesis. In other words, **the yeast cells are metabolically engineered/modified by osmotic stress with desired characteristic of high Ethanol specific productivity.** Additional proof of enhanced fermentation of yeast in salt conditions comes from enhanced leavening potential of yeast in dough in presence of NaCl (Oda and Tonomura 1993).

Metabolic engineering involves directed modulation of metabolic pathways for over production of metabolite or improvement of cellular properties by optimization of genetic and regulatory processes within cells. In our experiments, modulation of fermentation process is aimed as a response to salt stress. Microbial fermentation has been widely used in industrial production of biotechnological compounds including biofuels (bioethanol). The rapidly growing biotechnology market requires an efficient bioprocess platform including both efficient production cells and efficient process for biochemical and biofuels production. Metabolic engineering is often used to develop high-producing cells needed for the process. Metabolic engineering of bio-factories especially yeast has potential to increase the fermentation production providing stable and sustainable production of bioethanol (Nielsen 2015). Metabolic process engineering (MPE) is another advanced technology that alters or manipulates metabolic pathway to produce the interested metabolites by controlling or manipulating bio-production process parameters used for fermentation process development (Yang and Liu 2015). The goal of metabolic process engineering is to achieve high-productivity, robust and scalable process through dynamic monitoring and investigating the interactions between cellular metabolism and environment/ process parameters. Fermentation can easily be disturbed by slight changes in some process parameters, which leads to variable product quality or quantity.

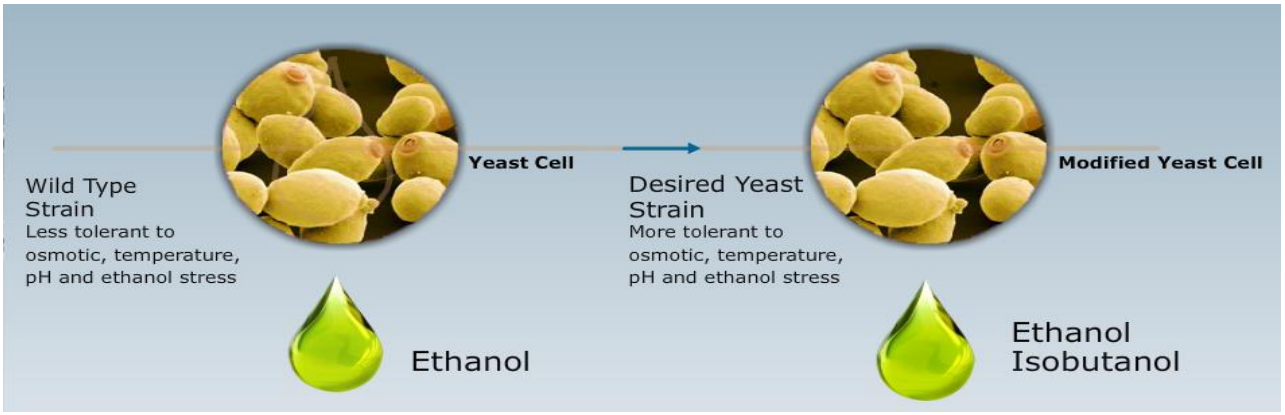


Figure 4: Cellular response to stress with enhanced ethanol production in stress tolerant yeast.

3.7. Bioethanol Production Cost

Negative environmental consequences of fossil fuels and concerns about petroleum supplies have led to the search for renewable transportation biofuels. To be a viable alternative, a biofuel should provide a net energy gain, have environmental benefits, be economically competitive, and be producible in large quantities without competing with food supplies (Hill *et al.* 2006). Ethanol yields 25% more energy than the energy invested in its production. Ethanol can be used as transportation fuel or fuel additive (ethanol blending with petrol) as greenhouse gas emissions are reduced 12% by the production and combustion of ethanol compared to the fossil fuels. Until recent increases in petroleum prices, high production costs of ethanol biofuel made it unprofitable. Transportation biofuels such as cellulosic ethanol (2nd generation biofuel), if produced from waste biomass, could provide much greater supplies and environmental benefits than food-based biofuels. To meet increasing global demand for second generation bio-ethanol require diverse raw materials utilisation, process & engineered strains development (Balat 2009).

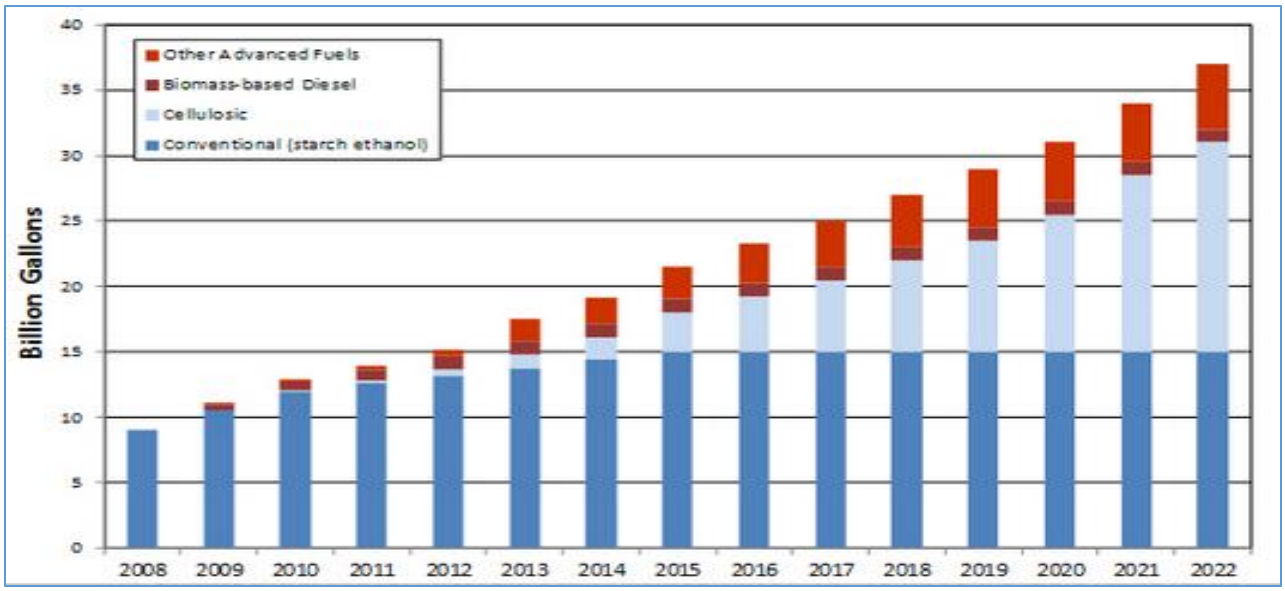


Figure 5: Renewable fuels' standard volumes per annum (Source: U.S. Department of Energy, 2013**)** {Projected increase in Cellulosic Ethanol demand and production }

Conventional Bio-ethanol Production Costs

Bioethanol production costs are determined by installed capital costs, feedstock costs (which are a function of farming costs, productivity and market supply/ demand), operation and maintenance costs and fermentation efficiency. However, total production costs for conventional bioethanol products are dominated by feedstock costs (Balat 2009). Our research targets improving fermentation efficiency as a result of enhanced & faster fermentation process by application of salt stress adapted robust yeast strain.

The global prices for food-based biofuel feedstocks has been increasing from 2000 to 2012 leading to increase in net production costs (World Bank 2013). Therefore, shifting to lignocellulosic ethanol that utilizes waste biomass as feedstock will help in reduction of ethanol fuel costs (McAloon *et al*. 2000; National renewable Energy Lab, US 2000).

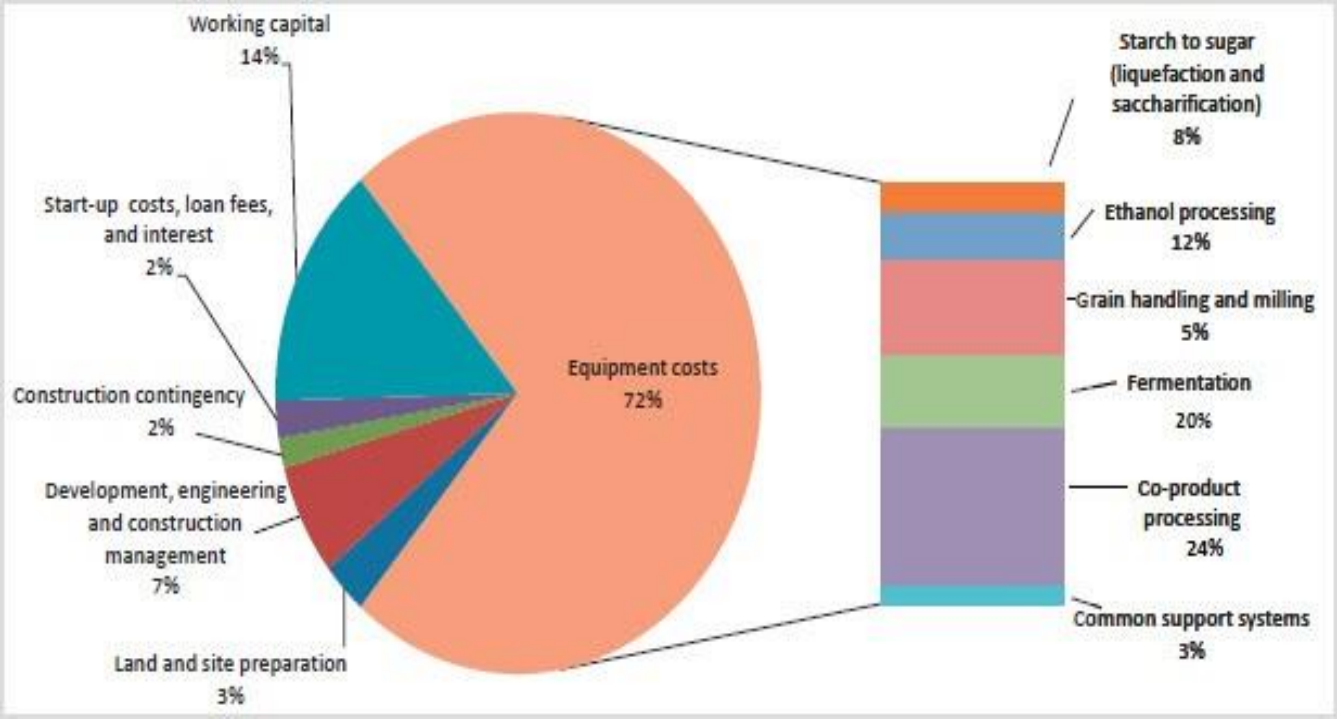


Figure 6: Total installed capital cost breakdown for conventional ethanol plant in the United States. (Sources: Iowa State University 2013 and Kwiatkowski *et al*. 2006)

4.

MATERIALS

&

METHODS

Materials:

1. Yeast strain: *Saccharomyces cerevisiae* CEN.PK 122 (diploid strain) from Peter Kötter, J.W. Goethe Universitat Frankfurt, Frankfurt, Germany

2. Culture Media (autoclaved):

a) YPD (Yeast Peptone 2% Dextrose): YPD liquid media, YPD-Agar plates, YPD (+3% NaCl)-Agar plates

b) CSL (Corn Steep Liquor): CSL liquid culture media (containing 10% glucose with varying CSL and salt concentrations), CSL-Agar Plates (containing 10% glucose with varying CSL and salt concentrations)

3. Glasswares and Other Materials:

a) Flasks- 100 ml flasks for cell culture & 250 ml, 500ml, 1L flasks for media preparation

b) Filter Assembly (0.22 µm polystyrene membrane filter) for filter sterilising & storing nutrient stocks

c) Syringes, 0.22 µm & 0.3 µm Nylon syringe filters for filter sterilisation of culture supernatant for HPLC Analysis

d) Capped HPLC vials

4. Instruments:

a) Yeast Culture Incubator maintained at 30°C, 200 rpm (Orbital Incubator Shaker)

b) Centrifuge machine (Eppendorf Micro-centrifuge 5424R and Eppendorf Centrifuge 5810R)

c) HPLC Analyzer (Agilent Technologies 1260 Infinity)

d) HPLC organic acid analysis column - Aminex HPLX-87H Ion Exclusion Column from BIO-RAD

e) U.V/ Visible Spectrophotometer (Amersham Biosciences Ultrospec 3100 Pro)

f) Nikon Eclipse Ni Microscope System

g) pH Meter

h) Horizontal Laminar Air Flow Cabinet (LAF Hood)

i) Refrigerator : 4 °C Freezer for plates' storage, -20 °C Freezer for supernatant storage

4.1. RESPONSE OF UNADAPTED YEAST CELLS TO SALT

4.1.1. Preliminary test for growth of yeast in salt supplemented CSL (4.5%) culture media

1) In sterile LAF hood, inoculate one of the single colonies of *Saccharomyces cerevisiae* CEN.PK122 maintained on YPD (2% glucose) master stock plates to YPD (5% glucose) liquid media.
2) Incubate the culture at 30 °C, 200 rpm shaking and allow it to grow overnight in yeast culture incubator.
3) After overnight culturing, inside LAF extract 1ml of the culture in eppendorf.
4) Transfer it to cuvette and measure the O.D. at λ=550 nm with spectrophotometer blanking with fresh uncultured YPD media. O.D. corresponds to cell density of culture.
5) Prepare five flasks of 40ml of 4.5% CSL media with 10% glucose and different NaCl concentrations- 0%, 1%, 2%, 3%, 4%.
6) Calculate the volume of YPD culture corresponding 0.05 Seeding O.D. (in 40 ml media). In LAF, transfer this volume from YPD culture flask to CSL media with varying NaCl concentration.
7) Incubate the culture in yeast incubator for 18 hours.
8) Transfer 1ml of culture in different salt concentrations to separate eppendorfs, centrifuge at 12000 rpm/10 min/4 °C. Separate & store the supernatant at -20 °C. The cell pellet is suspended in 1ml YPD (2% glucose) by vortexing.
9) Measure the O.D. at 550 nm blanking with fresh YPD media. (dilution may be required)
10) Transfer 30 µl of CSL cultures (with 2%, 3%, 4% salt) on CSL (10% glucose) plates with 2%, 3%, 4% salt. Spread the culture onto the plate with help of a spreader. Incubate the CSL spread plates in incubator (30 °C) for 1-2 days until growth/ white yeast colony appears on the plate. Later, store the plates in freezer (4 °C).

4.1.2. Response of yeast to varying salt (NaCl) concentration in 1% CSL media

1) Pre-culture the yeast colony from YPD Master stock plate to sterile 40ml of 1% CSL + 0% NaCl + 10% glucose media. Maintain culture in yeast culture incubator (30 °C with constant shaking at 200rpm) overnight (17-20 hours). Subculture it in fresh 1% CSL media for same time (if required).
2) Inside LAF, transfer 1ml of the 1% CSL culture in eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Discard the supernatant and dissolve cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water.
3) Calculate the volume of CSL culture corresponding 0.1 Seeding O.D (in 40 ml media).
4) Prepare five flasks with sterile 40ml media of 1% CSL concentration containing 10% glucose and different NaCl concentrations- 0%, 1%, 2%, 3%, 4%.
5) In LAF, transfer the calculated volume corresponding to 0.1 seeding O.D. from 1% CSL + 0% NaCl preculture flask to varying NaCl concentrations- 0%, 1%, 2%, 3%, 4% flasks of 1% CSL.

Keep these culture flasks in incubator for 24 hours. Measure $O.D_{550\ nm}$ of the 1% CSL + varying NaCl concentration. cultures at every 2 hours interval from 0 to 8 hours. Take the final culture reading at 24hours. Store the supernatant at every time interval for HPLC analysis. HPLC analysis of supernatant at time point 8 hours of cultures with varying salt concentration is carried.

6) Prepare spread plates from each NaCl concentration culture flask on 1% CSL–Agar plates containing 10% glucose & different NaCl concentrations. Incubate the plates at 30 °C for few days until yeast colonies start growing. Then, store the plates in freezer (4 °C).

* For measuring the O.D. at 0, 2, 4, 6, 8, 24 hours: pellet the cells and dissolve in M.Q. water, blanking with M.Q. water. Cells suspended in water gives consistent O.D. at every time point and thus correct cell density.

Analysis of Glucose & Ethanol in supernatant of cultures using HPLC :

1) For determining Glucose consumption & Ethanol production, supernatants were analysed using HPLC to accurately measure glucose and ethanol. Since media is highly coloured so, biochemical assay may give error.
2) The culture supernatant is first filtered using 0.22 µm syringe filter to get rid of any debris/ insolubles that may clog HPLC column.
3) Different dilutions of supernatant is prepared in Milli-Q water to correctly estimate glucose and ethanol concentration as HPLC is most sensitive in detection of analyte at an approximate concentration of 1g/L. Load 300-400 µl of supernatant or diluted supernatant in HPLC vials. Cap & label them and place in HPLC machine for analysis. Make sample entry in HPLC associated computer system.
4) 10 µl of supernatant is injected into HPLC column. The analytes in supernatant are separated by the Aminex Ion Exclusion column of HPLC according to their Retention time (R_T) in column. Separated analytes are detected by Refractive Index Detector (RID) to give different peaks (analyte is identified by peak at a particular R_T unique for every compound). Depending on concentration of analyte, RID signal is amplified into area for each peak/ analyte. Analyte concentration is determined by comparing Area of Sample (determined by RID signal) at specific Retention time (R_T- specific for each analyte) and Area of Standard (known concentration of particular analyte) using formula : $Conc.\ of\ analyte\ in\ sample = \frac{\text{Area of sample} \times \text{Conc. of Standard} \times \text{dilution factor}}{Area\ of\ Standard}$
5) Glucose consumption & Ethanol production in supernatants of different cultures is calculated as:
   Glucose Consumed = Initial Glucose in media – Glucose in supernatant of sample at time point
   Ethanol Produced = Ethanol present in sample

* Area of Standards of Analytes of HPLC is mentioned in appendix

4.2. OPTIMISATION OF CSL CONCENTRATION IN MEDIA W.R.T. 3% SALT STRESS

4.2.1. Response of Unadapted Yeast Cells to Varying CSL Concentration in Presence of 3% Salt

1) Pre-culture the yeast colony from YPD Master stock plate to 40ml of 2% CSL media without any salt. Maintain culture in yeast culture incubator (30 °C with constant shaking at 200rpm) overnight (17-20 hours). Subculture it in fresh 2% CSL media for same time (if required).
2) Inside LAF, take out 1ml of the 2% CSL culture in eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Discard the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water.
3) Calculate the volume of CSL culture corresponding 0.1 Seeding O.D (in 40 ml media)
4) Prepare four flasks of sterile 40ml media with varying CSL (0.5%, 1%, 2%, 4.5%) concentration containing 10% glucose and 3% NaCl. In LAF, transfer the calculated volume corresponding to 0.1 seeding O.D. from 2% CSL preculture flask to 3% NaCl supplemented media with varying CSL concentrations (0.5%, 1%, 2%, 4.5%).
5) Incubate the varying CSL concentration culture flasks in yeast incubator for 24 hours. Measure $O.D_{550\ nm}$ of the varying CSL cultures at every 2 hours interval from 0 to 8 hours. Take the final culture reading at 24hours. Store the supernatant at every time interval for further analysis
6) Prepare spread plates from each CSL concentration culture flasks into respective CSL concentration –Agar plates containing 10% glucose and 3% Salt. Incubate the plates at 30 °C for few days until yeast colonies start growing. Then, store the plates in freezer (4 °C).
7) Analyse the supernatant for glucose and ethanol using HPLC at time point 24hours of cultures with varying CSL concentration.

* Measure the O.D. at 0-8hours in their culture media without pelleting cells. Blank with respective CSL concentration media. For 24hours O.D. reading, pellet the cells and dissolve in Milli-Q water. Here, blank is M.Q. water. This is done because the CSL media colour intensity decreases after 24hours due to utilization of media by the cells and blanking with fresh media (higher colour intensity) will give an error for cell density.

4.2.2. Selection of Yeast Cells on Agar Plates of Different CSL Concentration Containing 3% Salt

1) Plate the culture growing in liquid media on Agar plates of respective CSL concentration

   From 0.5% CSL (+3% NaCl) liquid culture on 0.5% CSL –Agar plate (+3% NaCl)

   From 1% CSL (+3% NaCl) liquid culture on 1% CSL –Agar plate (+3% NaCl)

   From 2% CSL (+3% NaCl) liquid culture on 2% CSL –Agar plate (+3% NaCl)

   From 4.5% CSL (+3% NaCl) liquid culture on 4.5% CSL –Agar plate (+3% NaCl)

2) Incubate plates in incubator at 30 C for few days (2-3days) until colonies are observed.

* The colonies obtained on 2% CSL (3% Salt) mark the first Salt Tolerant/ adapted cells.

4.3. ADAPTATION OF YEAST CELLS ON MINIMAL 2% CSL MEDIA SUPPLEMENTED WITH 3% SALT (NACL)

4.3.1. Serial Adaptation of Yeast to 3% Salt Stress

1) Prepare flasks with sterile 20ml media of 2% CSL concentration containing 10% glucose and 3% NaCl (called as C2S3 media) used for serial adaptation experiment.
2) In order to adapt cells to high salt stress, pick a colony of First Salt Adapted yeast growing on CSL-Agar plate (+3% NaCl) as in experiment 4.2.2
3) Culture the first salt tolerant yeast in 10 ml liquid 2% CSL + 3% NaCl media in test tube. This is pre-culture. Incubate the culture at 30 °C on yeast shaker for 1-2 days till good growth (turbidity) is observed in the media.
4) Inside LAF, take out 1ml of the cultures into eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Separate the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water. Calculate the volume of CSL culture corresponding 0.1 Seeding O.D (in 20 ml media).
5) In LAF, transfer the calculated culture volume of preculture (corresponding to 0.1 seeding O.D) to 20 ml fresh C2S3 media flask. Label it as C1' cultures. Incubate flask at 30 °C with constant shaking in incubator for 24 hours.
6) After 24hours, pipette out 1 ml of C1' cultures into eppendorf, pellet the cells, suspend in 1 ml M.Q. water and store the supernatant at -20 °C. Measure $O.D._{550nm}$ of suspended cells of C1 and C1' by blanking it with M.Q. water.
7) Subculture C1' culture into C2S3 media at 0.1 seeding O.D. Label it as C2' culture. Maintain the culture in Yeast incubator for 24 hours. C2 is subsequently subcultured to C3' to C4'. There is 4 passages/ subcultures in this stage of serial adaptation.

8) Transfer and spread C4' culture on C2S3 (10% glucose) plate, C2S3 (2% glucose) plate and YPD (with 3% salt) respectively. Incubate the CSL spread plates in incubator (30 °C) for 1-2 days until growth/ white yeast colony appears on the plate. Later, store the plates in freezer (4 °C). This is Serial Adapted-1 (Sr1) yeast.
9) Similarly perform next serial adaptation process by picking colony from CSL plate of Sr1 and repeatedly sub-culturing at seeding O.D 0.1 every 24 hours for 5 passages by repeating steps 3-10.
10) Plate the last subculture on C2S3 (10% glucose) plate, C2S3 (2% glucose) plate and YPD (with 3% salt). Incubate the CSL spread plates in incubator (30 °C) for 1-2 days until growth/ white yeast colony appears on the plate. Later, store the plates in freezer (4 °C). This is Serial Adapted-2 (Sr2) yeast.
11) Similarly perform next serial adaptation by picking colony from CSL plate of Sr2 and repeatedly sub-culturing at seeding O.D 0.1 every 24 hours for 10 passages by repeating steps 3-10.
12) Plate the last subculture on C2S3 (10% glucose) plate, C2S3 (2% glucose) plate and YPD (with 3% salt). Incubate the CSL spread plates in incubator (30 °C) for 1-2 days until growth/ white yeast colony appears on the plate. Later, store the plates in freezer (4 °C). This is Serial Adapted-3 (Sr3) yeast.

* Liquid cultures are maintained with 2% CSL and 3% NaCl concentration along with 10% glucose.
* Sr1 is Serial Adaptation-1 in salt stress, Sr2 is Serial Adaptation-2 in salt stress, Sr3 is Serial Adaptation-3 in salt stress.
* C2S0 is salt free 2% CSL media, C2S3 is 2% CSL media containing 3% Salt (NaCl)

4.3.2. Selection and Maintenance of Salt Adapted Yeast on Agar Plates (with 3% Salt)

1) Prepare spread plates of liquid cultures of different serial adaptations (Sr1, Sr2 & Sr3 adapted to 3% NaCl stress) on 2 types of Agar plates-

   YPD (2% glucose) –Agar plates containing 3% NaCl – 1:100 culture dilution

   2% CSL (+2% glucose or 10% glucose) –Agar plate containing 3% NaCl – undiluted sample

2) Incubate the plates at 30 °C for 2-3 days until yeast colonies start growing. Then, store the plates in freezer (4 °C).

* Select single colonies from the plates & re-plate on same media composition plate once in a month.
* Serial Adaptation of yeast to salt stress is by repeatedly culturing cells in liquid media with 2% CSL & 3% NaCl.
* YPD Plates are for maintaining particular Serial Adaptation yeast i.e., growth in next culturing remains same as of culture before plating on agar plate

  CSL Plates are for sequential adaptation process i.e., growth in next culturing improves from culture before plating to become next serially adapted yeast.

4.3.3. Comparative Growth and Fermentation Characteristics of Different Serial Adapted and Unadapted Yeast in Salt Supplemented Industrial Media.

1) Precultures were simultaneously grown from different serial adaptations (Sr1 & Sr2) and unadapted yeast cells in order to compare their cell growth and fermentation capacity at different time intervals.

   1st preculture (C2S0 Unadapted) – From unadapted yeast colony on YPD master plate inoculated into liquid 2% CSL + 0% NaCl media (in 10 ml tube).

2nd preculture (C2S3 Sr1) - From the yeast colony of Sr1 Adaptation maintained on 2% CSL-Agar plate (+ 3% NaCl) inoculated into liquid 2% CSL + 3% NaCl media (in 10 ml tube).

3rd preculture (C2S3 Sr2) - From the yeast colony of Sr2 Adaptation maintained on 2% CSL-Agar plate (+ 3% NaCl) into liquid 2% CSL + 3% NaCl media (in 10 ml tube).

2) All pre-cultures were cultured in incubator at 30 °C. Since, C2S3 cultures are slow growing, allow them to grow for 24-48 hours to obtain substantial O.D. or cells for seeding.
3) Inside LAF, take out 1ml of the C2S0 unadapted, C2S3 Sr1 and C2S3 Sr2 cultures into eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Separate the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water. Calculate the volume of culture corresponding 0.1 seeding O.D (in 20 ml media) for unadapted cultures, Sr1, Sr2.
4) Prepare 3 flasks with sterile 20 ml media (2% CSL + 10% glucose + 3% NaCl).
5) In LAF, transfer the calculated culture volume from unadapted, Sr1 Adapted and Sr2 Adapted cultures into 2% CSL + 3% NaCl media. Label them as C1, C1', C1'' cultures. Incubate them at 30 °C with constant shaking in incubator for 42 hours.
6) Pipette out 1 ml of C1, C1' and C1'' of unadapted, Sr1 Adapted, Sr2 Adapted cultures from culture flasks into eppendorf, pellet the cells, suspend in 1 ml M.Q. water and store the supernatant at -20 °C. Measure $O.D._{550nm}$ of suspended cells of culture by blanking it with M.Q. water. This step is performed at time point of 8hours, 16 hours, 24 hours and 42 hours of culturing.
7) Subculture C1' culture of Sr1 Adaptation into 20 ml 2% CSL + 3% NaCl media at 0.1 seeding O.D. Label it as C2' culture of Sr1. Maintain the culture in Yeast incubator. Read the $O.D._{550nm}$ of 1ml C2 culture by blanking with M.Q. water at 8 hours and 24 hours respectively. Separate the supernatant from cell pellet at respective time point. Store the supernatant at -20 °C.

8) Subculture C2' culture into 20 ml 2% CSL + 3% NaCl media at 0.1 seeding O.D. Label it as C3' culture. Read the O.D. $_{550nm}$ of 1ml C2 culture by blanking with M.Q. water at 8 hours, 16 hours and 24 hours respectively.

9) Repeat step-8 for preparing C4' culture on 2% CSL + 3% NaCl by subculturing C3' and measure O.D at 8hours.

10) Preserve supernatant at -20 °C of all cultures of unadapted yeast, Sr1 Adapted & Sr2 Adapted yeast at different time points.

11) Perform HPLC analysis of supernatant of cultures of unadapted yeast, Sr1 Adapted & Sr2 Adapted yeast at different time points.

12) Compare the growth and fermentation characteristics (Glucose consumption and ethanol production)

4.4. RELATIONSHIP BETWEEN GROWTH AND FERMENTATION OF YEAST CELLS

1) Precultures of unadapted yeast and Sr2 salt adapted yeast cells were grown for 2days to obtain sufficient cells for seeding O.D 5.0

   1st Preculture: Unadapted yeast cells (Control) cultured in salt free 2% CSL media

   2nd Preculture: Sr2 salt adapted yeast cells cultured in 2% CSL media (containing 3% NaCl)

2) Inside LAF, take out 1ml of each preculture into eppendorf, centrifuge at 12000 rpm/10 min/4 °C. Separate the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D at 550 nm with spectrophotometer blanking with M.Q. water. Calculate the volume of culture corresponding 5.0 seeding O.D (in 20 ml media) for unadapted and adapted cultures.

3) Pipette out the volume of precultures in falcons, Centrifuge to separate cells. Discard the supernatant. Wash the cells with CSL free media. Again centrifuge to pellet down cells & remove the supernatant.

4) Prepare CSL free media with & without salt. In LAF, transfer the calculated culture volume from unadapted & adapted yeast washed cells into CSL free media (only nutrients & glucose).

   S0 Control: Unadapted (Control) yeast cells in CSL free liquid media without salt seeded at O.D 5

   S0 Sr2 Adapted: Sr2 salt adapted yeast cells in CSL free media without salt seeded at O.D 5

   S3 Sr2 Adapted: Sr2 salt adapted yeast cells in CSL free media with 3% salt seeded at O.D 5

5) The cell cultures from these 3 culture conditions were isolated at different time points till 24 hours, the supernatant was separated from cells by centrifugation. The cell growth is measured in terms of $O.D_{550\ nm}$ of cell pellet dissolved in M.Q. water at different time points till 24hours. The supernatant was analyzed using HPLC to quantify Ethanol produced and Glucose consumed at 8 & 16 hours.

* Sr2 Adapted is 2nd generation of Serially Adapted yeast to salt stress

4.5. COMPARATIVE ANALYSIS OF GROWTH & FERMENTATION CHARACTERISTICS OF SALT ADAPTED YEAST CELLS IN PRESENCE AND ABSENCE OF SALT STRESS IN INDUSTRIAL MEDIA

4.5.1. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at Low Seeding O.D (0.1)

1) Control yeast cells maintained on salt free 2% CSL-Agar plate was inoculated into salt free CSL liquid media (C2S0), Sr2 salt adapted yeast cells maintained on 2% CSL plate (+3% salt) and Unadapted yeast maintained on YPD plates were inoculated into 3% salt containing CSL media (C2S3). Culture for 2days to obtain sufficient cells for precultures.
2) Precultures of control, adapted and unadapted yeast were simultaneously grown from liquid cultures of step-1.

   1st preculture (Control cells) – From C2S0 Control into liquid 2% CSL + 0% NaCl media (in flask)

   2nd preculture (Sr2 Adapted cells) - From the C2S3 Sr2 salt adapted culture into liquid 2% CSL + 3% NaCl media (in flask).

   3rd preculture (Unadapted cells) - From C2S3 unadapted culture into liquid 2% CSL + 3% NaCl media (in flask).
3) All pre-cultures were cultured in incubator at 30 °C. Since, C2S3 cultures are slow growing, allow them to grow for 24-48 hours to obtain substantial O.D. or cells for seeding.
4) Inside LAF, take out 1ml of the precultures into eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Separate the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water. Calculate the volume of culture corresponding 0.1 seeding O.D (in 20 ml media) for control cultures, Sr2 salt adapted cultures and unadapted cultures.

5) Prepare 2 flasks with sterile 20 ml salt free 2% CSL media (C2S0) and other 2 flasks with 3% salt containing 2% CSL media (C2S3).

6) In LAF, transfer the calculated culture volume from control culture into C2S0 liquid media, Sr2 adapted culture into C2S0 & C2S3 liquid media and unadapted culture into C2S3 liquid.

   1st Culture (C2S0 Control): Control yeast cells from C2S0 Control preculture in CSL media without salt seeded at O.D 0.1

   2nd Culture (C2S0 Sr2 Adapted): Sr2 salt adapted yeast cells from C2S3 Sr2 Adapted preculture in CSL media without salt seeded at O.D 0.1

   3rd Culture (C2S3 Sr2 Adapted): Sr2 salt adapted yeast cells from C2S3 Sr2 Adapted preculture in CSL media with 3% salt seeded at O.D 0.1

   4th Culture (C2S3 Unadapted): Sr2 salt adapted yeast cells from C2S3 unadapted preculture in CSL media with 3% salt seeded at O.D 0.1

   Incubate them at 30 °C shaker incubator for 96hours.

7) Pipette out 1 ml of each culture from culture flasks into eppendorf, pellet the cells, suspend in 1 ml M.Q. water and store the supernatant at -20 °C. Measure $O.D._{550nm}$ of suspended cells of culture by blanking it with M.Q. water. This step is performed at different time points till 96 hours.

8) The supernatant was analyzed using HPLC to quantify Ethanol produced and Glucose consumed at different time points.

* Sr2 Adapted is 2nd generation of Serially Adapted yeast to salt stress

* C2S0 is salt free 2% CSL media, C2S3 is 2% CSL media containing 3% Salt (NaCl)

4.5.2. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at Moderate Seeding O.D (1.0)

1) Precultures of control and adapted yeasts were simultaneously grown. Precultures are recultured in order to get actively growing & dividing cells in exponential phase of growth.

   1st preculture: Control (normal) yeast cell maintained on salt free 2% CSL-Agar plate into liquid 2% CSL + 0% NaCl media

   2nd preculture: Sr2 salt adapted cells maintained on 2% CSL plate (+3% salt) into liquid 2% CSL + 3% NaCl media

2) All pre-cultures were cultured in incubator at 30 °C. Since, C2S3 cultures are slow growing, allow them to grow for 24-48 hours to obtain sufficient cells for seeding.

3) Inside LAF, take out 1ml of the precultures into eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Separate the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water. Calculate the volume of culture corresponding seeding O.D 1.0 (in 20 ml media) for control cultures & Sr2 salt adapted cultures.

4) Prepare 2 flasks with sterile 20 ml salt free 2% CSL media (C2S0) and one flask with 3% salt containing 2% CSL media (C2S3).

5) Pipette out the calculated volume of precultures in eppendorf, Centrifuge to separate cells. Discard the supernatant. Wash the cells with fresh CSL media. Again centrifuge to pellet down cells & remove the supernatant.

6) 3 culture sets of control & adapted yeast was cultured in absence & salt stress for 72hours at 30 °C.

   1st Culture (C2S0 Control): Control yeast cells from preculture-1 in 2% CSL media without salt seeded at O.D 1.0

2nd Culture (C2S0 Sr2 Adapted): Sr2 salt adapted yeast cells from preculture-2 in 2% CSL media without salt seeded at O.D 1.0

3rd Culture (C2S3 Sr2 Adapted): Sr2 salt adapted yeast cells from preculture-2 in 2% CSL media with 3% salt seeded at O.D 1.0

7) Pipette out 1 ml of each culture from culture flasks into eppendorf, pellet the cells, suspend in 1 ml M.Q. water and store the supernatant at -20 °C. Measure cell growth in terms of $O.D_{550nm}$ of suspended cell pellet of culture sets by blanking it with M.Q. water. This step is performed at different time points till 72hours.
8) The supernatant was analyzed using HPLC to quantify Glucose consumption, Ethanol production and Glycerol production at different time points.
9) Compare the growth & fermentation profile (Glucose consumption, Ethanol production and Glycerol production) of salt adapted yeast in absence & presence of salt w.r.t. control yeast culture.

* Sr2 Adapted is 2nd generation of Serially Adapted yeast to salt stress
* C2S0 is salt free 2% CSL media, C2S3 is 2% CSL media containing 3% Salt (NaCl)

4.5.3. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at High Seeding O.D (2.5)

1) Precultures of control and Sr2 & Sr3 adapted yeasts were simultaneously grown. Precultures are recultured in order to get actively growing & dividing cells in exponential phase of growth.

   1st preculture: Control (normal) yeast cell maintained on salt free 2% CSL-Agar plate into liquid 2% CSL + 0% NaCl media

   2nd preculture: Sr2 salt adapted cells maintained on 2% CSL plate (+3% salt) into liquid 2% CSL + 3% NaCl media

   3rd preculture: Sr3 salt adapted cells maintained on 2% CSL plate (+3% salt) into liquid 2% CSL + 3% NaCl media

2) All pre-cultures were cultured in incubator at 30 °C. Since, C2S3 cultures are slow growing, allow them to grow for 24-48 hours to obtain sufficient cells for seeding.
3) Inside LAF, take out 1ml of the precultures into eppendorf, centrifuge- 12000 rpm/10 min/4 °C. Separate the supernatant and suspend cell pellet in 1ml Milli-Q water. Vortex and transfer cell suspension in cuvette to measure the O.D. at 550 nm with spectrophotometer blanking with M.Q. water. Calculate the volume of culture corresponding seeding O.D 2.5 (in 20 ml media) for control, Sr2 salt adapted & Sr3 salt adapted cultures.
4) Prepare 3flasks with sterile 20 ml salt free 2% CSL media (C2S0) and one flask with 3% salt containing 2% CSL media (C2S3).
5) Pipette out the calculated volume of precultures in eppendorf, Centrifuge to separate cells. Discard the supernatant. Wash the cells with fresh CSL media. Again centrifuge to pellet down cells & remove the supernatant.
6) 4 culture sets of control & adapted yeast was cultured in absence & salt stress for 48hours at 30 °C.

1st Culture (C2S0 Control): Control yeast cells from preculture-1 in 2% CSL media without salt seeded at O.D 2.5

2nd Culture (C2S0 Sr2 Adapted): Sr2 salt adapted yeast cells from preculture-2 in 2% CSL media without salt seeded at O.D 2.5

2nd Culture (C2S0 Sr3 Adapted): Sr3 salt adapted yeast cells from preculture-3 in 2% CSL media without salt seeded at O.D 2.5

3rd Culture (C2S3 Sr2 Adapted): Sr3 salt adapted yeast cells from preculture-3 in 2% CSL media with 3% salt seeded at O.D 2.5

7) Pipette out 1 ml of each culture from culture flasks into eppendorf, pellet the cells, suspend in 1 ml M.Q. water and store the supernatant at -20 °C. Measure cell growth in terms of $O.D_{550nm}$ of suspended cell pellet of culture sets by blanking it with M.Q. water. This step is performed at different time points till 48hours.
8) The supernatant was analyzed using HPLC to quantify Glucose consumption, Ethanol production and Glycerol production at different time points.
9) Compare the growth & fermentation profile (Glucose consumption, Ethanol production and Glycerol production) of Sr2 & Sr3 salt adapted yeast in absence & presence of salt w.r.t. control yeast culture (in absence of salt).

* Sr2 Adapted & Sr3 Adapted are 2nd generation & 3rd generation of Serially Adapted yeast to salt stress respectively.

* C2S0 is salt free 2% CSL media, C2S3 is 2% CSL media containing 3% Salt (NaCl).

4.5.4. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at High Seeding O.D (5.0)

Experiments were conducted in 2 replicates. Repeat steps 1-9 of previous experiment with different culture sets.

Culture sets for Replicate 1 :

1st Culture (C2S0 Control): Control (normal) yeast cells maintained on salt free 2% CSL– Agar plates are inoculated in same 2% CSL media without salt at seeding O.D 5.0

2nd Culture (C2S0 Sr2 Adapted): Sr2 salt adapted cells maintained on 2% CSL–Agar plates (+ 3% NaCl) are inoculated in liquid media in salt free 2% CSL media at seeding O.D 5.0

3rd Culture (C2S3 Sr2 Adapted): Sr2 salt adapted cells maintained on 2% CSL–Agar plates (+ 3% NaCl) are inoculated in 2% CSL liquid media with salt at seeding O.D 5.0

1) The cell growth is measured in terms of $O.D_{550\ nm}$ at different time points till 56hours.
2) The supernatant was analyzed using HPLC to quantify Glucose consumption, Ethanol production and Glycerol production at different time points.
3) Compare the growth & fermentation profile (Glucose consumption, Ethanol production and Glycerol production) of Sr2 salt adapted yeast in absence & presence of salt w.r.t. control yeast culture (in absence of salt).

Culture sets for Replicate 2 :

1st Culture (C2S0 Control): Control (normal) yeast cells maintained on salt free 2% CSL– Agar plates are inoculated in same 2% CSL media without salt at seeding O.D 5.0

2nd Culture (C2S0 Sr2 Adapted): Sr2 salt adapted cells maintained on 2% CSL–Agar plates (+ 3% NaCl) are inoculated in liquid media in salt free 2% CSL media at seeding O.D 5.0

3rd Culture (C2S0 Sr3 Adapted): Sr3 salt adapted cells maintained on 2% CSL–Agar plates (+ 3% NaCl) are inoculated in liquid media in salt free 2% CSL media at seeding O.D 5.0

4th Culture (C2S3 Sr3 Adapted): Sr3 salt adapted cells maintained on 2% CSL–Agar plates (+ 3% NaCl) are inoculated in 2% CSL liquid media containing salt at seeding O.D 5.0

1) The cell growth is measured in terms of $O.D_{550\ nm}$ at different time points till 48hours.
2) The supernatant was analyzed using HPLC to quantify Glucose consumption, Ethanol production and Glycerol production at different time points.
3) Compare the growth & fermentation profile (Glucose consumption, Ethanol production and Glycerol production) of Sr2 & Sr3 salt adapted yeast in absence & presence of salt w.r.t. control yeast culture (in absence of salt).

* Sr2 Adapted & Sr3 Adapted are 2nd generation & 3rd generation of Serially Adapted yeast to salt stress respectively.

* C2S0 is salt free 2% CSL media, C2S3 is 2% CSL media containing 3% Salt (NaCl).

4.6. CELLULAR CHARACTERISTICS OF ADAPTED AND UNADAPTED YEAST CELLS IN DIFFERENT CULTURE CONDITIONS (+/- SALT)

4.6.1. Observation of Colour of Adapted & Unadapted Yeast Cells Cultured in +/- Salt

1) Isolate cell culture of different culture sets –C2S0 Control, C2S0 Adapted, C2S3 Adapted for different experiment sets of seeding O.D 1.0, 2.5 & 5.0 in 10 ml falcons.
2) Centrifuge the culture to pellet the yeast cells
3) Observe the colour of different cultures and capture image.

4.6.2. Observation of Cell Size of Adapted & Unadapted Yeast cultured in +/- Salt

1) Isolate 1ml cell culture of different culture sets –C2S0 Control, C2S0 Adapted, C2S3 Adapted for different experiment set of seeding 5.0 in eppendorfs.
2) On a clean slide, transfer 20µl of culture, spread it on slide, put a coverslip area covered by culture.
3) Place the slide on stage of microscope. First observe yeast under low power objective lens (10X) followed by high power objective lens (100X). Microscopic observation of yeast under 100X is by oil immersion method. For this, put one drop of oil on coverslip of slide. Touch the 100X objective lens to oil surface and observe cells in microscope.
4) Captivate the picture of yeast cells, measure cell dimensions (length & width) of C2S0 Adapted, C2S3 Adapted yeast and Unadapted C2S0 control. Compare cell sizes of different culture sets.

* For different cultures, use different slides.
* Yeast identified by budding
* C2S0 is salt free 2% CSL media, C2S3 is 2% CSL media containing 3% Salt (NaCl).

5.

RESULTS

&

DISCUSSIONS

5.1. RESPONSE OF UNADAPTED YEAST CELLS TO SALT

5.1.1. Preliminary Test for Growth of Yeast in Salt Supplemented CSL (4.5%) Culture Media

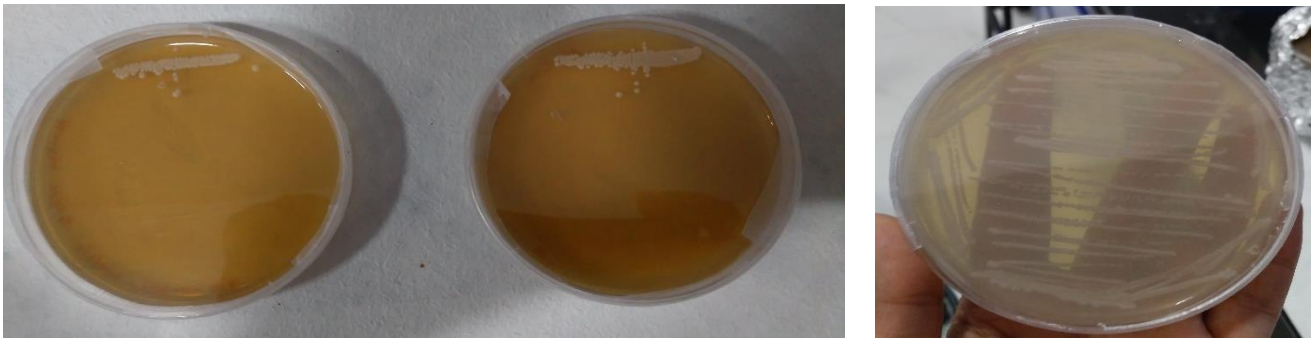


Figure 7: Replicates of Master Stock plates of S*accharomyces cerevisiae* CEN.PK 122 (diploid) maintained on YPD (2% glucose)-Agar Plate.

Saccharomyces cerevisiae cells were cultured in 4.5% CSL media with 10% glucose and different NaCl (0%, 1%, 2%, 3%, 4%) concentrations for 18 hours. Since, CSL media is dark coloured, contains debris and is turbid in nature so, cannot predict cell growth by direct turbidity observation. To find the cell density of cultures with different salt concentrations, O.D. at λ=550 nm was measured with spectrophotometer after pelleting the cells. The cell pellet contained a lot of CSL media debris and thus did not give correct O.D./cell density. An inference is made that the CSL media should be formulated, autoclaved and centrifuged to obtain debris-free CSL media. Also, addition of glucose to media after autoclaving to prevent charring of glucose releasing compounds that may be inhibitory to yeast cell growth.

The CSL cultures (with 2%, 3%, 4% NaCl) was plated on CSL-Agar plates with 10% glucose & different NaCl (2%, 3%, 4%) concentrations and incubated for 2 days at 30 °C. The following results were obtained as demonstrated in Fig. 8 :

4.5% CSL + 2% NaCl plates – white lawn of yeast cells observed.

4.5% CSL + 3% NaCl plates – few white coloured single colonies of yeast were observed.

4.5% CSL + 4% NaCl plates – no yeast colony was able to grow at 4% salt concentration.

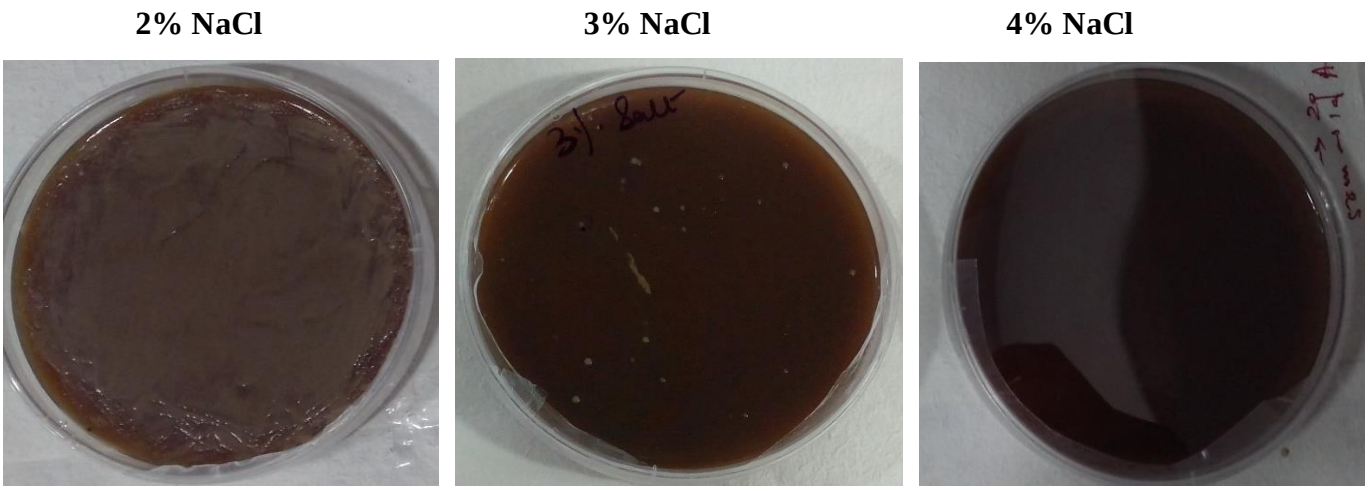


Figure 8: Growth of Yeast cells on 4.5% CSL Plates with 2%, 3%, 4% NaCl concentration respectively.

5.1.2. Response of Yeast to Varying Salt (NaCl) Concentration in 1% CSL Media

The yeast cells were precultured on debri-free 1% CSL media lacking NaCl (also act as control) from YPD stock plate. Subsequently 1% CSL + 0% NaCl preculture was inoculated into 1% CSL media with different NaCl concentrations (0%, %, 2%, 3%, 4%) at 0.1 seeding O.D. Cultured at 30 °C for 24 hours and measured $O.D._{550\ nm}$ reading of varying NaCl concentration at 0, 2, 4, 6,8, 24 hours and estimated and glucose consumption & ethanol production at 8 hours as shown in Table-2 and Table-3 respectively.

NaCl Conc. (+1% CSL)	**O.D. at λ=550 nm**					
	0 hr	**2 hr**	**4 hr**	**6 hr**	**8 hr**	**24 hr**
0% NaCl (Control)	0.115	0.130	0.180	0.234	0.275	0.915
1% NaCl	0.124	0.129	0.135	0.170	0.189	0.674
2% NaCl	0.144	0.153	0.155	0.188	0.215	0.480
3% NaCl	0.130	0.150	0.146	0.155	0.156	0.312
4% NaCl	0.129	0.140	0.148	0.152	0.149	0.205

Table 1: Effect of salt concentration on yeast cell growth in 1% CSL media.
{1 O.D corresponds to 3×10^7 cells/ml of culture ; Control culture media = 1% CSL+ 0% NaCl }

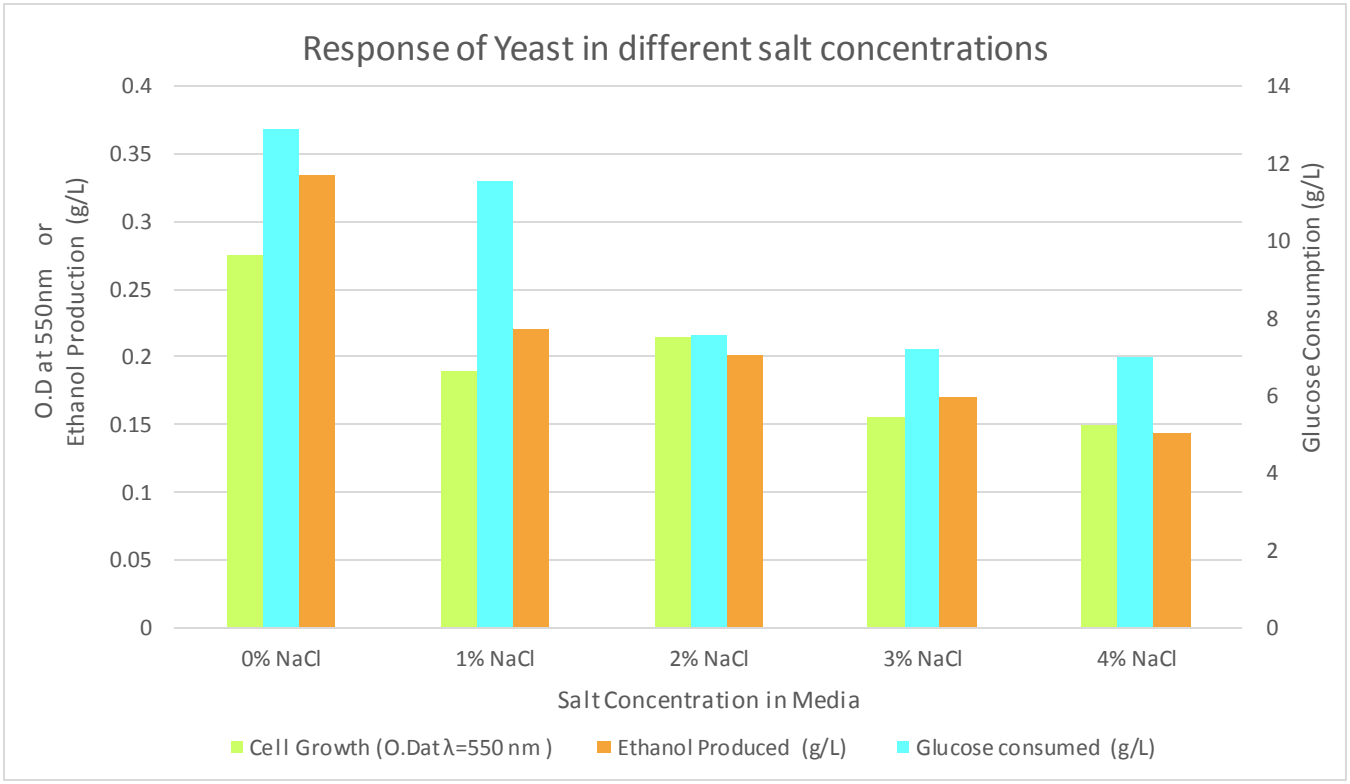
NaCl Conc. (+1% CSL)	**Time Point : 8 hrs**	
	Glucose consumed (g/L)	**Ethanol Produced (g/L)**
0% NaCl (Control)	12.88	0.334
1% NaCl	11.57	0.220
2% NaCl	7.55	0.202
3% NaCl	7.20	0.170
4% NaCl	7.02	0.144

Table 2: Effect of salt concentration on fermentation profile at 8 hours of culture.
{Initial Glucose conc. = 100g/L (10%) }

In Table-1, it is observed that there is decrease in cell density and cell growth as the NaCl concentration increases in the 1% CSL media as clearly evident from 24 hours reading. Also, there is 50% decrease of cell growth in 2 % NaCl and 65 % reduction in 3% NaCl concentration while comparing with 0% NaCl concentration (control). Cells are not able to grow in high salt supplemented 1% CSL media.

In Table 2, it is observed that with increasing salt concentration, the Glucose consumption and Ethanol production also decreases attributed to lower growth. But, Ethanol:Glucose ratio is highest in 3% salted condition. Therefore, selection of 3% salt concentration for further salt stress experiments.

No yeast cell colonies is able to grow on 1% CSL –Agar plates (with 10% glucose) with different salt concentration, not even the 0 % NaCl + 1% CSL culture. This confirms that 1% CSL cannot be used for culturing yeast cells and thus choosing higher (2% CSL or more) as minimal yeast adaptation media.



Graph 1: Effect of different salt concentration on cell growth, glucose consumption & ethanol production of yeast in 1% CSL media (containing 10% glucose).

5.2. OPTIMISATION OF CSL CONCENTRATION IN MEDIA W.R.T. 3% SALT STRESS

5.2.1 Response of Unadapted Yeast Cells to Varying CSL Concentrations in Presence of 3% Salt

The yeast cells were precultured in debri-free 2% CSL media lacking NaCl from YPD stock plate. Subsequently cultured 2% CSL + 0% NaCl preculture into 3% NaCl supplemented media with varying CSL concentrations (0.5%, 1%, 2%, 4.5%) at 0.1 seeding O.D. Incubated the varying CSL conc. culture flasks at 30 °C for 24 hours and measured $O.D._{550\ nm}$ reading of varying CSL concentration at 0, 2, 4, 6, 8, 24 hours and estimated and glucose consumption & ethanol production at 24 hours as shown in Table-3 and Table-4 respectively.

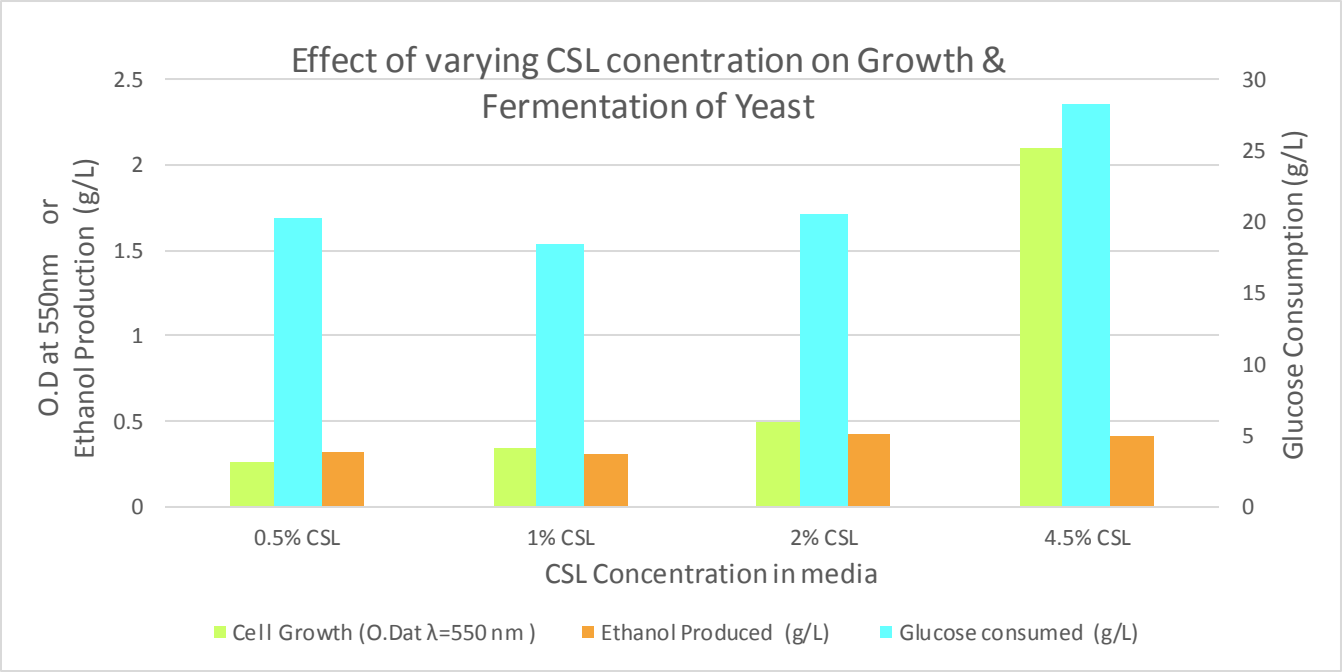
CSL Conc. (+3% NaCl)	O.D. at λ=550 nm					
	0 hr	2 hr	4 hr	6 hr	8 hr	24 hr
0.5% CSL	0.170	0.181	0.193	0.22	0.231	0.264
1% CSL	0.162	0.171	0.205	0.227	0.273	0.340
2% CSL	0.106	0.163	0.235	0.284	0.351	0.491
4.5% CSL	0.140	0.156	0.252	0.455	0.795	2.100

Table 3: Effect of CSL concentration on yeast cell growth in presence of 3% Salt.
{1 O.D. corresponds to 3×10^7 cells/ml of culture
Control culture media = 2% CSL+ 0% NaCl → O.D. = 1.12 after 24 hours of culturing}

CSL Conc. (+3% NaCl)	Time Point : 24 hrs		
	Glucose consumed (g/L)	Ethanol Produced (g/L)	Ethanol : Glucose ratio
0.5% CSL	20.23	0.320	0.0158
1% CSL	18.50	0.310	0.0167
2% CSL	20.52	0.420	0.0201
4.5% CSL	28.20	0.415	0.0147

Table 4: Effect of CSL concentration on fermentation profile at 24 hours of culture.
{Initial Glucose conc. = 100g/L (10%) }

From Table 3 & 4, it was observed that there is an increase in cell density and cell growth as the CSL concentration increases from 0.5% to 4.5% in media containing 3% NaCl. With increasing CSL concentration, glucose consumption increases whereas ethanol productivity is almost similar for 24 hours of culture which may improve in further stages of culturing. At 3% salt stress, in 2% CSL media, with lesser glucose consumption and lesser growth similar quantity of ethanol is formed when compared to 4.5% CSL media. Thus, there is better adaptation of cells, higher ethanol yield (specific ethanol production per cell) and higher glucose conversion in 2% CSL as compared to 4.5% CSL. Therefore, due to optimal media utilization of cells in 2 % CSL (+3% NaCl), it is chosen as minimal media for further adaptation and industrial implications. Even at higher glucose consumption, very less ethanol is produced, since cells are still in adapting stage and glucose is utilized to combat salt stress. Once, cells are adapted to salt stress, Glucose to ethanol conversion and Ethanol yield of cells will improve.



Graph 2: Effect of varying CSL concentration on cell growth, glucose consumption & ethanol production of yeast w.r.t 3% salt stress (containing 10% glucose).

5.2.2. Selection of Yeast cells on Agar Plates of Different CSL Concentrations containing 3% Salt

White coloured, spherical shaped yeast colonies appear on 2% CSL + 3% NaCl and 4.5 % CSL + 3 % NaCl plates. This shows 2% CSL is the minimal CSL required for the cells to cope up with 3% salt stress and further grow. So, next experimentations are to adapt the yeast cells on 3% NaCl stress in 2% CSL (10% glucose) minimal media in order to test Maintenance Energy hypothesis i.e., salt tolerant/ adapted yeast cells are capable of fermenting more sugar (glucose) to ethanol along with more ATP generation in order to maintain active cell metabolism and sustain life.

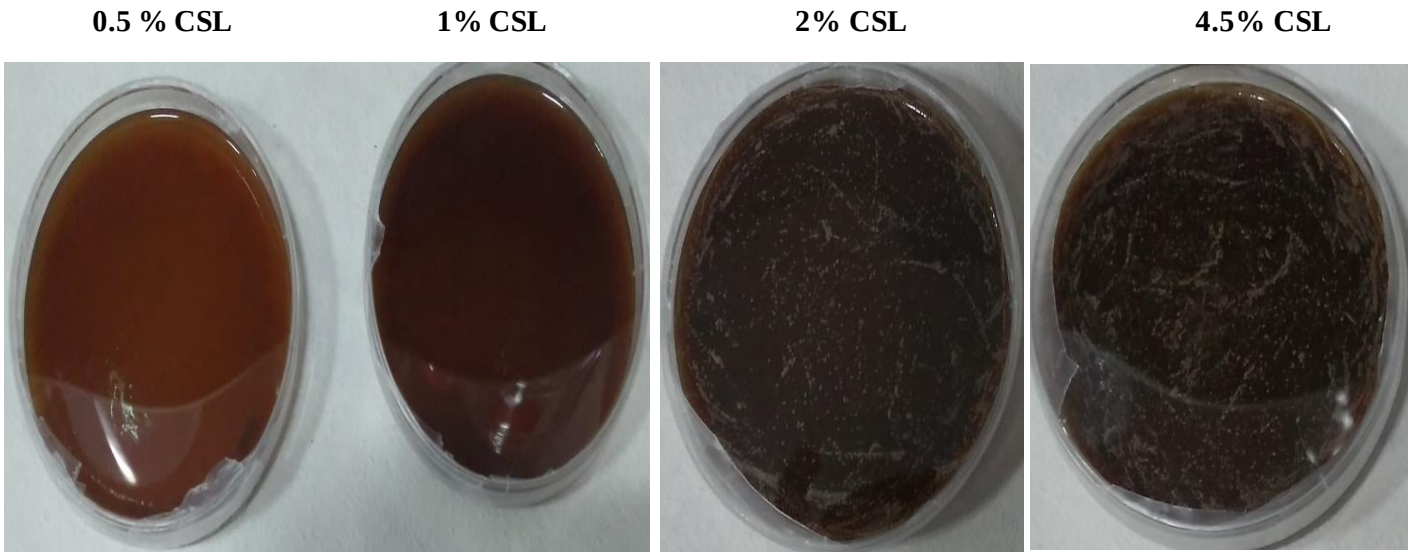


Figure 9: Growth & Selection of yeast on solid agar media with varying CSL concentration in 3% NaClstress.

5.3. ADAPTATION OF YEAST CELLS ON MINIMAL 2% CSL MEDIA SUPPLEMENTED WITH 3% SALT (NACL)

5.3.1. Serial Adaptation of Yeast to 3% Salt Stress

In order to adapt yeast - *Saccharomyces cerevisiae* CEN.PK 122 (diploid) is continuously cultured in minimal industrial culture media- 2% CSL (Corn Steep Liquor Media) + 10% glucose + 3% salt (NaCl) stress. The media was kept minimal (2% CSL) so that the cells adapt in Salt stress and not merely grow. If there is higher concentration of CSL, yeast cells will be able to grow even in high salt stress because of higher nutrients supplemented from media to cells to sustain life. Here, yeast cell may not adapt to the condition and loose its tolerance property as soon as the stress condition is removed. Therefore, it requires an optimization of CSL concentration in culture media (estimated to be 2% CSL) such that the cells do not die and cell growth is not a mere result of extra nutrients in the culture media but yeast cells get adapted to higher NaCl concentration i.e., metabolically engineered by Salt Stress. Higher glucose (10%) concentration was used for culturing because yeast majorly utilizes glucose as carbohydrate source and the industrial grade media has approximately same level of glucose optimized for high ethanol production.

The yeast colonies as obtained on 2% CSL (with 3% NaCl) plate in previous experiments are First Tolerant Yeast Cells. The largest colony is picked and cultured and sub-cultured for 8 passages in liquid 2% CSL media containing 3% NaCl till a considerate growth is obtained. This culture is named **Serial Adaptation-1 (Sr1)**. The culture is maintained on 2% CSL- Agar plate (+3% NaCl). Again the largest colony of Sr1 is picked and re-cultured for 8 passages in salt supplemented media, labelled as **Serial Adaptation-2 (Sr2)** culture, followed by maintenance on CSL-Agar plate ((+3% NaCl). Serial Adaptation is repeated from Sr2 and preserved on plates called as **Serial Adaptation-3 (Sr3)** culture.

5.3.2. Selection and Maintenance of Salt Adapted Yeast on Agar Plates (with 3% Salt)

In initial culturing of yeast cells in 3% salt media, the adapted cells are selected by the fact, non-adapted (salt intolerant) yeast cells die and are reduced in number while the adapting cells survive and transforms into Salt tolerant yeast. Hence, reducing a large population of yeast cells to a small population of 3% salt tolerant cells. With subsequent culturing, the salt tolerant cells starts dividing and so cell growth increases. The high Salt tolerant cells are thereby selected and thereafter maintained on CSL-3% salt containing Agar plates and YPD- 3% salt containing Agar plates. These plates are covered with enough number of salt tolerant yeast colonies as seen in Fig. 10. The tolerant yeast cells are maintained on 2% CSL + 2% glucose + 3% NaCl to allow better survival of yeast cells and low metabolic activity while storage. The Adapted cell cultures are able to grow well on Agar plates when plated without dilution while growth is rarely observed if plated with dilution of liquid culture. Thus, there is better survival and adaptability at high cell concentration.

This way the yeast cells are metabolically engineered/modified by osmotic stress and tested for the desired quality of enhanced ethanol specific productivity (to be checked in further experiments).

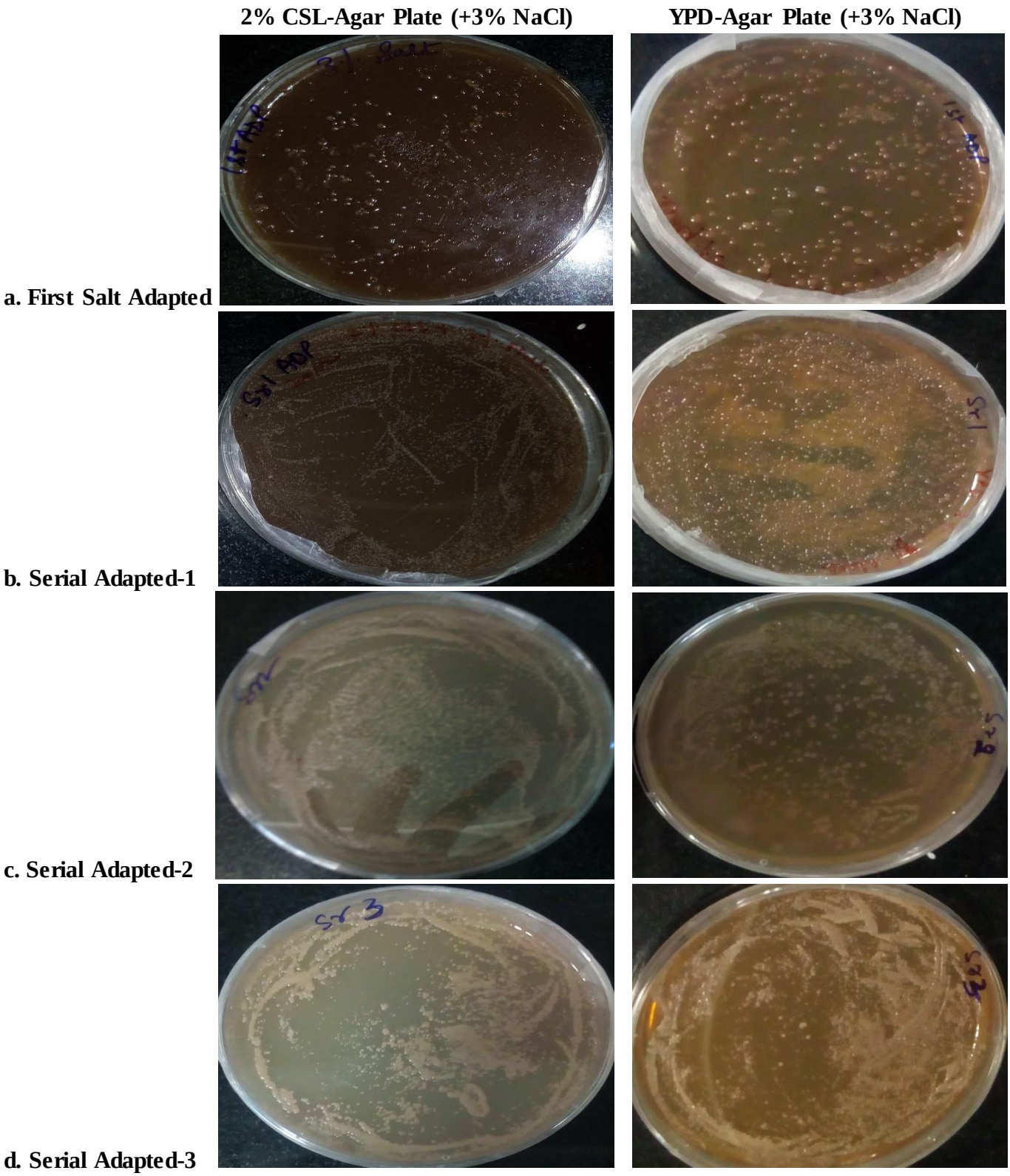


Figure 10: Different generations of Salt Adapted Yeast maintained on 2% CSL- Agar (3% NaCl) plate and YPD- Agar (3% NaCl) plate.

5.3.3. Comparative Growth and Fermentation Characteristics of Different Serial Adapted and Unadapted Yeast in Salt Supplemented Industrial Media

The unadapted cells were cultured from colony of YPD plates and adapted cells are cultured form colony of Sr1 and Sr2 Adapted 2% CSL (+3% salt) plates in 3% Salt containing CSL. Cell growth, Glucose consumption and Ethanol production of C1 (unadapted), C1' (Sr1 Adapted) and C1'' (Sr2 Adapted) is compared.

Cell growth in Sr1 adapted and Sr2 adapted culture is approximately 5 times and 7 times the cell growth of unadapted culture at 24 hours. The reason behind this significant increase is that the yeast cells in Sr1 and Sr2 has adapted to 3% NaCl stress because they were taken from 2% CSl + 3% NaCl plate. Hence, repeated culturing of yeast cells in minimal media in presence of NaCl has gradually improved their growth in salt stress, whereas unadapted culture has never experienced NaCl stress before and so, were not able to grow well in salt stress. C1' yeast cell culture of Sr1 Adaptation is then sequentially sub-cultured to C2', C3', C4'. The cell growth ($O.D._{550nm}$) in C1', C2', C3', C4'at same time intervals is approximately same; thereby, confirming that **yeast cells tend to maintain salt (NaCl) tolerance**.

Glucose consumption and Ethanol production also improves with Serial Adaptation as reflected by Graph-3 & 4. Therefore fermentation improves with adaptation of cells.

The cell growth and fermentation improves after every cycle of plating as evident from Sr2 Adapted culture and Sr1 Adapted culture (in Table-5 & 6). There is no difference in cell growth in subcultures of same serial adaptation. Therefore, selection of salt adapted yeast on CSL plating containing salt is an important step in Adaptation of cells to make them salt tolerant and ferment better.

Duration of culturing	O.D. at λ=550 nm of Cultures					
	Unadapted	Sr 1 Adapted				Sr 2 Adapted
	C1	C1'	C2'	C3'	C4'	C1''
0 hrs	0.11	0.1	0.128	0.1	0.1	0.112
8 hrs	-	-	0.362	0.401	0.420	0.312
16 hrs	0.431	1.245	-	1.12	-	1.85
24 hrs	0.502	2.71	2.52	2.55	-	4.15
42 hrs	3.712	5.28				7.89

Table 5: Effect of serial adaptation on yeast cell growth in 3% NaCl with 2% CSL media

{1 O.D corresponds to 3 $\times 10^7$ cells/ml of culture

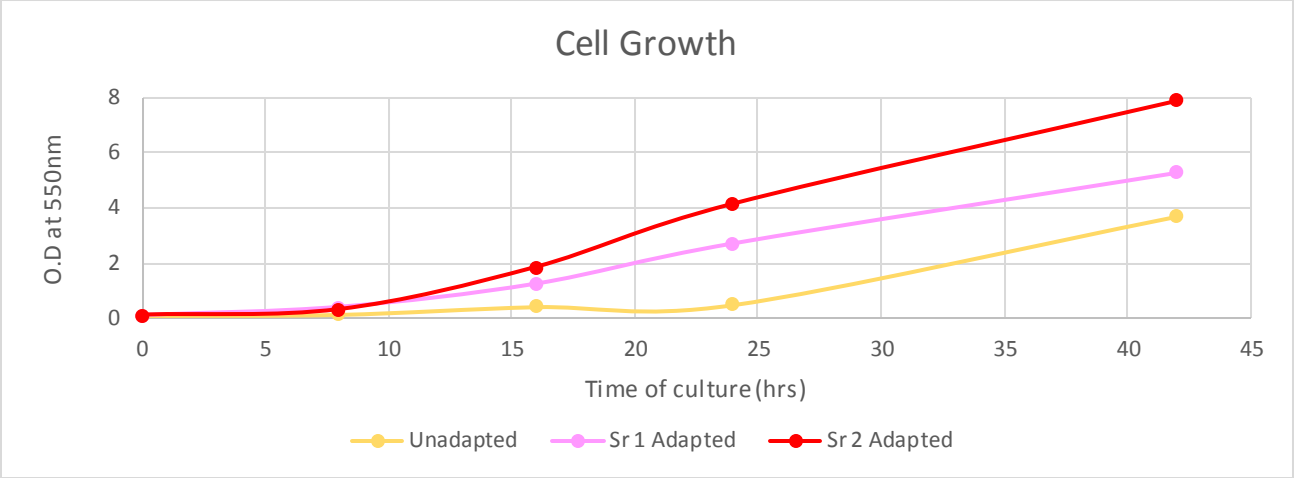
Sr1Adapted & Sr2 Adapted are first & second generation of serially adapted yeast to salt stress}

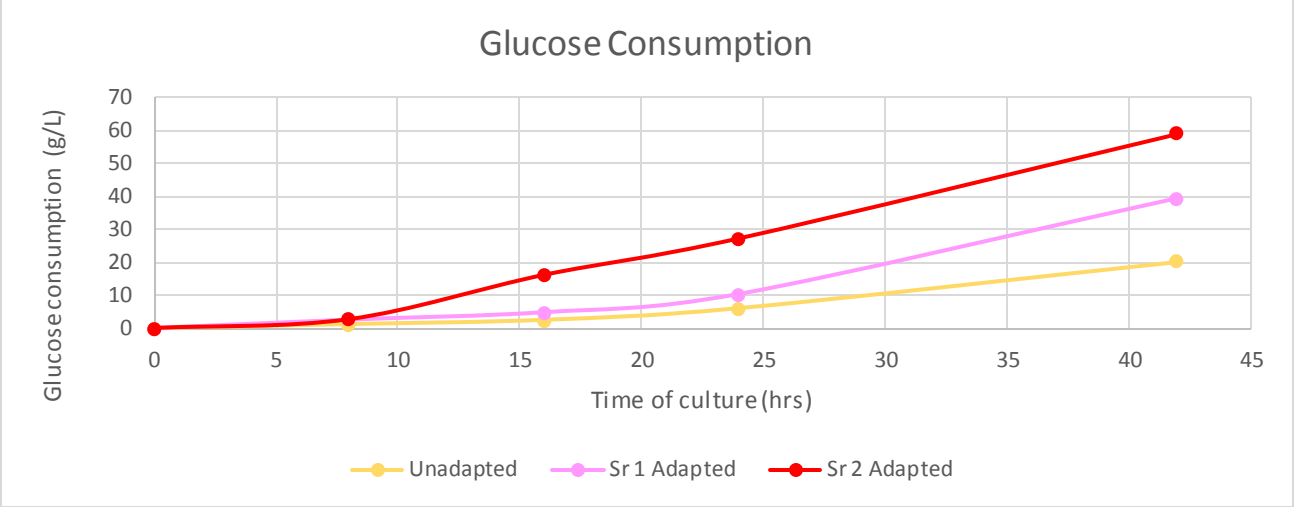
Duration of culturing	Cultures											
	Unadapted		Sr 1 Adapted								Sr 2 Adapted	
	C1		C1'		C2'		C3'		C4'		C1''	
	Glu cons. (g/L)	Eth cons. (g/L)	Glu cons. (g/L)	Eth cons. (g/L)	Glu cons. (g/L)	Eth cons. (g/L)	Glu cons. (g/L)	Eth cons. (g/L)	Glu cons. (g/L)	Eth cons. (g/L)	Glu cons. (g/L)	Eth cons. (g/L)
0 hrs	0	0	0	0	0	0	0	0	0	0	0	0
8 hrs	-	-	-	-	2.55	0.12	-	-	2.40	0.137	2.70	0.343
16 hrs	2.50	0.44	4.80	1.44	-	-	5.0	1.35	-	-	16.20	3.09
24 hrs	6.05	0.70	10.35	3.67	8.43	3.35	9.02	3.01	-	-	27.31	4.55
42 hrs	20.10	6.78	39.45	15.10							59.10	16.10

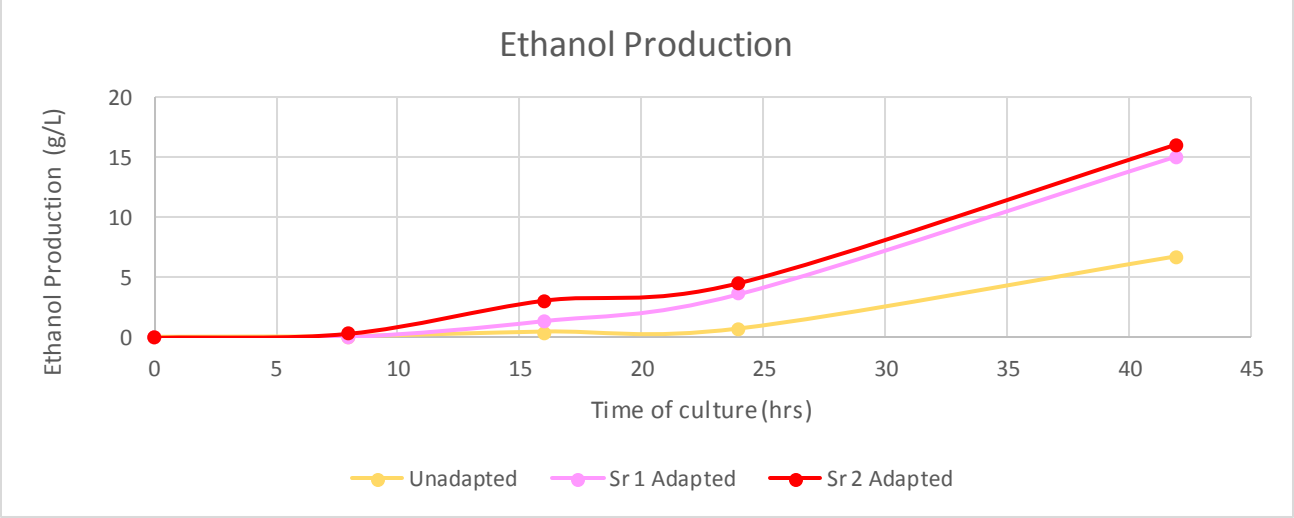
Table 6: Effect of serial adaptation on fermentation profile of yeast in 3% NaCl with 2% CSL media

{Initial Glucose Conc. = 100g/L (10%)

Sr1 Adapted & Sr2 Adapted are first & second generation of serially adapted yeast to salt stress}







Graph 3: Comparative Growth, Glucose Consumption & Ethanol Production in different Serial Adapted and Unadapted yeast in salt stress.

{ Sr1 Adapted & Sr2 Adapted are first & second generation of serially adapted yeast to salt stress }

5.4. RELATIONSHIP BETWEEN GROWTH AND FERMENTATION OF YEAST CELLS

Yeast cells were cultured in CSL free media in presence (3% NaCl) and absence of salt stress at high seeding O.D (5.0). Growth, Glucose consumption and Ethanol production were estimated at different time intervals. 3 Sets of culture are: S0 Control – Unadapted cells growing in salt free nutrient media (-CSL), S0 Sr2 Adapted – Sr2 Adapted cells growing in salt free media (-CSL) and S3 Adapted – Sr2 Adapted cells growing in 3% NaCl media (-CSL).

In all sets of cultures, there was negligible growth or ethanol production w.r.t time even when culture is seeded at high yeast cell concentration. Cells utilize small amount of glucose but cannot grow in absence of CSL. It infers that without CSL, the growth of yeast cells is arrested and remains unchanged in presence or absence of salt stress. There is requirement of Corn Steep Liquor (CSL) in media for growth of yeast to ferment glucose to ethanol since, CSL contains amino acids, vitamins and factors responsible for growth.

No growth of yeast cells corresponds to negligible ethanol production in absence of CSL. Similarly, higher growth corresponds to higher ethanol production and glucose consumption as demonstrated in previous experiments. Therefore, fermentation and ethanol production is growth dependent.

Time	O.D. at λ=550 nm		
	S0 Control	S0 Sr2 Adapted	S3 Sr2 Adapted
0 hr	4.40	4.7	4.48
4 hr	5.14	4.84	4.52
8 hr	4.58	4.68	4.67
16 hr	4.56	4.24	4.78
24 hr	4.45	4.27	4.77

Table 7: Growth of yeast in absence of CSL in various experiment sets.

{ Sr1 Adapted & Sr2 Adapted are first & second generation of serially adapted yeast to salt stress
S0 is salt free media, S3 is media with 3% salt }

Time	S0 Control		S0 Sr2 Adapted		S3 Sr2 Adapted	
	Glu cons. (g/L)	Eth Prod. (g/L)	Glu cons. (g/L)	Eth Prod. (g/L)	Glu cons. (g/L)	Eth Prod. (g/L)
0 hr	0	0	0	0	0	0
8 hr	5.65	0.77	6.07	0.324	7.43	0.957
16 hr	10.41	1.91	10.25	0.307	9.09	1.90

Table 8: Fermentation profile of yeast in absence of CSL in various experiment sets.

{ Initial Glucose concentration = 100 g/L
Sr1 Adapted & Sr2 Adapted are first & second generation of serially adapted yeast to salt stress
S0 is salt free media, S3 is media with 3% salt }

5.5. COMPARATIVE ANALYSIS OF GROWTH & FERMENTATION CHARACTERISTICS OF SALT ADAPTED YEAST CELLS IN PRESENCE AND ABSENCE OF SALT STRESS IN INDUSTRIAL MEDIA

The growth and fermentation characteristics (Glucose consumption, Ethanol production & Glycerol formation) of salt tolerant/ adapted yeast is compared with that of Control. Yeast cells were cultured in 2% CSL (industrial media) in presence (3% NaCl) and absence of salt stress at different seeding O.D (low, moderate & high). Various experiment sets are:

Experiment Set	Culture Sets				
	C2S0 Control	C2S0 Sr2 Adapted	C2S0 Sr3 Adapted	C2S3 Sr2/ Sr3 Adapted	C2S3 Unadapted
Low seeding density (O.D 0.1)	√	√		√	√
Moderate seeding density (O.D 1)	√	√		√	
High seeding density (O.D 2.5 & 5)	√	√	√	√	

C2S0 Control – Unadapted cells growing in 2% CSL in absence of salt

C2S0 Sr2 Adapted – Sr2 salt Adapted cells growing in 2% CSL in absence of salt

C2S0 Sr3 Adapted – Sr3 salt Adapted cells growing in 2% CSL in absence of salt

C2S3 Sr2/ Sr3 Adapted – Sr2/ Sr3 salt Adapted cells growing in 2% CSL in presence of salt (3% NaCl)

C2S3 Unadapted – Unadapted cells growing in 2% CSL in presence of salt (3% NaCl)

5.5.1. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at Low Seeding O.D (0.1)

The cell density is measured in terms of $O.D_{550\ nm}$ at different time points till 96 hours. Increase in O.D indicates cell growth. Important fermentation characteristics such as Glucose consumption and Ethanol production were analyzed by quantifying these analytes in the supernatant of culture using HPLC (High Performance Liquid Chromatography).

The Cell growth is observed maximum in C2S0 Control culture i.e., Unadapted cells growing in absence of salt stress. There is comparative cell growth in C2S0 Adapted and C2S3 Adapted cultures at higher time point i.e., adapted yeast cell shows similar growth in presence or absence of salt. Minimum cell growth is observed in C2S3 Unadapted Culture grown in 3% salt stress media.

Time	O.D. at λ=550 nm			
	C2S0 Control	C2S0 Sr2 Adapted	C2S3 Sr2 Adapted	C2S3 Unadapted
0 hr	0.130	0.080	0.056	0.128
2 hr	0.206	0.156	0.159	0.162
4 hr	0.276	0.226	0.190	0.188
6 hr	0.484	0.406	0.231	0.192
8 hr	0.530	0.698	0.312	0.190
24 hr	10.600	5.896	3.860	0.370
26 hr	12.160	6.400	4.760	0.484
28 hr	14.88	6.450	5.210	0.440
30 hr	13.600	6.940	6.640	0.750
48 hr	16.460	8.870	8.821	3.400
51 hr	19.085	7.08	8.661	4.060
72 hr	20.100	9.020	8.200	5.100
96 hr	22.520	9.620	8.700	7.220

Table 9: Comparative Growth of Sr2 salt adapted Yeast (+/-salt stress) w.r.t control at 0.1 seeding O.D.
{O.D 1 → 3×10^7 cells/ml of culture
C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt), C2S3 Unadapted- non salt tolerant yeast in CSL media (+3% salt) }

From Table-10, the Glucose consumption and ethanol production is maximum in case of C2S0 control culture where normal yeast cells were grown in absence of salt stress. The fermentation profile is almost similar in C2S0 Adapted and C2S3 Adapted Culture since, the source of cell is Salt Adapted Yeast grown in absence and presence of salt stress respectively. Therefore, cells once adapted to salt stress are unaffected by the media composition at lower cell density. Unadapted cells consume minimum glucose and produce minimum ethanol.

At low seeding O.D, the pattern is observed for

Growth/Fermentation: **C2S0 Control > C2S0 Sr2 Adapted ≈ C2S3 Sr2 Adapted > C2S3 Unadapted**

Control utilizes almost all glucose (116g/L) within 48 hours to produce 38g/L ethanol, whereas adapted culture growing in presence and absence of salt ferment slowly, converting approximately 50-60 g/L glucose into 15-18 g/L ethanol which is half of control. But, the Ethanol Yield (Ethanol produced per O.D or cell) and Glucose Conversion per cell at higher time interval of 48-96hrs is higher in case of salt adapted cells growing in +/- of salt as compared to control as seen in Table-10 & 11. Also, Unadapted cells growing in salt stress shows very slow growth for initial 48 hours that improves on longer culturing to 96 hours. The Unadapted cells take time to adapt and then grow. Although glucose consumption & ethanol production is lower initially, however lesser number of yeast cells are able to convert entire glucose present in media into 36g/L ethanol (equivalent to control whose cell density is higher) in 96 hours. The Ethanol Yield per cell and Glucose consumption per cell of C2S3 Unadapted has been significantly higher than control cells.

Thus, **salt stress induces metabolic changes in yeast that enhances the Ethanol Productivity of yeast cell** as confirmed from salt adapted cell and unadapted cell's fermentation characteristic. Further experiments are done to confirm this pattern at higher seeding O.D.

Time	C2S0 Control		C2S0 Sr2 Adapted		C2S3 Sr2 Adapted		C2S3 Unadapted	
	Glu cons. (g/L)	Sp. Glu cons. (g/L/O.D)	Glu cons. (g/L)	Sp. Glu cons. (g/L/ O.D)	Glu cons. (g/L)	Sp. Glu cons. (g/L/O.D)	Glu cons. (g/L)	Sp. Glu cons. (g/L/O.D)
0 hr	0	0	0	0	0	0	0	0
2 hr	19.53	-	19.69	-	21.56	-	12.68	-
8 hr	20.2	-	19.93	-	21.304	-	17.14	-
24 hr	57.29	5.40	27.91	4.74	29.10	7.53	21.35	-
30 hr	83.42	6.13	37.90	5.46	33.60	5.06	19.46	-
48 hr	114.79	6.97	52.39	5.91	67.97	7.07	43.16	12.69
51 hr	115.51	6.05	56.32	7.95	72.00	8.31	49.30	12.14
96 hr	116.00	5.15	83.80	8.71	86.67	9.96	112.10	15.53

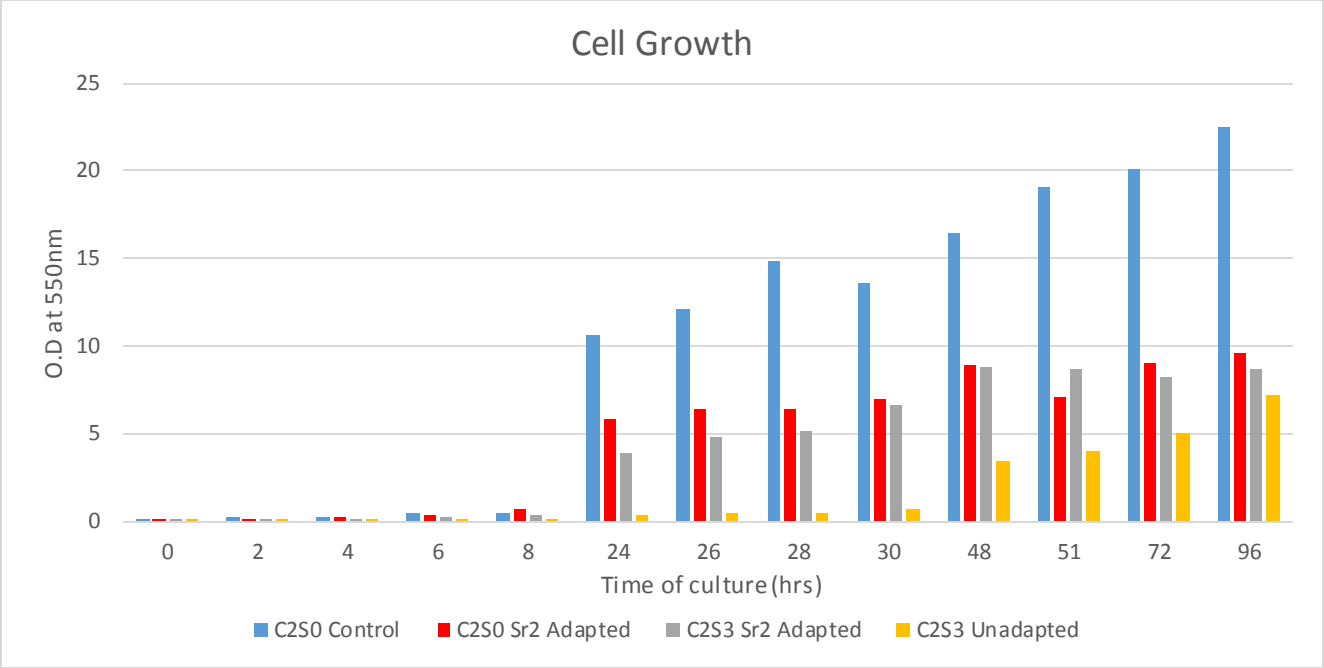
Table 10: Comparative Glucose consumption of Sr2 salt adapted Yeast (+/-salt stress) w.r.t control at 0.1 seeding O.D.

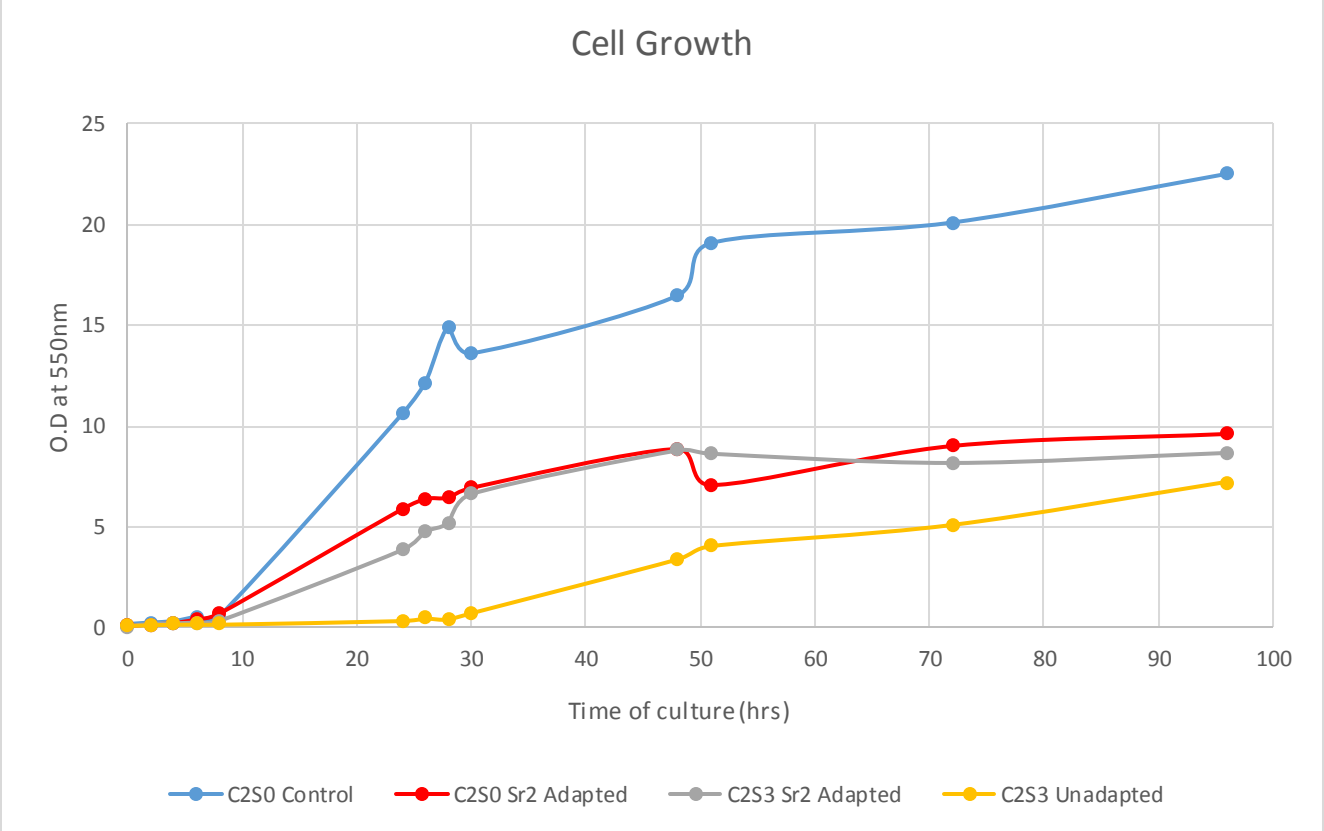
{Initial Glucose in C2S0 media = 116g/L , Glucose in C2S3 media = 119g/L (approx. 10%)
C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt), C2S3 Unadapted- non salt tolerant yeast in CSL media (+3% salt) }

Time	C2S0 Control		C2S0 Sr2 Adapted		C2S3 Sr2 Adapted		C2S3 Unadapted	
	Eth Prod. (g/L)	Sp. Eth Prod. (g/L/cell)	Eth Prod. (g/L)	Sp. Eth Prod. (g/L/cell)	Eth Prod. (g/L)	Sp. Eth Prod. (g/L/cell)	Eth Prod. (g/L)	Sp. Eth Prod. (g/L/cell)
0 hr	0	0	0	0	0	0	0	0
2 hr	0.11	-	0.242	-	0.255	-	0.206	-
8 hr	0.597	-	0.652	-	0.343	-	0.282	-
24 hr	15.72	1.48	5.68	0.963	4.37	1.13	0.88	-
30 hr	28.39	2.09	7.89	1.137	8.75	1.318	1.6	-
48 hr	38.00	2.308	15.114	1.70	18.40	2.086	9.52	2.80
51 hr	34.80	1.82	16.20	2.29	21.00	2.42	11.35	2.79
96 hr	33.10	1.47	23.38	2.43	25.50	2.93	36.50	5.055

Table 11: Comparative Ethanol productivity of Sr2 salt adapted Yeast (+/-salt stress) w.r.t control at 0.1 seeding O.D

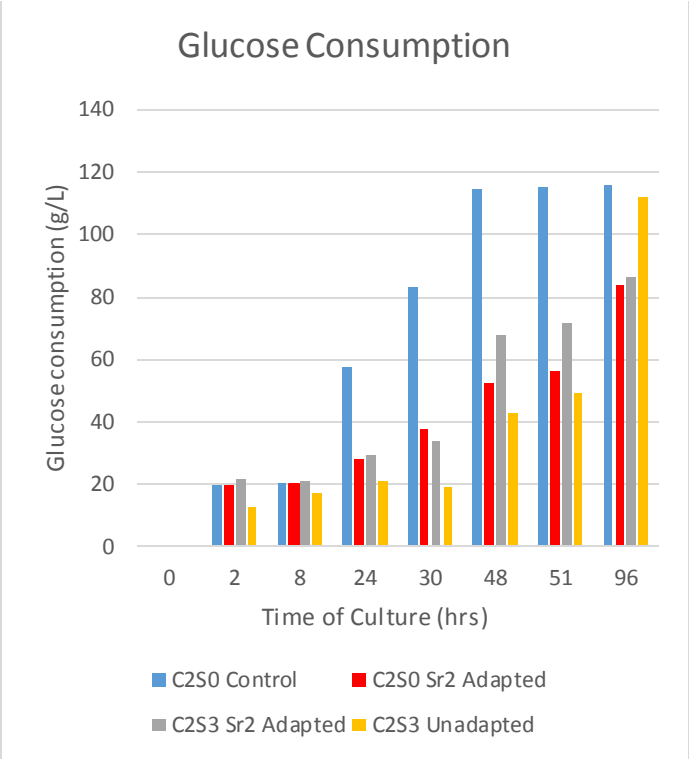
{ C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt), C2S3 Unadapted- non salt tolerant yeast in CSL media (+3% salt) }

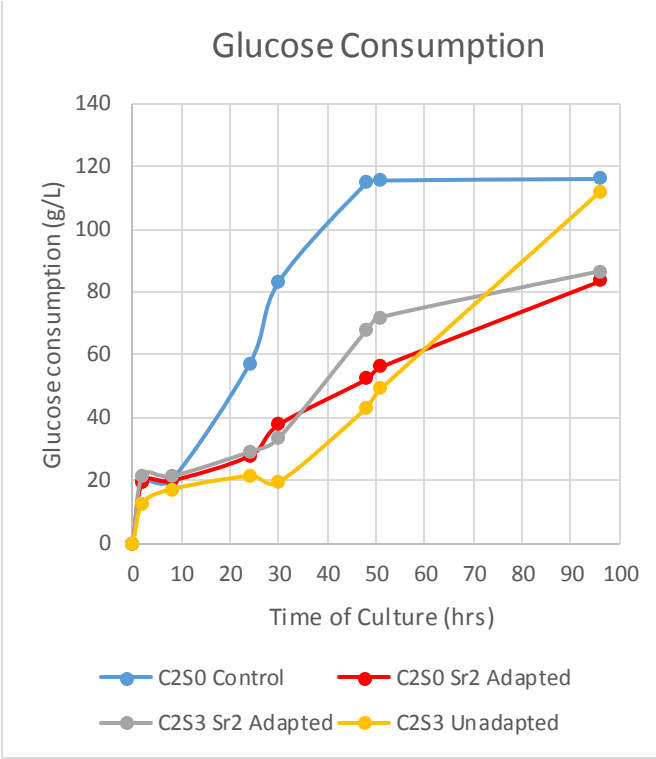


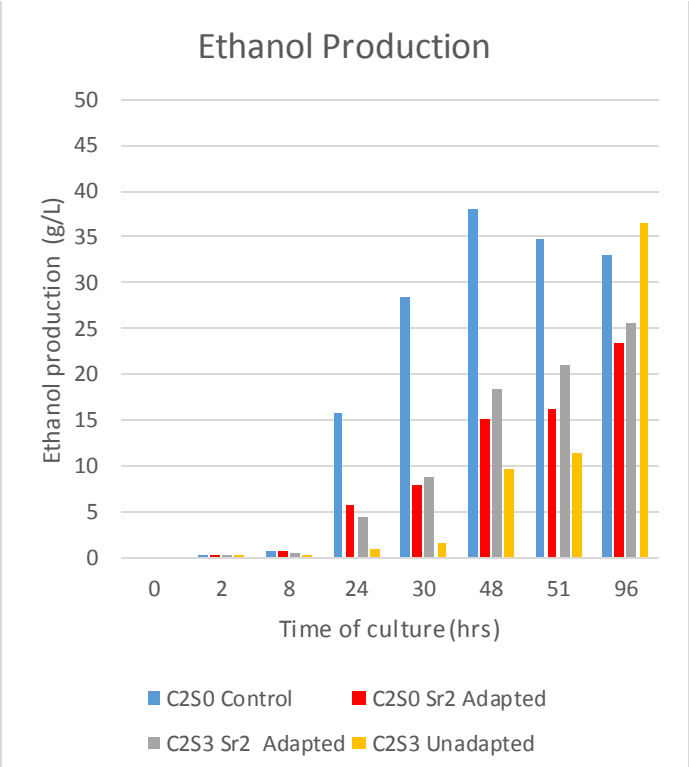


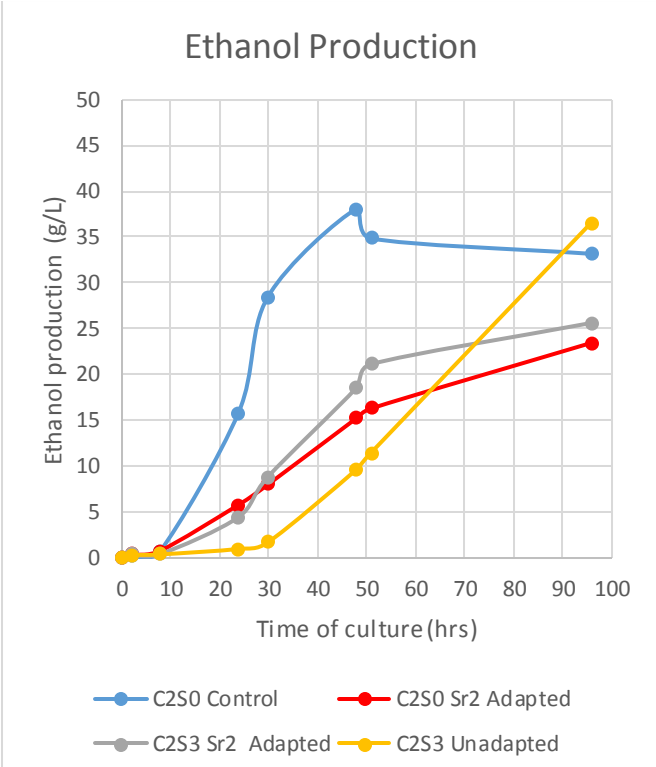
Graph 4: Comparative Growth of Sr2 salt adapted Yeast (+/-salt stress) and unadapted yeast (+ salt stress) w.r.t control at 0.1 seeding O.D.

{C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt), C2S3 Unadapted- non salt tolerant yeast in CSL media (+3% salt) }









Graph 5: Comparative Fermentation profile (Glucose consumption & Ethanol production) of Sr2 salt adapted Yeast (+/-salt stress) and unadapted yeast (+ salt stress) w.r.t control at 0.1 seeding O.D.

5.5.2. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at Moderate Seeding O.D (1.0)

The cell density is measured in terms of $O.D_{550\ nm}$ at different time points till 72 hours. Increase in O.D indicates cell growth. Important fermentation characteristics such as Glucose consumption, Ethanol production and Glycerol levels were analyzed by quantifying these analytes in the supernatant of culture using HPLC.

The cell growth is observed maximum in C2S0 Control culture i.e., Unadapted cells growing in absence of salt stress followed by C2S0 Sr2 Adapted growing in absence of salt (after adaptation to salt) and C2S3 Sr2 Adapted growing in salt stress.

Cell Growth order: **C2S0 Control > C2S0 Sr2 Adapted > C2S3 Sr2 Adapted**

Time	**O.D. at λ=550 nm**		
	C2S0 Control	**C2S0 Sr2 Adapted**	**C2S3 Sr2 Adapted**
0 hr	1.08	0.91	1.03
4 hr	1.29	1.09	1.40
8 hr	2.53	2.12	1.62
24 hr	8.80	7.13	1.90
32 hr	10.08	8.36	2.02
48 hr	11.86	8.76	3.55
56 hr	12.32	9.80	4.72
72 hr	11.90	10.34	6.49

Table 12: Comparative Growth of Sr2 salt adapted Yeast (+/-salt stress) w.r.t control at 1.0 seeding O.D.
{O.D 1 → 3×10^7 cells/ml of culture
C2S0 Control- unadapted yeast in salt free CSL media, C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt) }

The fermentation characteristics involves Glucose consumption, Ethanol production and Glycerol production by control and adapted cells in presence and absence of salt stress in liquid media.

At moderate seeding O.D 1.0 :

Glucose Consumption/ Ethanol Production: **C2S0 Control > C2S0 Sr2 Adapted > C2S3 Sr2 Adapted**

Glycerol Production (Initial stage): **C2S0 Sr2 Adapted > C2S3 Sr2 Adapted > C2S0 Control**

Glycerol Production (later stage): **C2S3 Sr2 Adapted > C2S0 Sr2 Adapted > C2S0 Control**

Control utilizes almost all glucose (104 g/L) in 48-72 hours to produce 46 g/L ethanol, whereas adapted culture growing in absence of salt ferment slower, converting approximately 50 g/L glucose into 21g/L ethanol which is half of control as seen in Table-12 & 13. For initial 48 hours, Sr2 Adapted yeast cultured in salt stress shows lower growth & fermentation than Sr2 Adapted cells growing in absence of salt that improves on longer culturing to 72 hours. The reason for lower ethanol production can be attributed to higher production of glycerol in initial stages to combat salt stress. Glycerol produced cell is significantly higher in C2S3 Sr2 Adapted cells compared to control & C2S0 Adapted cell growing without salt. Glycerol production keep on increasing in C2S3 Adapted cells due to salt stress and reaches maximum at 72 hours. Glycerol production is almost same in control cells because they are not subjected to salt stress. C2S0 Sr2 Adapted cells are previously adapted to salt therefore, produces good amount of glycerol despite lack of salt stress in media.

It is observed from experiments 5.5.1 & 5.5.2 (Table-11 & 13),

At low seeding O.D (at 24 hours) : 5.6g/L Ethanol is produced by Glucose consumption of 27g/L

At moderate seeding O.D (at 24 hours) : 10g/L Ethanol is produced for 29g/L Glucose consumed.

At low seeding O.D (at 48 hours) : 15g/L Ethanol is produced for 50g/L Glucose consumed.

At moderate seeding O.D (at 48 hours) : 21g/L Ethanol is produced from 53g/L Glucose consumed.

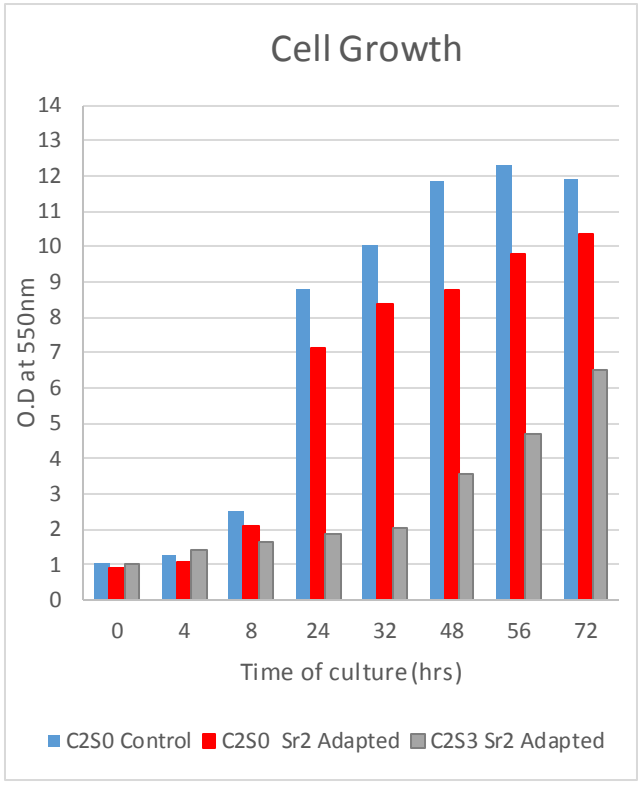
This indicates **higher ethanol production from same amount of glucose consumed by Adapted cells** in salt free media attributed to higher seeding density. Thus, **Glucose conversion to Ethanol increases at higher cell density.**

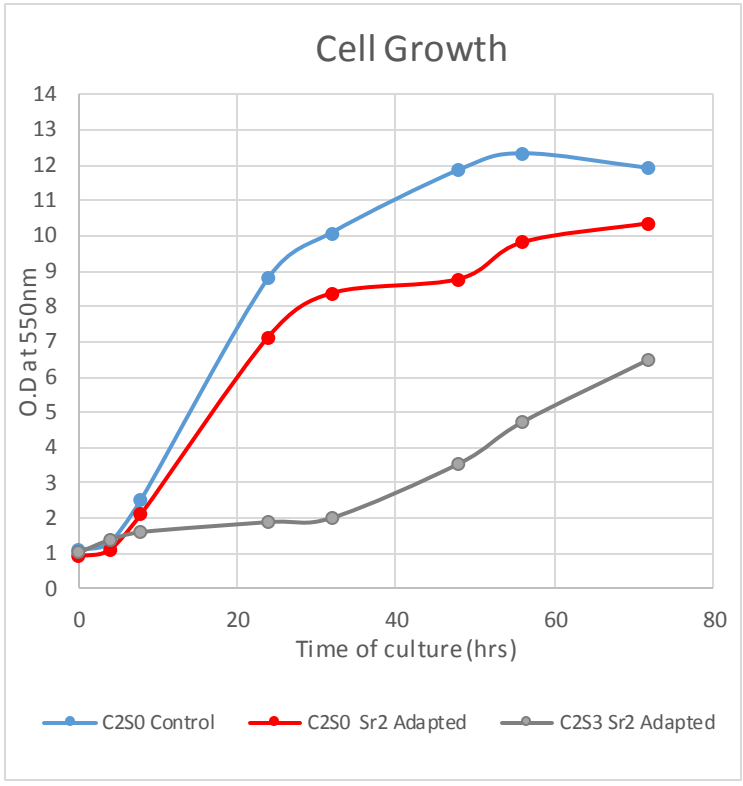
Further experiments are done to confirm this pattern at higher seeding O.D.

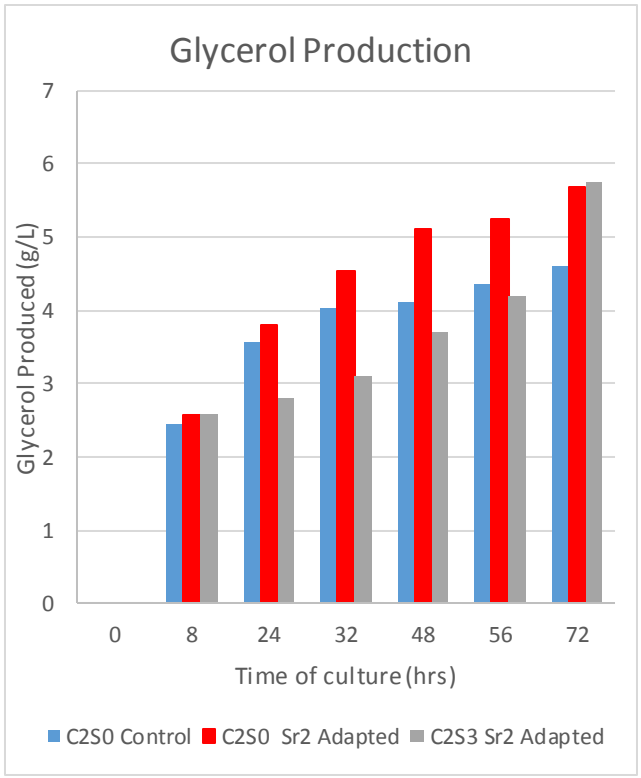
Time	C2S0 Control			C2S0 Sr2 Adapted			C2S3 Sr2 Adapted		
	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)
0 hr	0	0	0	0	0	0	0	0	0
8 hr	12.45	3.04	2.45	3.40	1.50	2.58	4.81	1.03	2.60
24 hr	39.53	15.85	3.56	29.05	10.07	3.80	9.80	1.50	2.82
32 hr	55.58	25.90	4.04	32.61	15.26	4.55	11.31	1.86	3.10
48 hr	90.50	39.37	4.11	53.15	21.72	5.11	15.34	5.02	3.72
56 hr	99.68	41.20	4.37	58.20	24.00	5.25	21.14	7.80	4.20
72 hr	104.55	46.54	4.61	69.10	28.81	5.68	42.54	15.20	5.75

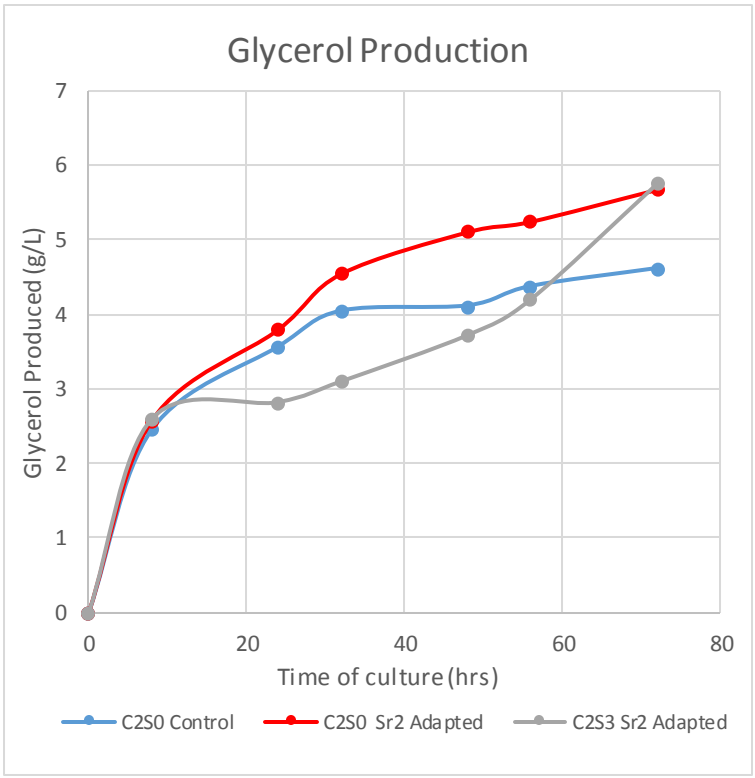
Table 13: Comparative Fermentation Profile of salt Sr2 adapted Yeast (+/-salt stress) w.r.t control at 1.0 seeding O.D.

{Initial Glucose in C2S0 Control = 104.7 g/L, C2S0 Adapted = 104 g/L, C2S3 Adapted = 105.2g/L (approx. 10%) C2S0 Control- unadapted yeast in salt free CSL media, C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt) }

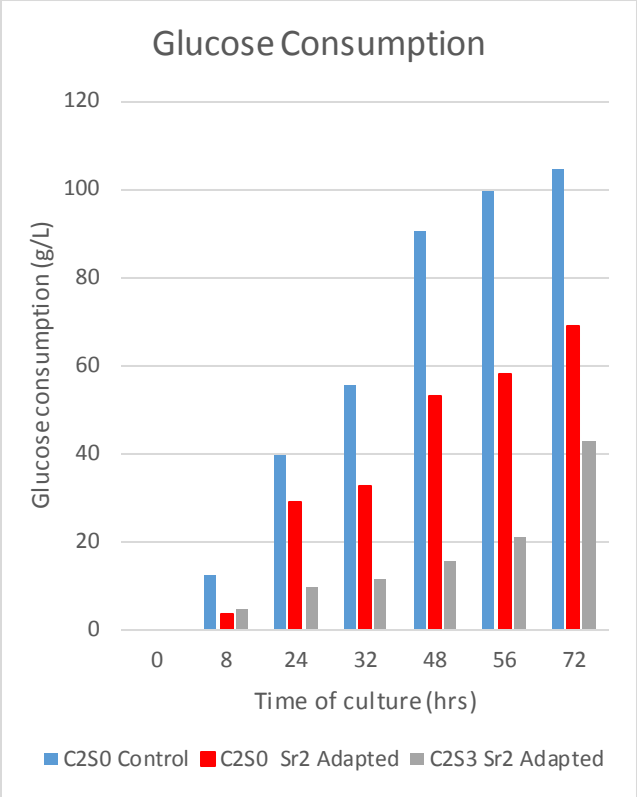


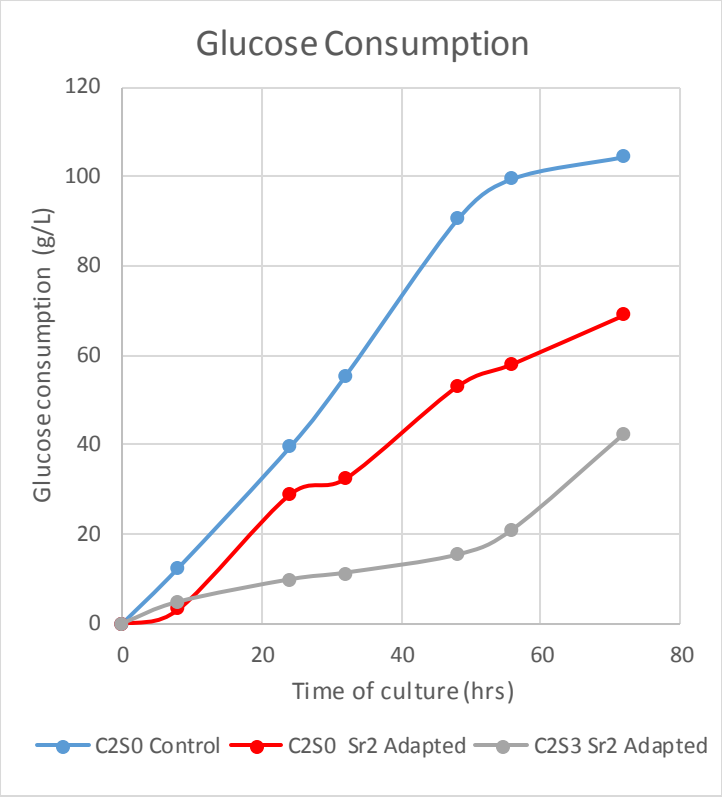


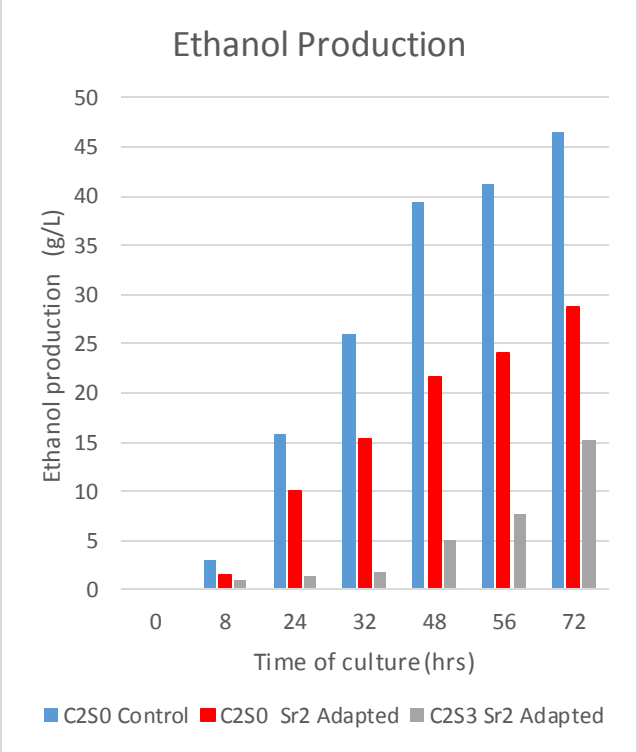


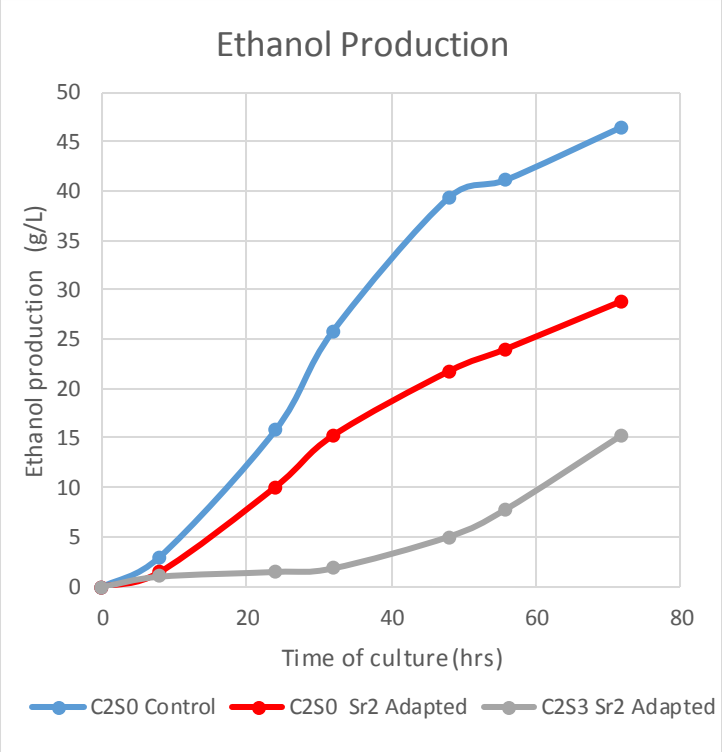


Graph 6: Comparative Growth and Glycerol production level of Sr2 salt adapted Yeast (+/-salt stress) w.r.t control at 1.0 seeding O.D.









Graph 7: Comparative Fermentation profile (Glucose consumption & Ethanol production) of Sr2 salt adapted Yeast (+/-salt stress) w.r.t control at 1.0 seeding O.D.

5.5.3. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at High Seeding O.D (2.5)

The cell density is measured in terms of $O.D_{550\ nm}$ at different time points till 48 hours. Increase in O.D indicates cell growth. Important fermentation characteristics like Glucose consumption, Ethanol production and Glycerol levels were analyzed by quantifying these analytes in the supernatant of culture using HPLC.

The cell growth is observed maximum in C2S0 Adapted culture i.e., adapted cells growing in absence of salt stress followed by C2S0 Control cells growing in absence of salt and C2S3 Sr2 Adapted growing in salt stress. At high seeding O.D., adapted cells are able to grow better than control cells in salt free media. Sr3 Adapted has more growth than Sr2 Adapted culture since, Sr3 is one generation better adaptation of Sr2 culture. However, adapted cells growing in presence of 3% Salt stress has lowest growth of all. This pattern is observed only at higher seeding O.D & not at low / moderate seeding O.D. Cell Growth order: **C2S0 Sr3 Adapted > C2S0 Sr2 Adapted > C2S0 Control > C2S3 Sr3 Adapted**

Time	O.D. at λ=550 nm			
	C2S0 Control	C2S0 Sr2 Adapted	C2S0 Sr3 Adapted	C2S3 Sr3 Adapted
0 hr	2.18	2.64	2.54	2.49
16 hr	9.53	10.42	11.61	4.08
20 hr	10.32	12.01	14.72	5.29
24 hr	11.52	15.60	18.94	8.02
48 hr	11.17	14.58	18.01	12.58

Table 14: Comparative Growth of salt Sr2 & Sr3 adapted Yeast (+/-salt stress) w.r.t control at 2.5 seeding O.D.
{O.D 1 → 3×10^7 cells/ml of culture
C2S0 Control- unadapted yeast in salt free CSL media, C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S0 Sr3 Adapted- 3rd generation of salt adapted yeast in salt free CSL media, C2S3 Sr3 Adapted- 3rd generation of salt adapted yeast in CSL media (+3% salt) }

Glucose consumption and Ethanol production follows the same pattern of cell growth as fermentation is growth dependent. At high seeding O.D 2.5 :

Glucose Consumption/ Ethanol Production:

C2S0 Sr3 Adapted > C2S0 Sr2 Adapted > C2S0 Control > C2S3 Sr3 Adapted

Glycerol Production : **C2S3 Sr3 Adapted > C2S0 Sr2 Adapted > C2S0 Sr3 Adapted > C2S0 Control**

There is a significant improvement in cell growth of C2S0 Sr2 & Sr3 Adapted cultures in comparison to control cells both growing in absence of salt stress. However, the glucose consumption and ethanol production at 24 hours is not very different in control and adapted cells cultured in absence of salt but is low for C2S3 Adapted cells in presence of salt. At 24 hours, C2S0 Sr2 adapted culture produces 1.1 times ethanol, Sr3 adapted culture produces 1.2 times ethanol as compared to control. At 48 hours, glucose is completely consumed by adapted cells in salt free media whereas slight glucose remains in control and C2S3 adapted cell. Highest ethanol is obtained in Sr3 Adapted cells which ferments slightly better than Sr2 Adapted cells on seeding at high cell density. Sr3 Adapted cells in salt stress improves fermentation producing 0.6 times ethanol w.r.t control at 24 hours to 0.97 times (almost similar) ethanol w.r.t control at 48 hours.

The glycerol level is highest in C2S3 Sr3 Adapted cell since it is growing in 3% salt stress while other cultures are presently not subjected to salt stress condition. Glycerol levels remains almost constant in control culture but increases in other cultures, most significantly increases in Sr3 Adapted cell (+Salt stress). Growth and Ethanol production is slightly higher in C2S0 Sr3 than C2S0 Sr2 Adapted culture, while Glycerol level in C2S0 Sr2 is higher than C2S0 Sr3 Adapted culture. Cells of Sr3 culture lowers their glycerol production and improves conversion of glucose to ethanol. This is the reason for higher

ethanol concentration coupled with lower glycerol levels. Thus, it can be inferred, Sr3 Adapted yeast is capable of better fermenting glucose to produce more ethanol attributed to its Salt Adaptation.

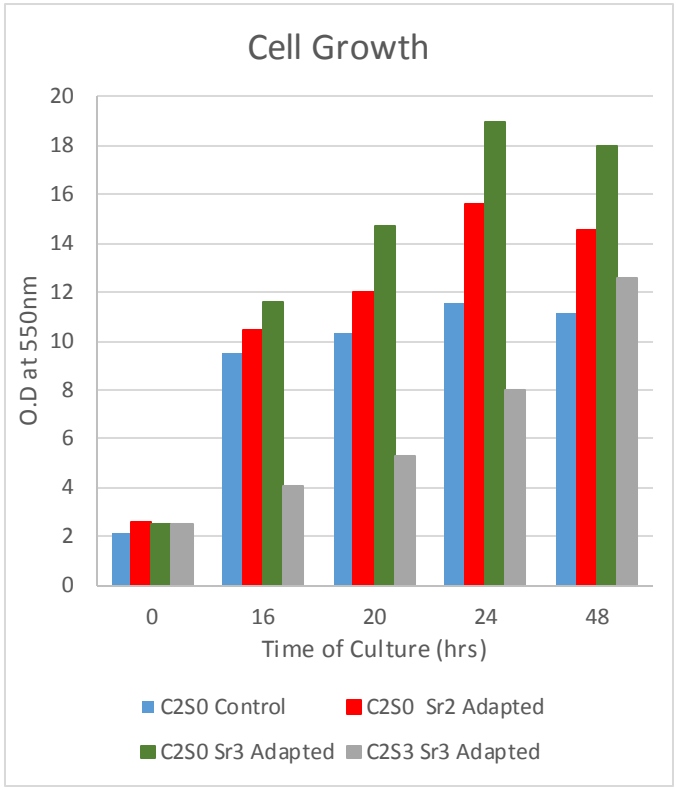
Further experiments are done on higher O.D. for response of Sr2 & Sr3 Adapted cells in presence and absence of salt.

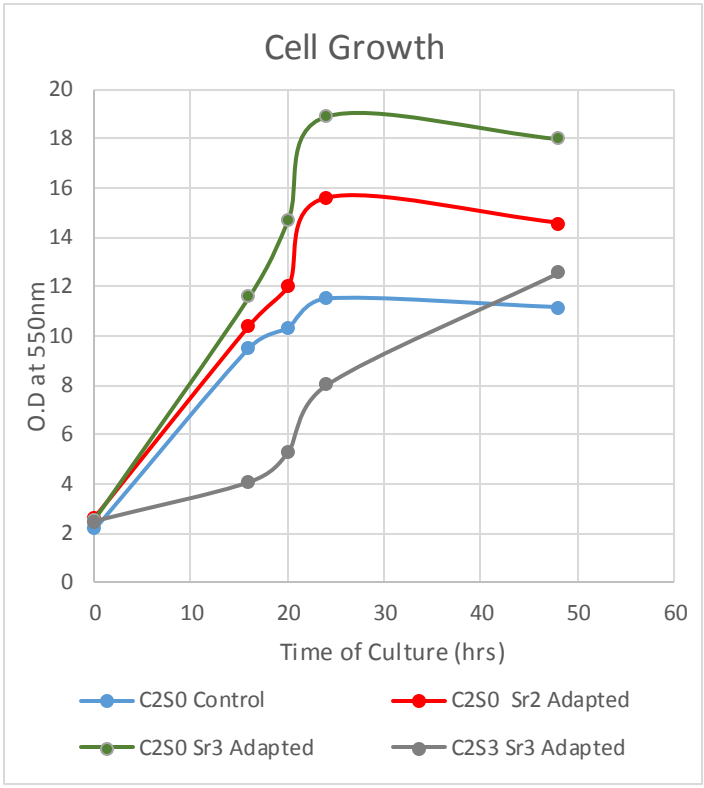
Time	C2S0 Control			C2S0 Sr2 Adapted			C2S0 Sr3 Adapted			C2S3 Sr3 Adapted		
	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)
0 hr	0	0	0	0	0	0	0	0	0	0	0	0
16 hr	31.62	12.55	3.60	32.50	13.10	4.61	34.33	12.64	3.70	16.20	5.29	4.39
20 hr	44.10	15.99	3.70	45.41	18.23	4.74	46.00	18.85	4.05	20.45	8.03	5.05
24 hr	50.87	20.11	3.79	54.00	22.47	5.20	60.30	25.25	4.43	32.71	12.10	5.77
48 hr	92.02	33.14	4.34	103.80	37.16	6.11	100.1	40.30	5.05	93.02	32.27	9.05

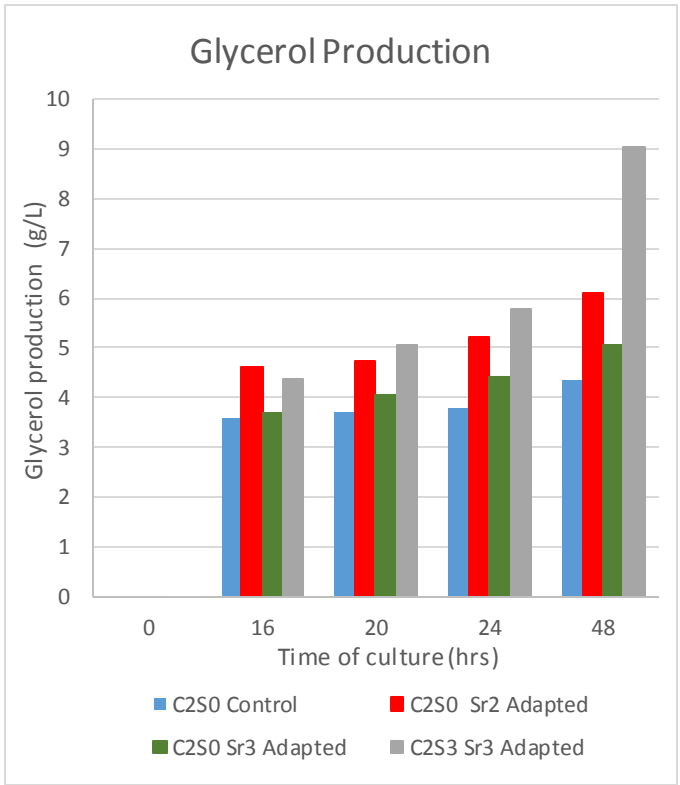
Table 15: Comparative fermentation profile of salt Sr2 & Sr3 adapted Yeast (+/-salt stress) w.r.t control at 2.5 seeding O.D.

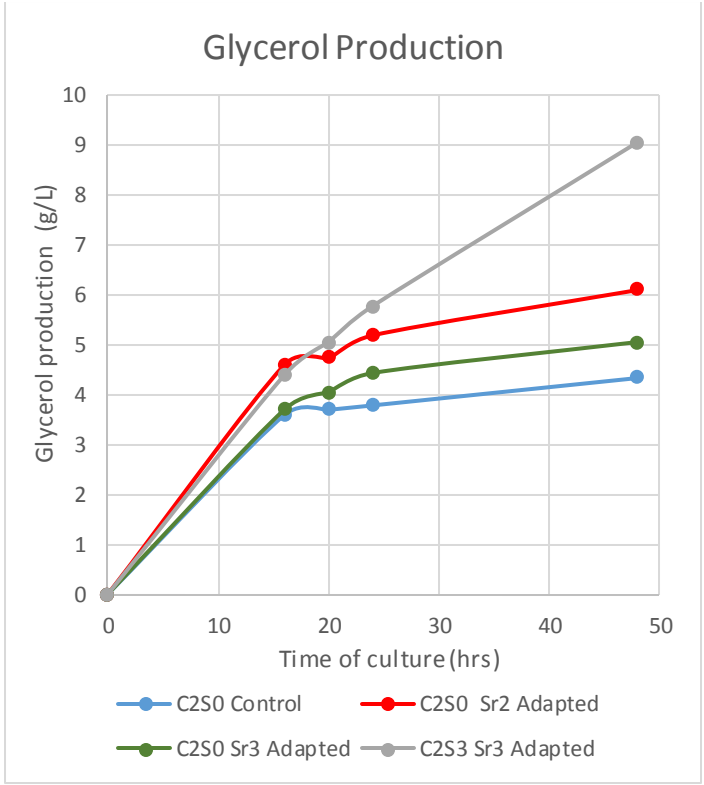
{Initial Glucose in C2S0 Control = 109.5g/L, C2S0 Sr2 Adapted = 109.4g/L, C2S0 Sr3 Adapted = 101g/L, C2S3 Adapted = 109.6g/L (approx. 10%)

C2S0 Control- unadapted yeast in salt free CSL media, C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S0 Sr3 Adapted- 3rd generation of salt adapted yeast in salt free CSL media, C2S3 Sr3 Adapted- 3rd generation of salt adapted yeast in CSL media (+3% salt) }

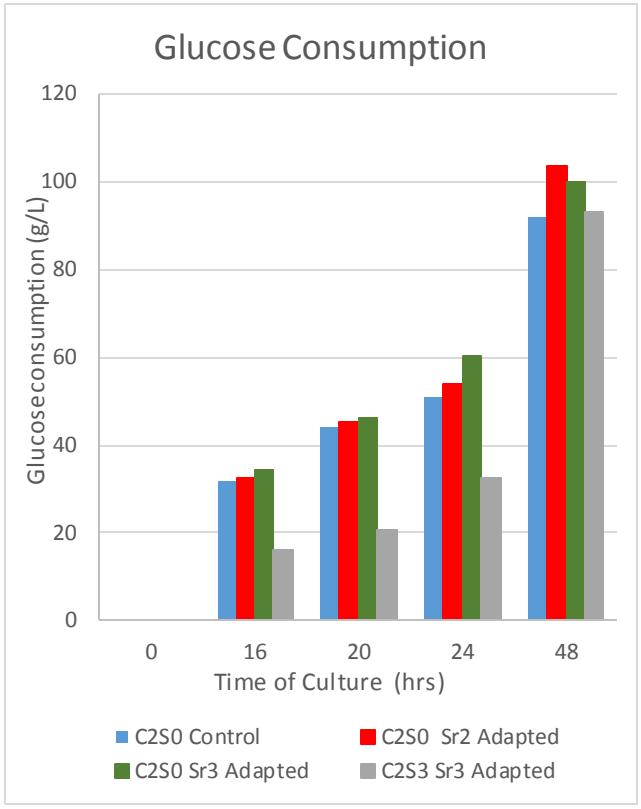


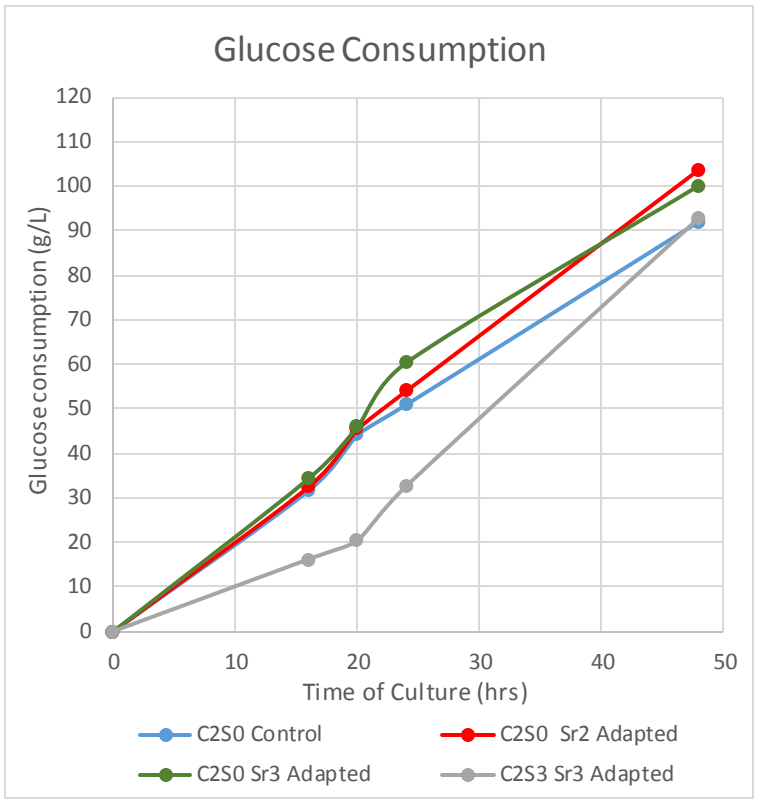


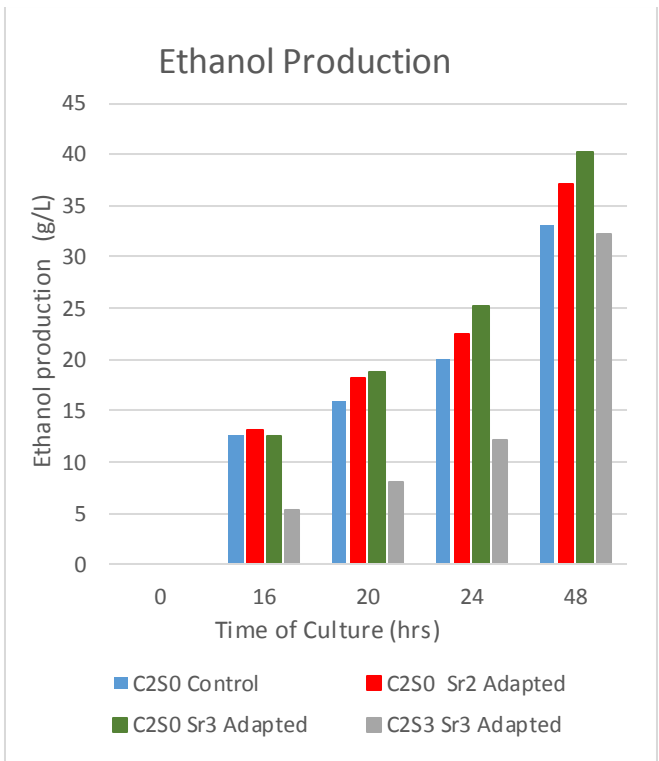


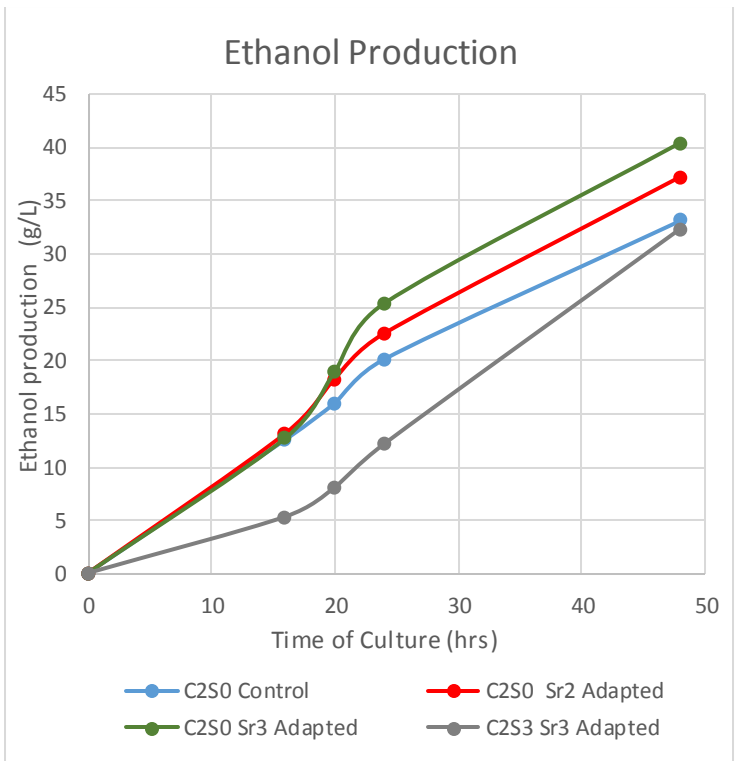


Graph 8: Comparative Growth and Glycerol production level of salt Sr2 & Sr3 adapted Yeast (+/-salt stress) w.r.t control at 2.5 seeding O.D.









Graph 9: Comparative Fermentation profile (Glucose consumption & Ethanol production) of salt Sr2 & Sr3 adapted Yeast (+/-salt stress) w.r.t control at 2.5 seeding O.D.

5.5.4. Growth & Fermentation Characteristics of Salt Adapted Yeast in Presence and Absence of Salt Stress at High Seeding O.D (5.0)

The cell density is measured in terms of $O.D_{550\ nm}$ at different time points till 48- 56 hours. Increase in O.D indicates cell growth. Important fermentation characteristics like Glucose consumption, Ethanol production and Glycerol levels were analyzed by quantifying these analytes in the supernatant of culture using HPLC. Two biological replicate experiments of higher seeding O.D were done to obtain significant data. This is the most important experiment to confirm enhanced growth and fermentation of salt adapted yeast cells in salt free industrial media.

REPLICATE EXPERIMENT 1

The cell growth is observed maximum in C2S0 Sr2 Adapted culture i.e., adapted cells growing in absence of salt stress followed by C2S0 Control cells growing in absence of salt and C2S3 Sr2 Adapted growing in salt stress as seen in Table 16. At high seeding O.D 5, adapted cells are able to grow better than control cells in salt free media as there is significant increase in growth at 16 and 24hours.

Cell Growth order: **C2S0 Sr2 Adapted > C2S0 Control > C2S3 Sr2 Adapted**

Time	O.D. at λ=550 nm		
	C2S0 Control	C2S0 Sr2 Adapted	C2S3 Sr2 Adapted
0 hr	4.30	4.46	4.28
4 hr	6.28	4.94	4.70
8 hr	8.94	7.24	4.92
16 hr	10.62	17.45	8.74
24 hr	12.02	18.72	9.32
48 hr	13.68	17.64	12.76
56 hr	15.64	17.61	10.8

Table 16: Comparative Growth of salt Sr2 adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate-1){ O.D 1 → 3×10^7 cells/ml of culture }

Fermentation Profile includes Glucose consumption, Ethanol production and glycerol production by yeast cells. The pattern as observed in Graph-10 & 11 :

Glucose Consumption/ Ethanol Production: **C2S0 Sr2 Adapted > C2S0 Control > C2S3 Sr2 Adapted**

Glycerol Production : **C2S3 Sr2 Adapted > C2S0 Sr2 Adapted > C2S0 Control**

At seeding O.D. 5, along with significant improvement in cell growth of C2S0 Sr2 Adapted cultures w.r.t control cells, there is also significant improvement in glucose utilization and ethanol production by Sr2 Adapted cells w.r.t control cells. In seeding O.D 5, C2S0 Sr2 adapted culture produces 1.8 times ethanol at 20 hours and 1.3 times ethanol at 24 hours with an average of 1.55 times ethanol production w.r.t control. In seeding O.D. 2.5 experiment, higher growth is observed but ethanol production is nearly same in C2S0 Adapted and control cells i.e., at 24 hours, C2S0 Sr2 adapted culture produces 1.1 times ethanol w.r.t control. Therefore, it suggests **Salt stress adaptation induces cellular & metabolic changes in Adapted yeast cells that enable them to enhance growth & ethanol fermentation process at higher cell density** after release of salt stress as compared to control. The ethanol production of C2S3 Adapted culture w.r.t control increases from 0.33 at 20 hours to 0.6 at 24 hours. The adapted cells in presence of salt are not fermenting equivalent to adapted cells in absence of salt but surely they improve with time. So, better adaptation is key to enhanced ethanol production.

At 48 hours, glucose is completely consumed by adapted cells and control in salt free media whereas slight glucose C2S3 adapted cell. With almost 100g/L Glucose consumed by both cultures, Sr2 Adapted produces more ethanol (42 g/L) than control cells (38g/L) at 48 hours. Thus, there is better conversion of glucose to ethanol in Adapted cultures which is a desired result for our experiment.

The glycerol level is highest in C2S3 Sr2 Adapted cell since it is growing in 3% salt stress while other cultures are presently not subjected to salt stress condition. At 56 hours, Glycerol levels of C2S0 control

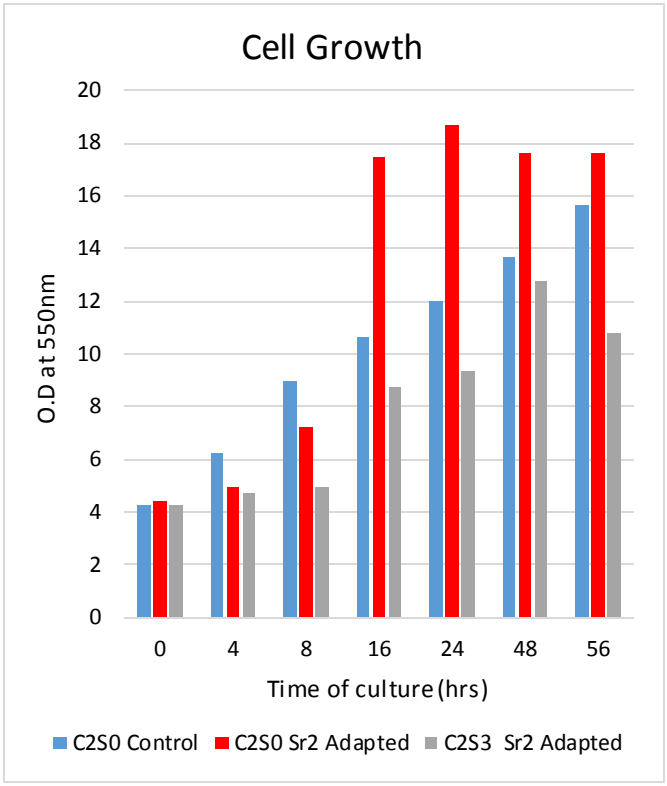
is 4.5g/L , C2S0 Adapted is 5.5g/L and C2S3 Adapted is 9.4g/L. Adapted cells growing in salt stress (C2S3) has almost double glycerol produced w.r.t control cultures growing in absence of salt as seen in Table-17. At this stage, there is better fermentation by C2S3 Adapted culture than previous time points. Thus, glycerol production mitigate the osmotic stress experienced by cells while growing in 3% salt.

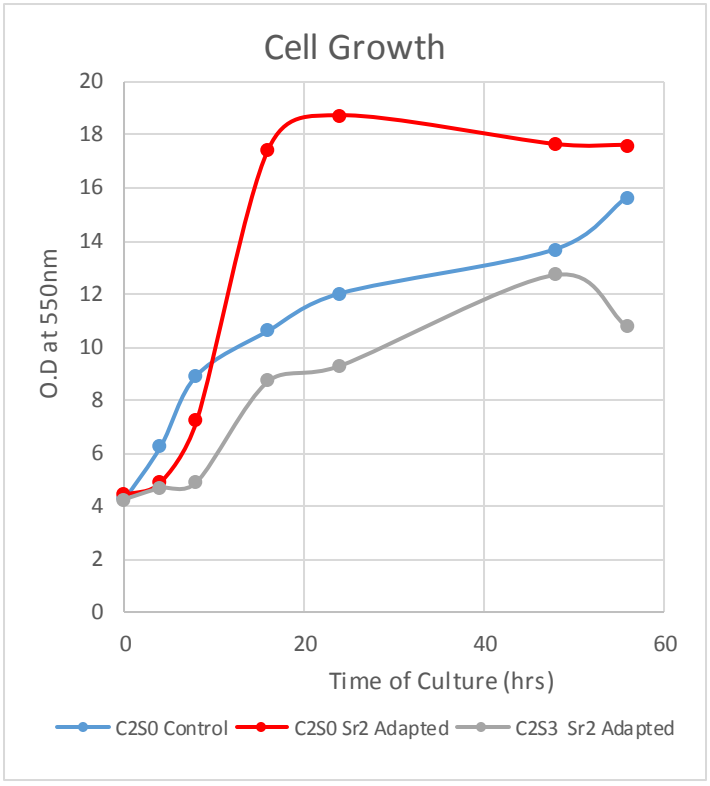
Time	C2S0 Control			C2S0 Sr2 Adapted			C2S3 Sr2 Adapted		
	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)
0 hr	0	0	0	0	0	0	0	0	0
4 hr	3.96	1.75	0.74	10.40	3.12	1.42	2.3	1.1	1.38
8 hr	16.40	6.08	2.35	20.76	5.91	3.30	12.1	2.5	2.99
16 hr	29.14	17.05	3.77	77.35	31.24	4.80	31.60	5.82	5.11
24 hr	57.1	26.43	3.94	91.40	34.69	5.21	44.87	15.99	6.41
48 hr	99.92	38.85	4.18	105.87	42.50	5.39	88.20	30.61	8.65
56 hr	103.33	40.31	4.52	105.90	43.27	5.43	91.75	33.50	9.40

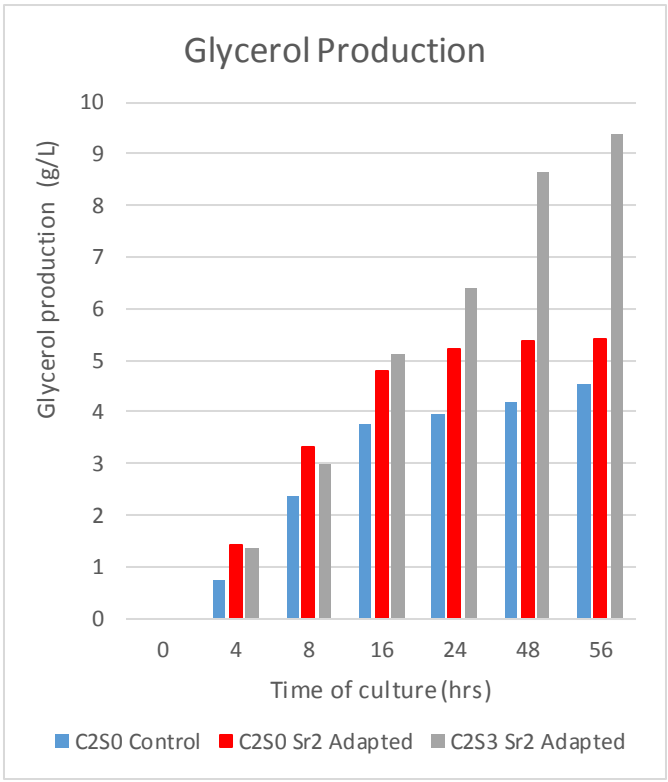
Table 17: Comparative fermentation profile of salt Sr2 adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 1)

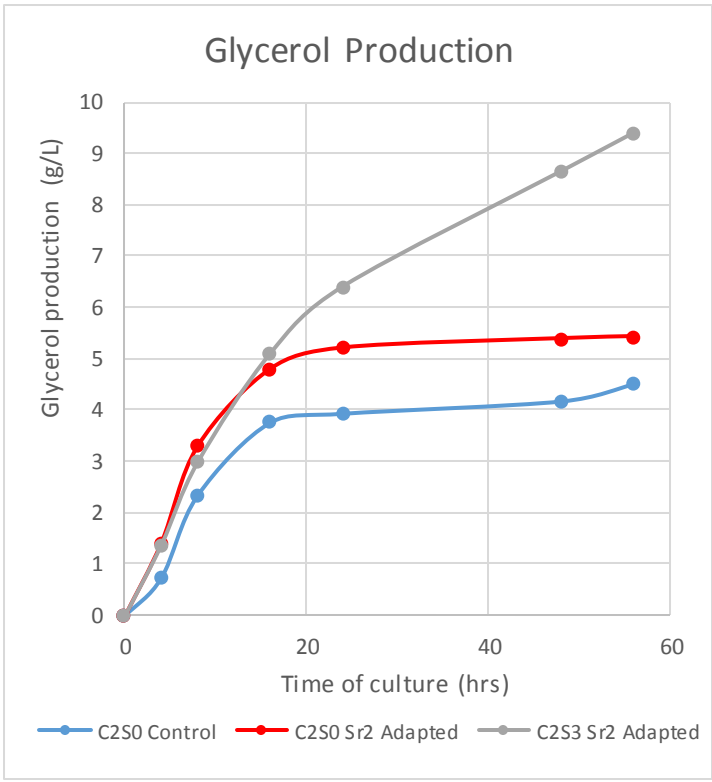
{Initial Glucose in C2S0 Control =103.6g/L, C2S0 Sr2 Adapted =105.9g/L, C2S3 Adapted =113.8g/L (approx.10%)

C2S0 Control- unadapted yeast in salt free CSL media, C2S0 Sr2 Adapted- 2nd generation of salt adapted yeast in salt free CSL media, C2S3 Sr2 Adapted- 2nd generation of salt adapted yeast in CSL media (+3% salt) }

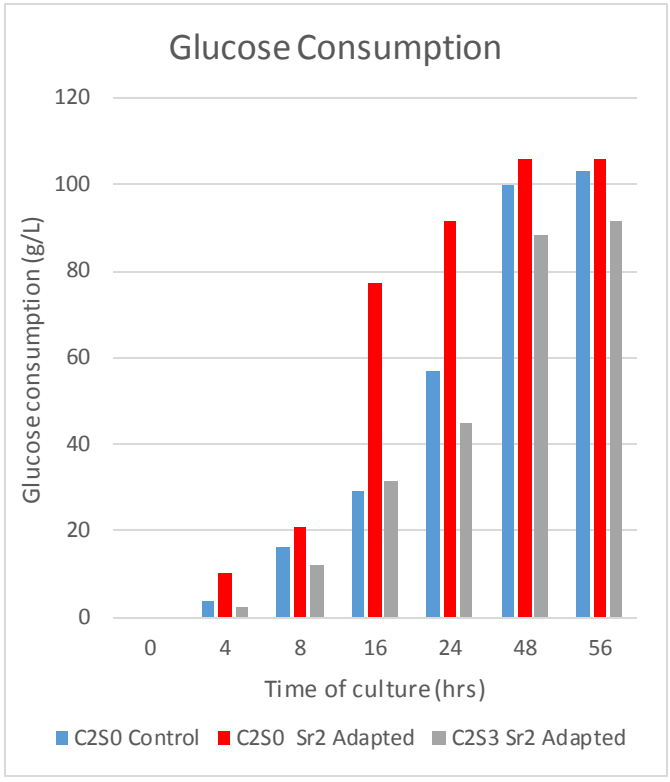


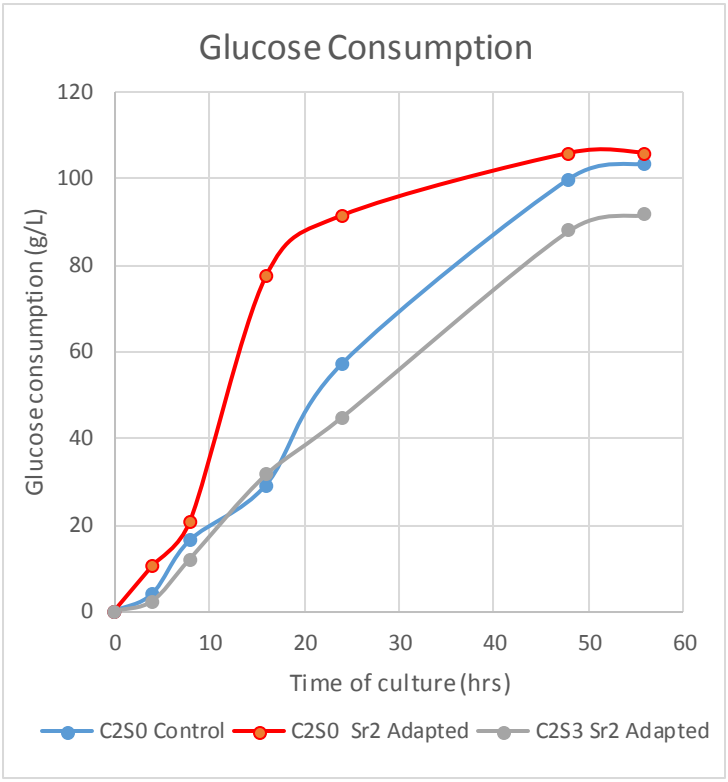


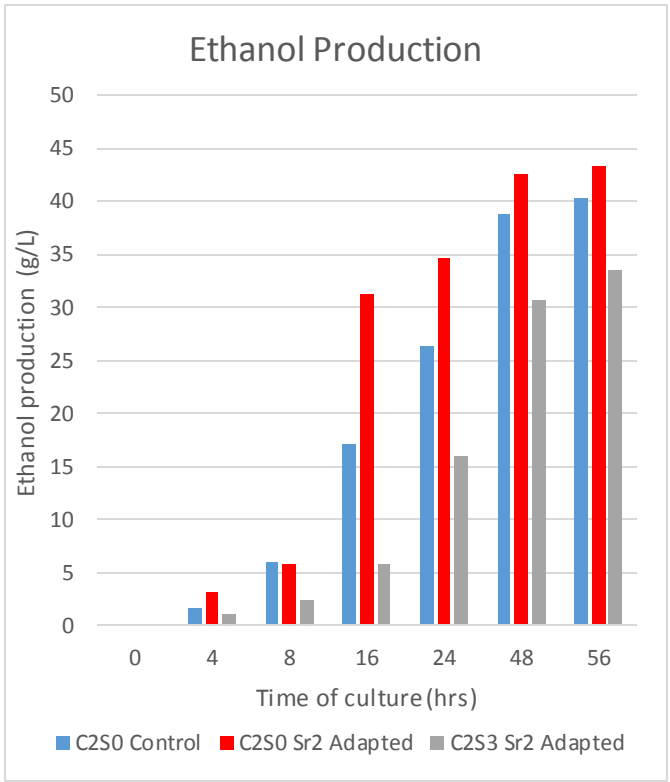


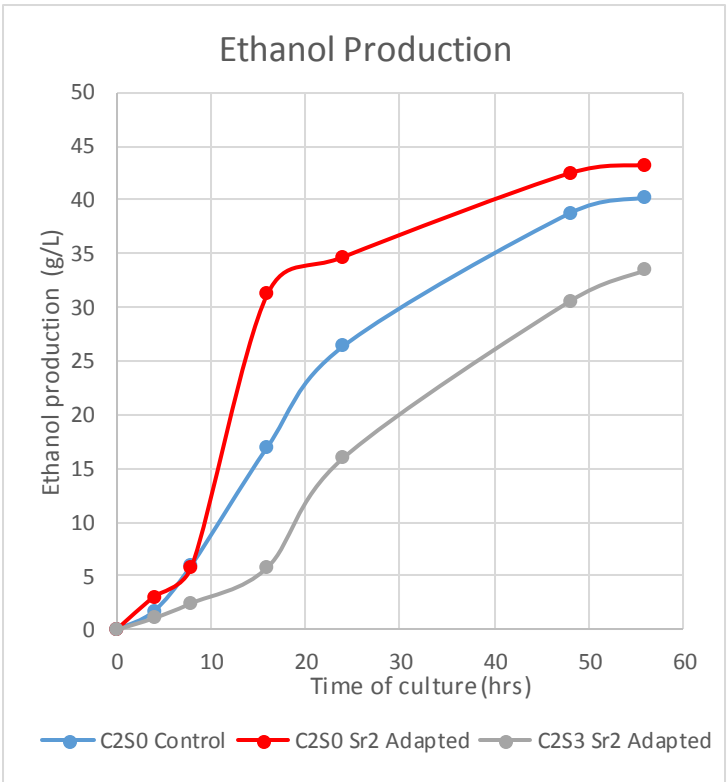


Graph 10: Comparative Growth and Glycerol production level of salt Sr2 adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 1)









Graph 11: Comparative Fermentation profile (Glucose consumption & Ethanol production) of salt Sr2 adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 1)

REPLICATE EXPERIMENT 2

From Table-18, it is observed there is significant increase in growth in Sr2 & Sr3 Adapted cells growing in absence of salt w.r.t control whereas Sr3 Adapted cell growing in presence of salt has almost equivalent growth to control at 24 hrs. At 24 hours, C2S0 Sr3 Adapted has 1.8 times and C2S0 Sr2 Adapted has 1.4 times growth as compared to control.

Cell Growth order: **C2S0 Sr3 Adapted > C2S0 Sr2 Adapted > C2S0 Control > C2S3 Sr3 Adapted**

Time	O.D. at λ=550 nm			
	C2S0 Control	C2S0 Sr2 Adapted	C2S0 Sr3 Adapted	C2S3 Sr3 Adapted
0 hr	4.88	5.16	4.82	4.78
16 hr	11.05	13.95	15.74	7.05
20 hr	12.62	15.63	20.02	8.48
24 hr	13.26	18.52	24.84	11.22
48 hr	13.76	18.36	21.40	12.22

Table 18: Comparative Growth of Sr2 & Sr3 salt adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 2)

{ O.D 1 → 3×10^7 cells/ml of culture }

Time	C2S0 Control			C2S0 Sr2 Adapted			C2S0 Sr3 Adapted			C2S3 Sr3 Adapted		
	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)	Glu cons. (g/L)	Eth prod. (g/L)	Glyc prod. (g/L)
0 hr	0	0	0	0	0	0	0	0	0	0	0	0
16 hr	42.90	16.22	3.90	56.40	21.60	5.18	47.61	20.28	4.40	28.17	8.93	5.05
20 hr	52.91	21.11	4.16	73.21	25.35	5.50	65.10	27.29	4.75	34.60	13.72	6.12
24 hr	66.82	23.70	4.55	87.37	31.51	5.81	92.62	36.85	5.50	44.27	19.19	7.36
48 hr	109.72	41.95	4.76	115.37	45.53	6.02	105.61	45.30	5.71	103.97	40.13	10.80

Table 19: Comparative fermentation profile of Sr2 & Sr3 salt adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 2)

{Initial Glucose in C2S0 Control = 111.2g/L, C2S0 Sr2 Adapted = 115.4g/L, C2S0 Sr3 Adapted = 105.6g/L, C2S3 Adapted = 100.4g/L (approx. 10%) }

The pattern as observed in Graph-12 & 13 :

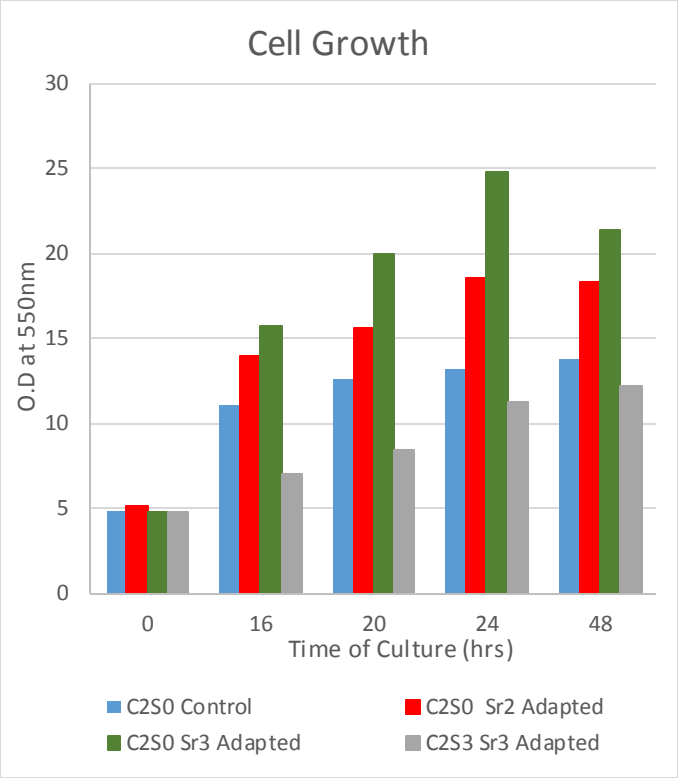
Glucose Consumption and Ethanol Production:

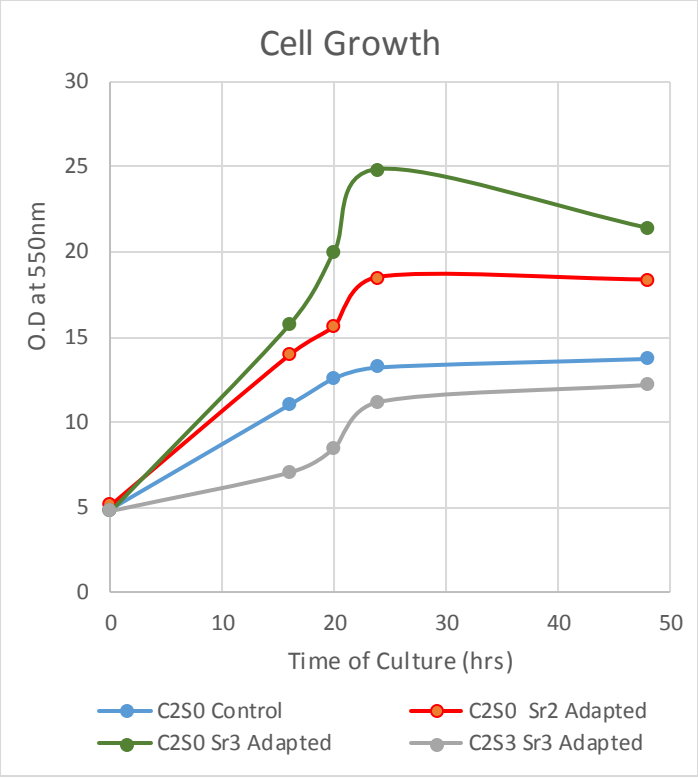
C2S0 Sr3 Adapted > C2S0 Sr2 Adapted > C2S0 Control > C2S3 Sr3 Adapted

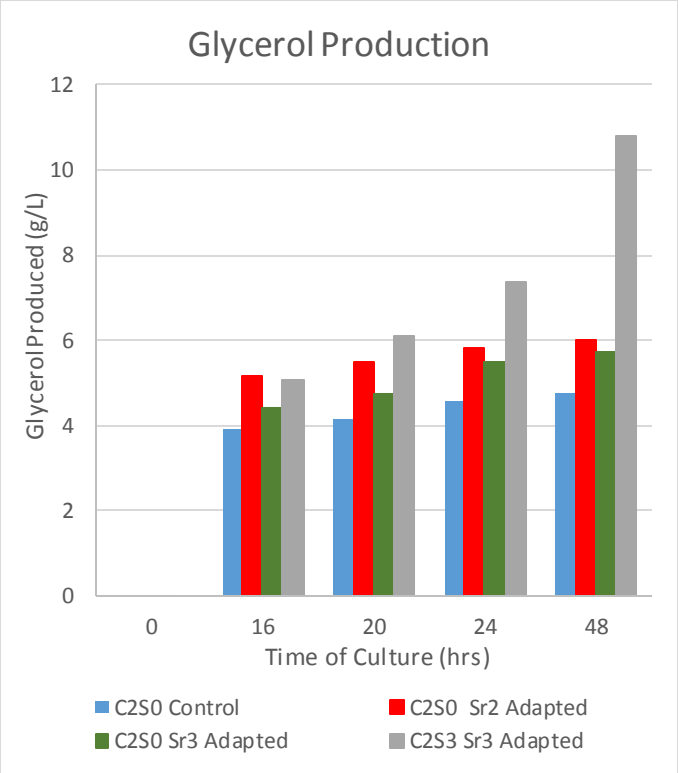
Glycerol Production: **C2S3 Sr3 Adapted > C2S0 Sr2 Adapted > C2S0 Sr3 Adapted > C2S0 Control**

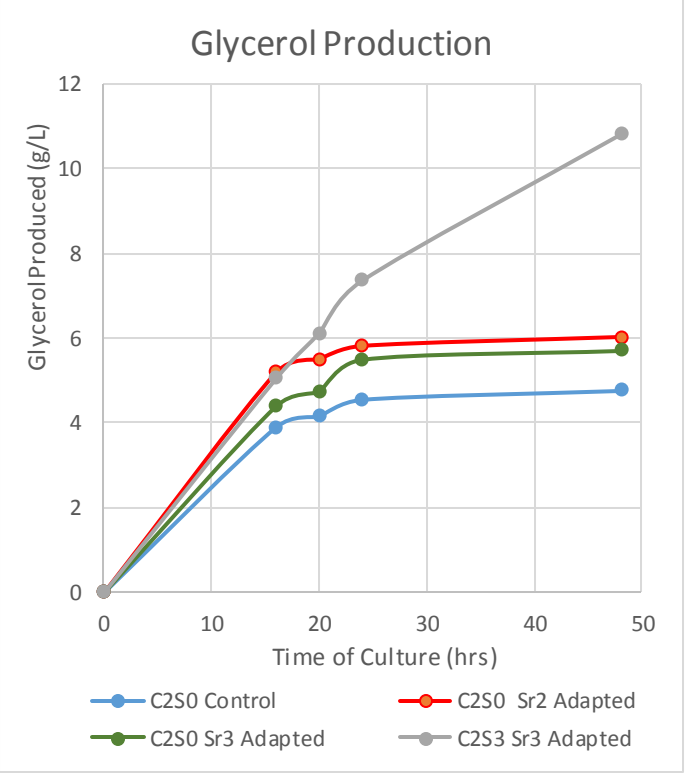
At 24 hours, along with significant increased growth in Adapted cultures (-salt), there is enhanced fermentation of Glucose to higher Ethanol as compared to control (-salt). Sr2 Adapted culture & Sr3 Adapted cultures produces 1.34 times and 1.6 times ethanol respectively in salt free media w.r.t to control's ethanol production. This is consistent with result obtained in replicate experiment 1. **Thus, Adapted cells ferment more glucose (higher glucose consumption) to produce more ethanol when cultured in absence of salt (after adaptation phase) in comparison to Control (Unadapted cells growing in salt free media)**. C2S3 Sr3 Adapted (adapted cell growing in 3% salt) has lowest growth and fermentation rate i.e., 0.8 times ethanol productivity w.r.t control at 24 hours. At 48 hours, 10% glucose is consumed by all culture sets with highest ethanol production by Sr3 Adapted culture (-salt).

The glycerol level is highest in C2S3 Sr3 Adapted cell as seen in Graph-12, since it is growing in 3% salt stress while other cultures are presently not subjected to salt stress condition. Glycerol levels remains almost constant in control culture, slightly increases in salt free adapted cultures & most significantly increases in Sr3 Adapted cell (+Salt stress). Growth and Ethanol production is higher in C2S0 Sr3 than C2S0 Sr2 Adapted culture, while Glycerol level in C2S0 Sr2 is higher than C2S0 Sr3 Adapted culture. Cells of Sr3 culture lowers their glycerol production and improves conversion of glucose to ethanol. This is the reason for higher ethanol concentration coupled with lower glycerol levels. Similar glycerol production pattern is observed in experimental setup with seeding O.D 2.5 as seen in Graph-8. Thus, it is inferred, **Sr3 salt adapted yeast is capable of better fermenting glucose to produce more ethanol attributed to its Salt Adaptation**

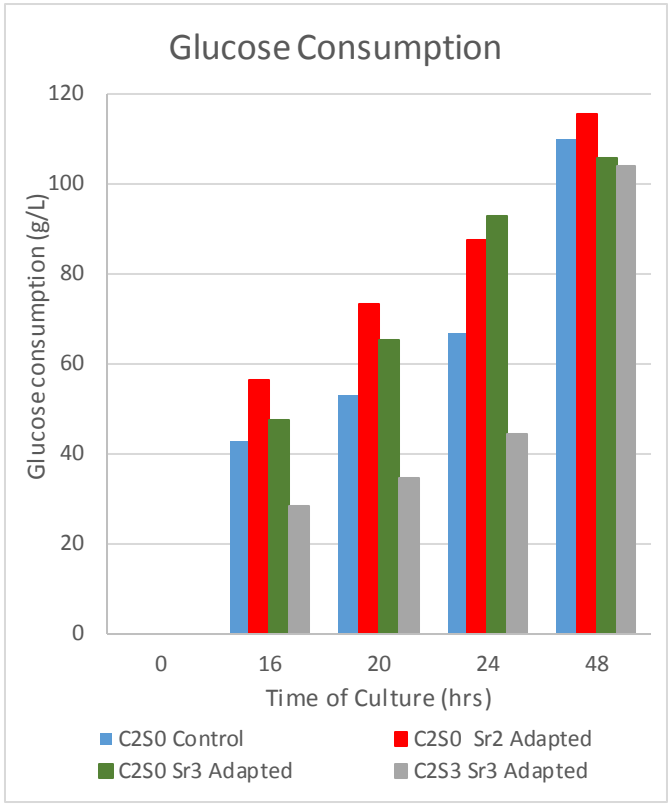


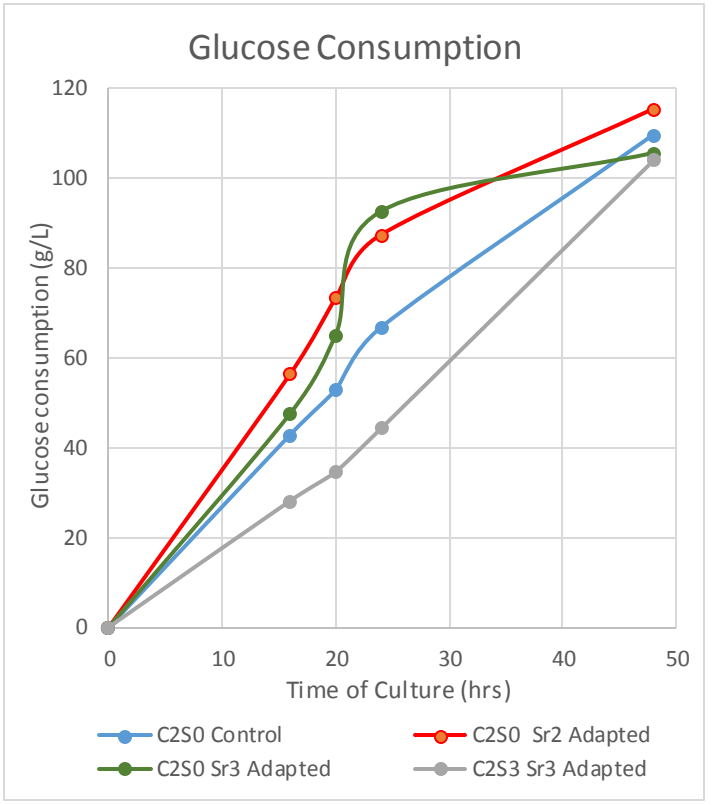


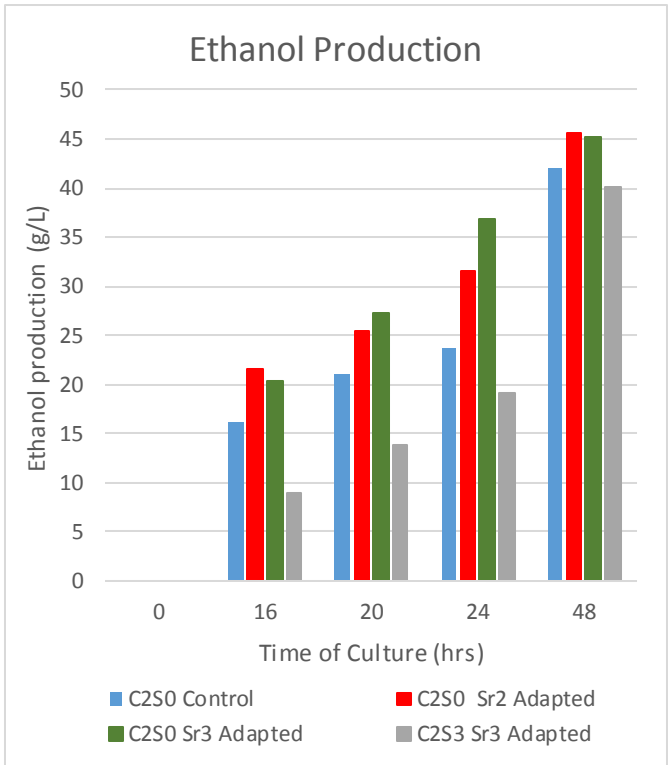


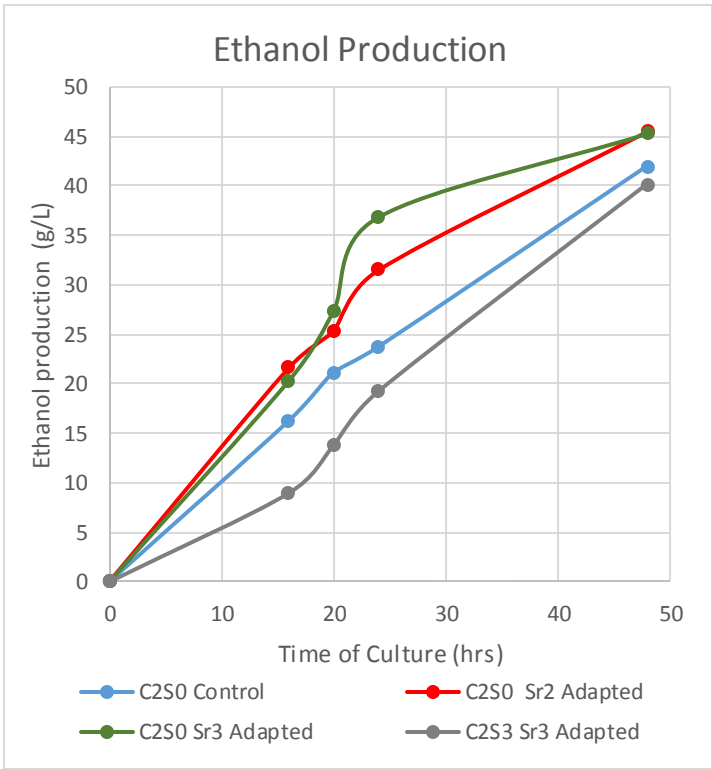


Graph 12: Comparative Growth and Glycerol production level of salt Sr2 & Sr3 adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 2)









Graph 13: Comparative Fermentation profile (Glucose consumption & Ethanol production) of salt Sr2 & Sr3 adapted Yeast (+/-salt stress) w.r.t control at 5.0 seeding O.D. (Replicate 2)

From the two biological replicate experiments of higher seeding O.D, similar results were obtained and growth & fermentation profile were confirmed.

Growth in Salt adapted (Sr2 & Sr3) cells is significantly higher than Control, cultured in salt free media. Sr2 salt adapted yeast has 1.4 times and Sr3 salt adapted yeast has 1.8 times growth of control at 24 hours. The fermentation process is faster in both Sr2 & Sr3 adapted cells while growing in absence of any salt (after they have successfully adapted earlier). Adapted cells (-salt) utilizes 90% of Glucose present in media in 24 hours, while control cells utilizes 60% of glucose content, thereby, producing more ethanol. **Adapted cells in salt free media produces on an average 1.5 times ethanol w.r.t. control** (unadapted cells in salt free media). **Therefore, we obtain more ethanol in less time from same composition industrial media using salt stress adapted cells, enhancing the fermentation process**. This is optimal utilization of industrial media.

Further observations shows on higher serial adaptation from Sr2 to Sr3, glucose consumption and ethanol production improves as **Sr3 Adapted cells produces more ethanol than Sr2 Adapted cells both in presence and absence of salt.** Growth or Ethanol production trend :

C2S0 Sr3 Adapted > C2S0 Sr2 Adapted (from replicate experiment 2)

C2S3 Sr3 Adapted > C2S3 Sr3 Adapted (from replicate experiment 1 & 2)

The Ethanol production of Sr2 to Sr3 Adaptation increases from 0.6 to 0.8 times w.r.t control at 24 hours growing in presence of salt. So, continuous adaptation improves fermentation process even in presence of salt.

Growth and Ethanol production is higher in C2S0 Sr3 than C2S0 Sr2 Adapted culture, while Glycerol level in C2S0 Sr2 is higher than C2S0 Sr3 Adapted culture. This trend is similar in both seeding O.D 2.5 and O.D 5 experiments seen in Graph-8, Graph-10 and Graph-12. Cells of Sr3 culture lowers their

glycerol production when cultured in salt free condition and improves conversion of glucose to ethanol. This is the reason for higher ethanol concentration coupled with lower glycerol levels. Thus, it is inferred, **Sr3 salt adapted yeast is capable of better fermenting glucose to produce more ethanol attributed to its Salt Adaptation**. Glycerol is produced in adapted cells by virtue of their salt adaptation so, once adapted to salt stress that enhances fermentation, glycerol is not necessary when cultured in salt free media. **It seems salt adaptation also enables yeast to sense the environment better.** Sr3 adapted cell is higher adaptation to Sr2 adapted cells and Sr3 adapted culture decreases glycerol better than Sr2 adapted culture in absence of salt stress, while Sr3 Adapted culture keep on producing higher glycerol in presence of salt stress. So, Sr3 decreases/ increases the glycerol production by sensing external environment in media and is not innate property of cell. **So, yeast is metabolically modified/ engineered by salt stress.**

The results suggest **Salt stress adaptation induces cellular & metabolic changes in Adapted yeast cells that enable them to enhance growth & ethanol fermentation process at higher cell density** after release of salt stress as compared to control. These trends of enhanced fermentation by adapted cells (-salt) is observed only at high seeding O.D. (2.5 & 5) and not at lower seeding O.D (0.1& 1), **suggesting molecular cross talk among salt adapted yeast cells.**

5.6. CELLULAR CHARACTERISTICS OF ADAPTED AND UNADAPTED YEAST CELLS IN DIFFERENT CULTURE CONDITIONS (+/- SALT)

5.6.1. Observation of Colour of Adapted & Unadapted Yeast Cells Cultured in +/- Salt

There is an observable difference in colouration of salt adapted cells cultured in presence and absence of salt (3%NaCl) stress vs control/ Unadapted cells cultured in absence of salt as indicated by Fig.11. For all 3 experimental setups (seeding O.D 1, 2.5, 5)

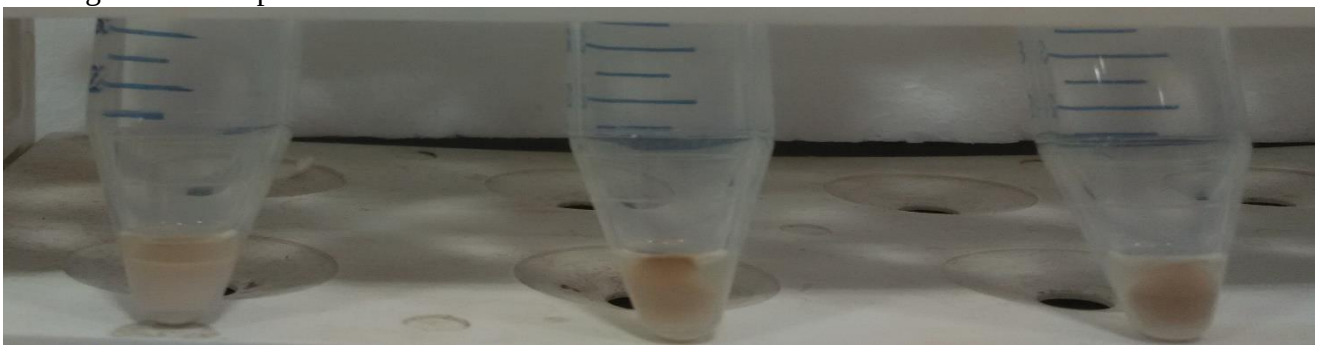
C2S0 Control (Unadapted yeast growing in 2% CSL without salt stress) – Cell pellet is white/off-white in colour.

C2S0 Adapted (Salt adapted yeast growing in 2% CSL without salt stress) – Cell pellet is light brown coloured (darker than C2S0 Control and lighter than C2S3 Adapted)

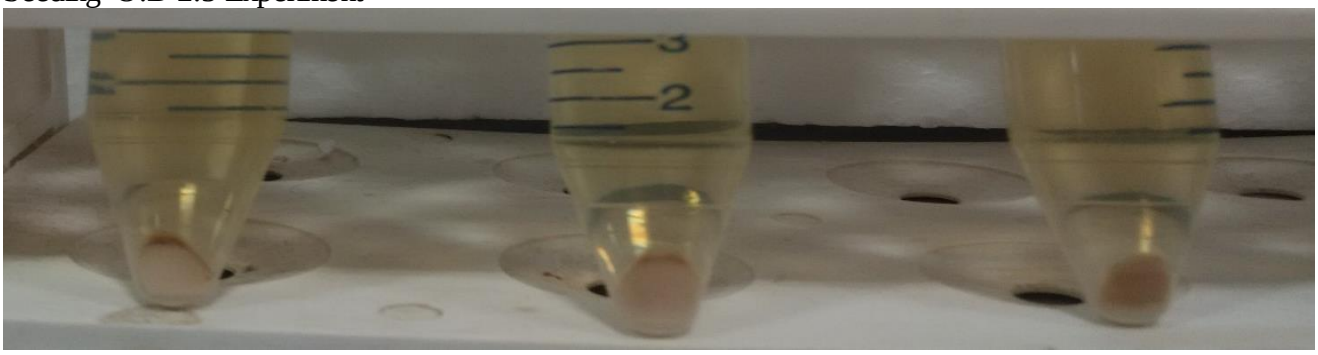
C2S3 Adapted (Salt adapted yeast growing in 2% CSL with salt stress) – Cell pellet is dark brown coloured.

Therefore, **NaCl salt stress induces some changes in salt tolerant/adapted yeast cells** as reflected by their colour difference. This can be confirmed by lipid analysis of yeast because change in cell colour indicates cell membrane (Phospholipid bi-layer) composition modification by osmotic salt stress. **The colour difference between C2S0 Control and C2S0 Adapted is significant in lower seeding O.D (1 & 2.5) culture while is neglible in higher seeding O.D 5 cultures.** This is complemented by the fact at higher seeding O.D, adapted yeast cells behave like control cells and much better in terms of fermentation efficiency to produce more ethanol. The Adapted cells growing in salt appears darkest in all 3 sets.

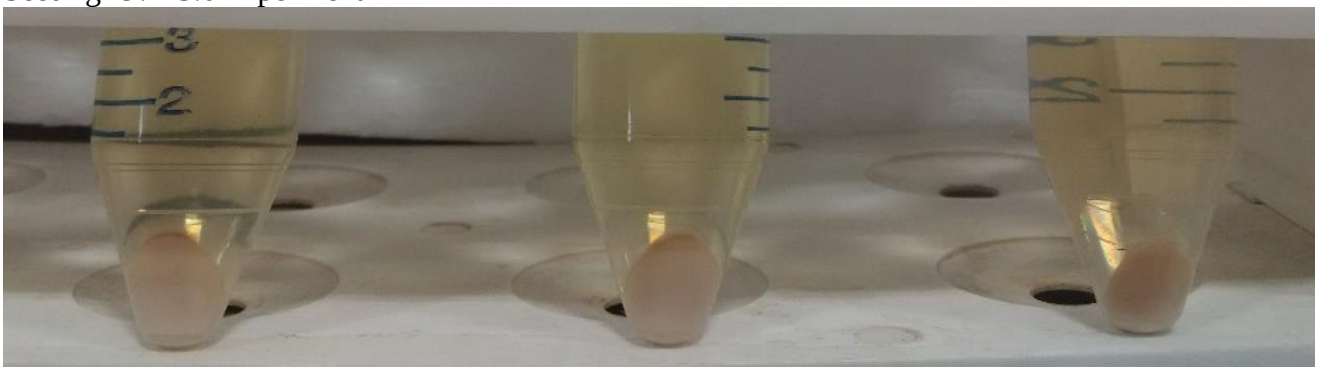
Seeding O.D 1.0 Experiment



Seeding O.D 2.5 Experiment



Seeding O.D 5.0 Experiment



C2S0 Control	C2S0 Sr2 Adapted	C2S3 Sr2 Adapted

Figure 11: Difference in colour of salt adapted (in +/- salt stress) and unadapted yeast (in – salt stress)in 3 experimental setup.

5.6.2. Observation of Cell Size of Adapted & Unadapted Yeast cultured in +/- Salt

The cell size of yeast was observed using compound microscope by oil immersion method under 100X objective lens to check if there is any difference in cell size of salt adapted and unadapted cell. The average cell size of Unadapted yeast cultured in absence of salt stress (C2S0 Control), Salt adapted yeast cultured in absence of salt (C2S0 Adapted) and Salt adapted yeast growing presence of salt (C2S3Adapted) is same with dimensions 5µm × 4µm. Salt stress does not affect yeast cell size.

C2S0 Control

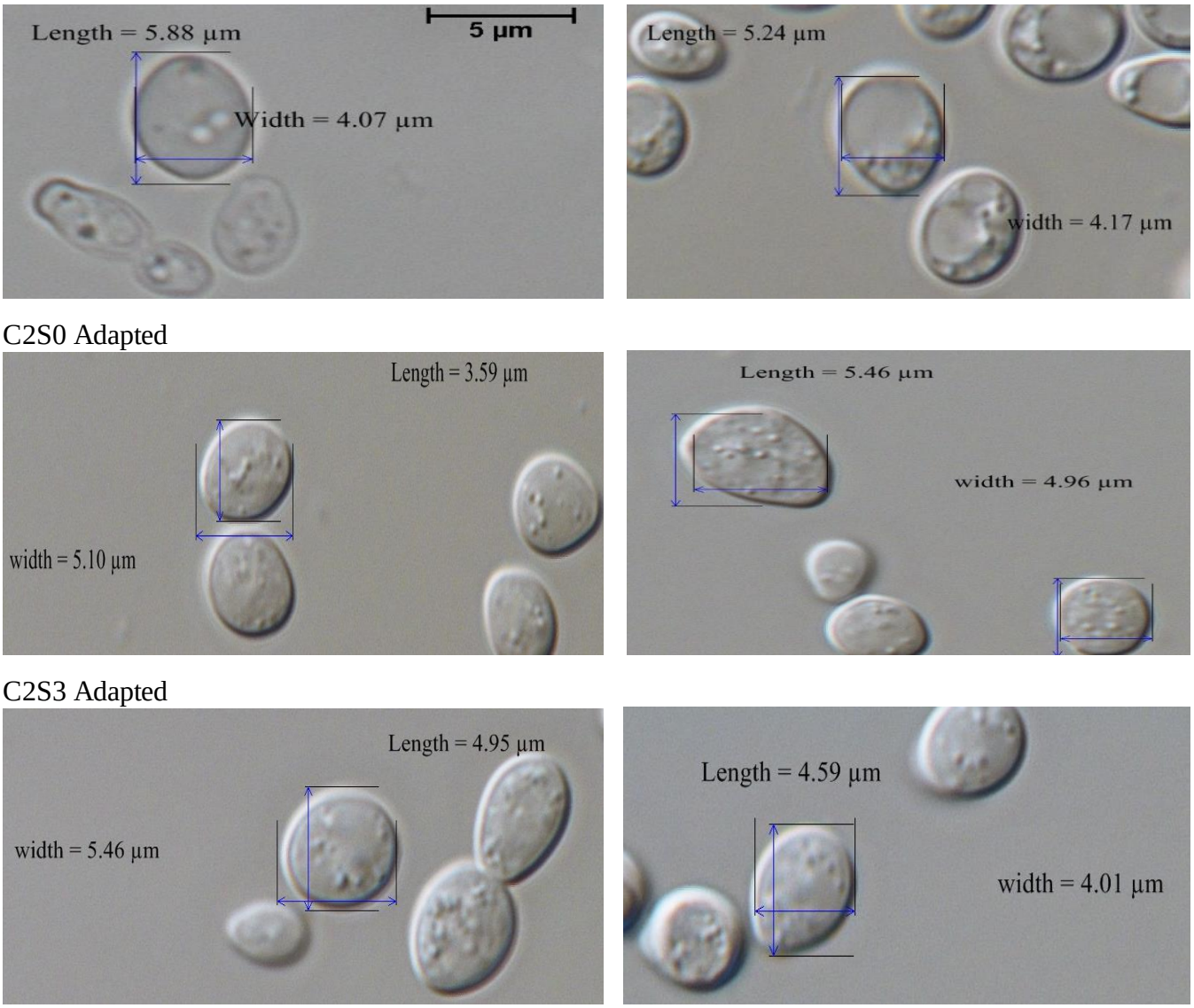


Figure 12: Cell Size of Salt adated yeast (in +/- salt stress) and unadapted yeast (control in –salt stress).

6.

CONCLUSION

Saccharomyces cerevisiae is the cheapest & commercially available yeast for fermentation process converting glucose substrate into ethanol product. Therefore, it has significant potential in mass production of bio-ethanol production from sugar molasses and corn steep liquor media (used in industrial media). The present research focuses on enhancing fermentation of *S. cerevisiae* CEN.PK 122 (diploid) strain for higher ethanol yield using salt stress in industrial media. This requires evaluation of growth and fermentation profile of adapted and unadapted yeast cells cultured in minimal CSL media with or without salt stress. We have optimized the CSL and salt concentration in media for salt stress adaptation as 2% CSL and 3% NaCl. Choice of 2% CSL over standardly used 4.5% CSL is to adapt the yeast cells on a minimal media. The media was kept minimal (2% CSL) so that the cells adapt in Salt stress and not merely grow. If there is higher concentration of CSL, yeast cells will be able to grow even in high salt stress because of higher nutrients supplemented from media to cells to sustain life. Here, yeast cell may not adapt to the condition and loose its tolerance property as soon as the stress condition is removed. Lower concentration of CSL (less than 2% CSL) cannot be used because growth is very low and cannot obtain colonies on Low CSL-Agar plates. Additionally, growth of yeast can be higher using 4.5% CSL but ethanol production is similar in 2% and 4.5% CSL containing media and yeast once adapted to salt in 2% CSL minimal media can always be supplemented with 4.5% CSL for further enhancement of fermentation. Lower CSL concentration also makes the process cheaper. Salt stress of 3% is employed keeping in mind the fact sea water (approximately 3.5% saline) can directly be used in media at industrial scale production, thereby eliminating the need of freshwater or purified salts. 10% Glucose is used for high density fermentation for high ethanol yields as required in an industrial process.

All living cells spend energy to maintain essential life processes called as Maintenance Energy. Enhanced ethanol production by yeast by salt stress is to test Maintenance Energy hypothesis that

suggests salt adapted/tolerant yeast cells are capable of fermenting more sugars to ethanol to generate more ATP in order to maintain active cell metabolism and sustain life in osmotic/ salt stress. There is an increased energy expenditure in stress condition which translates to slower reproduction and hence, reduced cell growth rates. But yeast cells are slowly able to adapt to salt stress with no decrease in cell concentration suggesting cells were able to prevent cell death at the cost of reduced reproduction and respond to stress with more energy expenditure. This explains enhanced ethanol specific productivity of the yeast cells as a Salt (NaCl) Stress response to immediately meet the ATP requirement of cell by increased rate of fermentation as claimed by the hypothesis. While adapting yeast to salt stress (3% NaCl), the cell growth and ethanol production of *S. cerevisiae* strain was less initially even with high glucose consumption but increased as time elapsed, with subsequent passaging and Serial adaptation as observed in Table 5. Sr2 Adapted and Sr3 Adapted are 2^{nd} generation and 3^{rd} generation of serial adaptation to salt stress and used in further experiments.

The comparative growth and fermentation profile of Salt tolerant (Salt adapted) and Intolerant (unadapted) yeast cells were studied in presence and absence & absence of salt stress in industrial media at various seeding O.D. (0.1, 1, 2.5 & 5). Different growth & fermentation trends (ethanol productivity) were observed in each experiment set:

At low seeding O.D 0.1: **C2S0 Control > C2S0 Adapted ≈ C2S3 Adapted**

At moderate seeding O.D 1: **C2S0 Control > C2S0 Adapted > C2S3 Adapted**

At high seeding O.D 2.5: **C2S0 Adapted ≥ C2S0 Control > C2S3 Adapted**

At high seeding O.D 5: **C2S0 Adapted > C2S0 Control > C2S3 Adapted**

This shows with increasing seeding O.D, there is a shift in growth and fermentation pattern. **At higher seeding O.D, the salt adapted cells are able to ferment 1.5 times to control when cultured in salt free media i.e, 50% more ethanol produced attributed to salt adaptation**. There is growth

improvement of adapted cells growing in both presence & absence of salt but no growth improvement in control cells at particular time interval when seeding O.D. changes. At 24 hours, cell growth of Control is around O.D 10-12 at all seeding O.Ds whereas cell growth of Adapted yeast (growing in salt free condition) increases from O.D 4 to 7 to 15 to 24 in seeding O.D 0.1, 1, 2.5 & 5 experiments respectively. This is complemented by increasing glucose consumption for ethanol production in C2S0 Adapted cultures with 5.6g/L ethanol to 10g/L ethanol to 25g/L ethanol to 35g/L ethanol at 0.1, 1, 2.5 & 5 seeding O.D respectively. There is not much increase in ethanol production of C2S0 control from 15g/L ethanol to 24g/L ethanol at seeding O.D. 0.1 & 5 respectively. There is chance of further improvement in glucose consumption and ethanol production on **higher serial adaptation of yeast to salt stress making fermentation process faster and more economical by optimal utilization of glucose & industrial media.**

These trends of enhanced fermentation by adapted cells (-salt) is observed in liquid cultures only at high seeding O.D. (2.5 & 5) and not at lower seeding O.D (0.1& 1). Similarly, salt adapted yeast survive and grow better on CSL plate containing 3% salt only when plated at high cell density while diluted salt adapted cultures rarely grow on salt containing solid media. This confirms **intercellular molecular cross talk among salt adapted yeast cells leading to better adaptability of yeast in salt stress and better fermenting capability resulting in enhanced ethanol specific productivity (ethanol production per cell). It seems salt adaptation also enables yeast to sense the environment better.** Sr3 adapted cell is higher adaptation to Sr2 adapted cells and Sr3 adapted culture decreases glycerol better than Sr2 adapted culture in absence of salt stress, while Sr3 Adapted culture keep on producing higher glycerol in presence of salt stress. So, Sr3 decreases/ increases the glycerol production by sensing external environment in media and is not innate property of cell. **So, yeast is metabolically engineered by Salt Stress.**

Higher cell growth (O.D.) is linked with higher ethanol production from glucose utilization and no growth is linked with negligible ethanol production**. Thus fermentation in yeast is growth linked process**. Two factors are crucial in improving fermentation efficiency of process: Glucose Conversion (Ethanol production to Glucose consumption ratio =E/G) and Ethanol Specific productivity (Ethanol production per cell per unit time). The Glucose conversion ratio (E/G) of Salt adapted yeast growing in absence of salt increases from 0.28 to 0.40 in case of moderate seeding O.D 1 to high seeding O.D 2.5 & 5, E/G ratio of salt adapted yeast growing in presence of salt also increases from 0.22 to 0.43 in seeding O.D 1 to seeding O.D 5, while E/G ratio of control cells (unadapted cells growing in absence of salt) remains almost same i.e., between 0.33-0.35 in low to high seeding experiments. Hence, **salt adaptation targets better conversion of glucose for enhanced ethanol productivity** by decreasing production of other fermentation products.

Improved fermentation and environment sensing of yeast strain by salt stress adaptation makes it ideal for use in industrial production of ethanol since crude industrial media may contains many impurities (including salts). One type of stress leads to protective response to other stress factors (John *et al.* 2012). The experimentation results of enhanced ethanol production by salt adapted yeast (in salt free media) need to be confirmed in fermentor. Further reusability of salt adapted yeast cells need to be checked since enhanced ethanol production is observed at high seeding O.D to meet the requirement of enormous amount of adapted yeast cells. Higher generation of salt adapted yeast ferments differently in presence and absence of salt stress. Adapted cells in presence of salt produces lesser ethanol and more glycerol to combat stress compared to adapted cells growing in absence of salt. Addition of osmotic protectant (glycerol) to industrial media may enable adapted cells to grow and ferment better in saline stress. This needs to be investigated in future. Further lipid analysis needs to done accounting for colour difference of salt adapted and unadapted yeast cells.

2nd generation Biofuel from Lignocellulosic biomass waste involves 2 processes: Breakdown of complex lignocellulose (LG) into monomeric sugars and fermentation of monomeric sugars into ethanol. A lot of emphasis and effort is put on efficient degradation of the LG complex. But, improving the efficiency of fermentation process is also necessary to yield more ethanol from the monomers. More specific productivity of end product (Bioethanol) is aimed in our experiments.

Nowadays, the petroleum products are running out of race due to unbalanced relation between supply and demand. The hike in petrol cost is mainly due to shortage of resources which leads to search for alternate fuel to replace fossil fuels. An eco-friendly bio-ethanol is one such alternate fuel. Combustion of ethanol results in relatively low emission of volatile organic compounds, carbon monoxide and nitrogen oxides. The emission and toxicity of ethanol are lower than those of fossil fuels such as petroleum, diesel etc. In developing countries like India, bioethanol can be utilized to run public transport buses as well as means of small mass transport vehicles that currently exhausts fossil fuels (Petrol/Diesel/CNG) and cause air pollution due to non-regulation of norms. The adverse climatic conditions (increasing pollution and CO_2 release) from continuous use of fossil derived fuels are the driving factors for the development of renewable sources of fuel- bioethanol. The need of the hour is to move to cleaner fuel alternative considering the deteriorating quality of air of metropolitan city in the country (New Delhi is rated as world's most polluted city). Moving to bio-ethanol as a fuel require huge ethanol production and therefore an efficient fermentation process as targeted in our research. The Government of India announced **"*National Policy on Biofuel" in 2008*** focusing on ***Ethanol Blending Policy (EBP***) which aims to achieve blending of ethanol with petrol. The policy was drafted and released in year 2011- 2012 with implementation from 2013. From 2013-16, *petrol blending with 5% ethanol* has been mandatory as the fuel has better calorific value and burns more efficiently. Since, grain (corn/edible plant) based ethanol is not allowed by the government in India, currently the

Government owned Oil Marketing Companies (OMCs) are fully dependent on sugar mills for procurement of sugar and molasses for use as industrial media for ethanol production. After three years of 5% ethanol blending with petrol, the country is set to achieve its next target of 10% blending of ethanol in petrol by 2017. The annual ethanol production nearly doubled from 67.5 crore litres in 2014-2015 to 120 crore litres in 2015-2016. **There is requirement of additional *260 crore litres of ethanol/year;* achieved by designing an improved/efficient fermentation process using desired yeast strain capable of enhanced ethanol productivity and varied substrate utilization under salt stress.** The present cost of ethanol in India is Rs.39/L. Fermentation process has 20% share in total production cost i.e, Rs.8/L. Our research boosts fermentation process by 50% by use of salt adapted yeast in industrial media so a deduction of cost by Rs.4/L. The targeted price of ethanol becomes Rs.35/L which can be further decreased to Rs.30/L by coupling technological innovation in other process of production. **Our research caters dual solution i.e., higher ethanol production to meet the rising demand of ethanol and decrease in ethanol price by improving fermentation efficiency of yeast in minimal industrial media**. Bio-ethanol as biofuel will lead to sustainable development of country, boosting the economy by less expenditure on buying of petroleum from other countries & selling fuel grade ethanol overseas besides curbing pollution levels by decreasing dependency on non-renewable fuels and paving way for green & clean future.

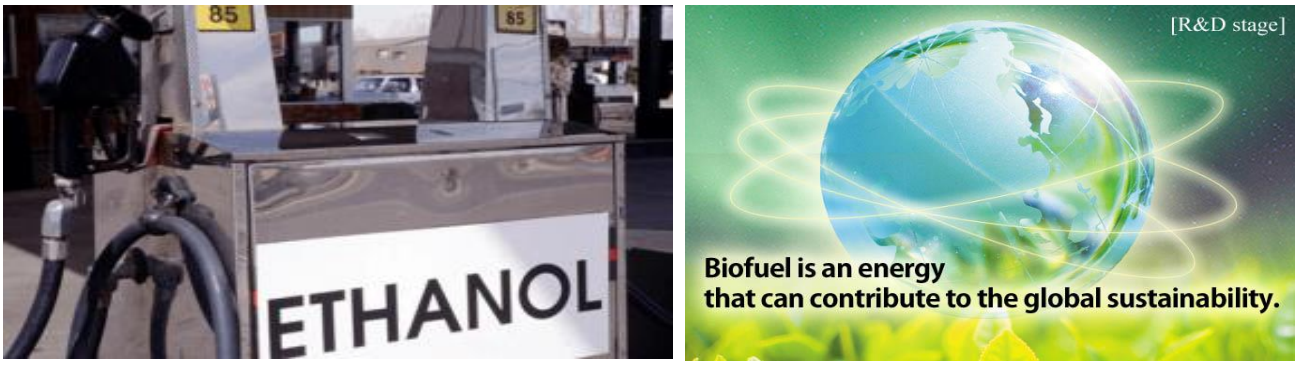


Figure 13: Bio-ethanol mass production in controlled fermentation is the key to sustainable future.

7.

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8.

APPENDIX

MEDIA COMPOSITION

1. YPD (Yeast extract Peptone Dextrose) Media – 100 ml

(i) YPD Liquid Media

1% Yeast Extract – 1g

2% Bactero-Peptone – 2g

2% Glucose – 2g

Dissolved in 70 ml M.Q water. Adjusted pH -5 with acid. Made up the final volume to 100 ml.

Autoclaved the media before culturing.

(ii) YPD Solid Media = YPD Agar Plates

Prepared YPD liquid media, adjusted pH- 5, made up the final volume to 100 ml.

Added 2% Agar – 2g. Autoclaved the media.

Poured the YPD-Agar media on petri plates and allow to cool/solidify. Cover the petriplate and seal with paraffin to prevent contamination during storage.

2. CSL (Corn Steep Liquor) Media – 100 ml

(i) CSL Liquid Media

4.5% (or variable) CSL – 4.5 g

0.23% Urea – 0.23 g

0.38% $MgSO_4.7H_2O$ – 0.38 g

0.003% CuSO4 – 3 mg

10% Glucose – 10 g (or 20 ml of 5X Glucose Stock after autoclaving the media)

3% (or variable) NaCl – 3 g

Dissolved in 50 ml M.Q water (do not add glucose). Adjusted pH -5 with acid. Made up the final volume to 80 ml.

Autoclaved the media before culturing. Centrifuged to get rid of insoluble debris and get clear media.

Added 20 ml 5X filter sterilized Glucose Stock Solution in LAF hood to make up the final volume to 100 ml.

* CSL concentration is varied (0.5%, 1%, 2%, 4.5%) for diff. experiments.

* NaCl concentration is varied (1%, 2%, 3%, 4%) for diff. experiments.

(ii) CSL Solid Media = CSL Agar Plates (10% glucose or 2% glucose plates)

Prepared CSL liquid media (do not add glucose), adjusted pH- 5, made up the volume to 80 ml.

Added 2% Agar – 2g. Autoclaved the media.

CSL (10% Glucose) Plate: Added 20 ml 5X Glucose Stock Solution to make up final vol. to 100 ml.

CSL (2% Glucose) Plate: Added 4 ml 5X Glucose Stock Solution, adjust remaining volume with M.Q water to make up final vol. to 100 ml.

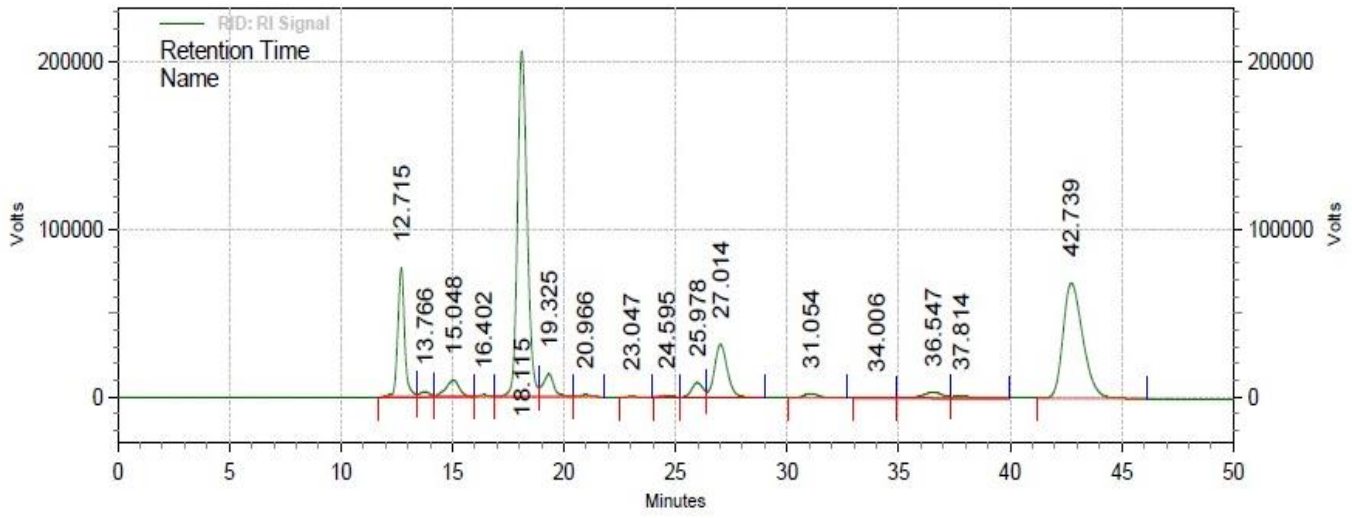
Poured the CSL-Agar media on petri plates, allow to solidify. Cover and seal with paraffin.

HPLC ANALYSIS

1. Calculation of Concentration of Analyte using Standard Area

Analyte	Retention Time (min)	Standard Area	Conc. of Standard
Glucose	18.15	29055174	1g/L
Glycerol	27.00	24205240	1g/L
Ethanol	43.20	12502983	1g/L

$$Conc.\ of\ analyte\ in\ sample = \frac{\text{Area of sample} \times \text{Conc. of Standard} \times \text{dilution factor}}{Area\ of\ Standard}$$



HPLC Peaks of Analyte