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SANTHOSH SUDHAKARAN

MUTAGENESIS OF *HALANAEROBIUM SACCHAROLYTICUM* SUBSP. *SACCHAROLYTICUM* AIMING FOR ANTIBIOTIC SENSITIVITY AND IMPROVED HYDROGEN AND 1,3-PROPANEDIOL TOLERANT STRAINS.

Master of Science thesis

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Examiners and topic approved by the Faculty of Natural Sciences on 9th January 2013

ABSTRACT

SANTHOSH SUDHAKARAN: Mutagenesis of *Halanaerobium saccharolyticum* subsp. *saccharolyticum* aiming for antibiotic sensitivity and improved hydrogen and 1,3-propanediol tolerance.

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Keywords: *Halanerobium sacchrolyticum* subsp. *sacchrolyticum*, Multi dug efflux pump, novobiocin, UV mutation, hydroxylamine Hydrogen, 1,3 – propanediol.

Halanerobium sacchrolyticum subsp. *sacchrolyticum* is a halophilic anaerobic fermentative bacteria, grows in high salt condition. Based on the salt concentration halophiles are classified into moderate halophiles and extreme halophiles. Hydrogen and 1, 3-propanediol was the essential product produced by this bacterium. Survivals of the *H. sacchrolyticum* on high salt concentrations were by salt in and salt out strategy, which maintains the osmotic balance.

Different antibiotics were tested for resistivity of *H. sacchrolyticum*; ampicillin, kanamycin and chloramphenicol were resistance to these bacteria. Whereas novobiocins with > 1 µg/ml were sensitive, thus inhibited the growth of bacteria, novobiocin concentration of < 1 µg/ml was resistant. Transfomation efficiency of Plasmid pMDS133 which contains NovR gene were analysed by natural transformation method. *H. sacchrolyticum* with < 1 µg/ml of novobiocin was subjected to two different types of mutation, UV and hydroxylamine mutagenesis. The multidrug efflux pump plays important role in excreting the toxic substances, since *H. sacchrolyticum* is a gram negative bacteria, the multi dug efflux pump is complex.

Mutation in *H. sacchrolyticum* increases the yield of hydrogen, were the optimal yield was obtained 2.4 mol/mol (glucose) and with initial 33 % of hydrogen tolerance 1.5 mol/mol (glucose) was obtained. 1, 3-propanediol tolerance was tested after mutagenesis, which inhibits the growth and affects in production of end metabolites. Screening of mutants was the most challenging part of the study; replica plating technique was used in selection of mutants, initially experimented with *E. coli* KRX strain and later with mutated *H. sacchrolyticum.*

PREFACE

This thesis has been carried out at Department of Chemistry and Bioengineering of Tampere University of Technology, Tampere, Finland.

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ABBREVIATIONS

ATP	Adenosine Tri Phosphate.
NADPH	Nicotinamide adnine Dinucleotide Phosphate Hydrogen.
PDA	Phenylenediamine
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
MDR	Multiple Dug Resistance
OM	Outer membrane layer
DNA	Deoxyibo Nucleic Acid
HA	Hydoxylamine
UV	Ulta Violet
CPD	Cyclobutane Pyimidine Dimers
OD	Optical Density
$CaCl_2$	Calcium Chloride
NaCl	Sodium Chloride
HCl	Hydrogen Chloride
1, 3-PD	1, 3 - Propanediol

1. Introduction

Halophiles are the bacteria which require high salt concentration for growth and cellular activity. Based on the salt concentrations, it can be classified as haloterant, moderately halophilic and extremely halophilic microorganisms. *H. sacchrolyticum* subsp. *sacchrolyticum* is an order of Haloanerobiales and Haloanerobiaceae are moderate halophilic bacteria which requires 0.5M to 2.5M of salt concentration for optimal growth. Some of the advantage of halophilic bacteria includes, ability to suppress the growth of other microorganisms due to high salt content, other biotechnological applications are, used in production of bacteriorhodopsin, enzymes, polymers and in hydrogen, ethanol, 1, 3- propanediol production. [1]

H. sacchrolyticum isolated at hypersaline environments like lagoons and lakes, it is oxygen sensitive, and therefore it requires anaerobic condition for growth, high salt concentration, and organic solute as carbon sources. [1]

Halophilic bacteria follow two strategies for controlling their osmotic balance, salt-in and salt-out strategy. Salt in strategy is utilizing potassium or sodium into cytoplasm. Salt-out strategy is excluding salt from cytoplasm and utilizing organic solutes like glycerol which is comparatively expensive.

Halophilic bacteria survive at high salt concentration by maintaining osmotic balance using two strategies. Salt-in strategy adapted by several methods 1) Replacing large hydrophobic residues on the protein surface i.e. reducing of hydrophobicity by using hydrophilic residues, example *Dihydrofolate reductase*. 2) Increase in acidic residues, by changing the charges or inter converting the mesophilic and halophilic form of proteins. 3) Using amino acids, for example the insertion of 30-amino acid at the N-terminal, which is rich in acidic amino acids, usage of above strategies does not interrupt the metabolic process. [2]

2. Theoretical background

2.1 Types of halophiles

Halophiles are generally categorized based on salt concentration. Slight Halophiles optimal growth is at 0.2 – 0.85 mol L^{-1} (2-5%) of Nacl, Moderate Halophiles grows maximum at 0.85-3.4 mol L^{-1} (5-20%) and extreme halophiles attains optimal growth at 4 - 5.1mol L^{-1} (20- 30%) of Nacl. These extreme halophiles have special characteristics compare with other halophiles, it has purple membrane which allows phototrophic growth and it contains sensory rhodopsins responsible for phototactic response. Halotolerant is a organism which has an ability to grow in absence of salt and it can also grow with high salt concentration. [3]

Very few Eukaryotic Halophiles can withstand high salt concentration, example *Tilapa* species. Some of the halophytic plants can survive at moderate salt concentration E.g. *Atriplex halimus* and *Mesembryanthemum crystallinum*. Even some of the rotifiers can survive at hypersaline environments, E.g. *Brachionus angularis, Macrostomum* species, *copecods and ostracods.* These above mentioned species are multicellular eukaryotic Halophiles. An alga which has optimal growth in moderate salt concentration, it utilizes organic solutes and amino acids to maintain their osmotic balance example *Dunaliella salina*. Diatoms are algae which also grows in hypersaline environments. *Amphora caffeaeformis, Nitzschia and Navicula* species are algae utilizes proline and oligossacharides to maintain osmotic balance, but still need to study extensively. Some of the fungi are chemoheterotrophic cell walled eukaryotes also have a capability to grow at hypersaline environment example *Polypaecilum pisce* and *Basipetospora halophilia.* [3]

Prokaryotes have ability to tolerate (grow) at hypersaline environment. Cynobacteria is prokaryotes can grow at high salt concentration from 2-5 mol L^{-1} of NaCl which considered as extreme halophiles. It utilizes glycine betaine as organic solute example *Aphonethece halophyt*ica which is unicellular cyanobacteria. Also filamentous cyanobacteria can grow in hypersaline environment at the concentration of 1 - 2.5 mol L^{-1} Nacl, example *Oscillatoria neglecta, O.limnetica, O.salina* etc, these are moderate halophiles.

Phototrohic bacteria which grow anaerobically has capability to adapt at hypersaline environment, it includes purple – sulfur bacteria, green- sulfur bacteria and non – sulfur bacteria. Green sulfur bacteria are moderate halophilic which utilizes trehalose and glycine betaine for osmotic balance; it is also slightly thermophilic example *Chloroflexus aurautiacus and C.limicola.* Halophilic purple sulphur bacteria deposits slufur granules responsible for floating, there are both moderate and extreme Halophiles example *Thiocapsa sucrose halophilia* is a moderate Halophiles which synthesize sucrose by utilizing glycine betaine, It also synthesizes glycine betaine and N-acteylglutaminylglutamine amide for osmoprotection. *Ectothiorhodospira halochloris* is an extreme halophiles use to synthesize ecotine and it utilizes glycine betaine and trehalose as organic solutes. [3]

2.2 Halophilic Fermentative bacteria

The order Haloanerobiales currently contains 25 fermentative species, all the halophilic fermentative bacteria are gram negative bacteria except one called Hb. tunisiense but the cell wall is gram negative. Halophiles has optimal growth at the temperature of 35°C to 45°C and maximum pH range for growth between 7 to 8. The first work was reported in 1977, Haloferax volcanii which has isolated from Dead Sea mud. Till now 12 haloarchaeal genomes have been sequenced, they contain one main chromosome and megaplasmids. This haloarchaeal genome contains high G+C content around 65% [2]. The phylogeny tree shown in Figure 1 shows types of halophilic fermentative bacteria and shows the relation of these bacteria compare to other non-fermentative bacteria, these comparison is based on the similarities of 16s rDNA sequence, which are commonly found in bacteria. [1]

In extreme conditions, every organism follows some mechanism to control intracellular metabolic pathways in order to survive. Halophilic fermentative bacteria accumulate metabolites through salt in, salt out strategies to maintain living condition. Halophilic organisms have better growth in starch rich medium where starch molecules were used as substrate for maintaining cellular metabolism. In recent studies, photosynthetic fermentation of halophilic bacteria in starch rich medium significantly noted for hydrogen production [7] and glycerol substrate medium also had better hydrogen production along with some end metabolites like acetate, 1,3 propanediol from anaerobic fermentation[4].

As glycerol will be soon considered as waste product from biodiesel industry, cost of glycerol may decrease in the future which opens a hopeful way to use glycerol as a substrate for hydrogen production. Moreover, hydrogen has already gained the expecting next generation fuel for all purpose. Increasing number of studies on halophilic fermentation may lead us to find more useful end products which support industries, technological or knowledge development. [4]

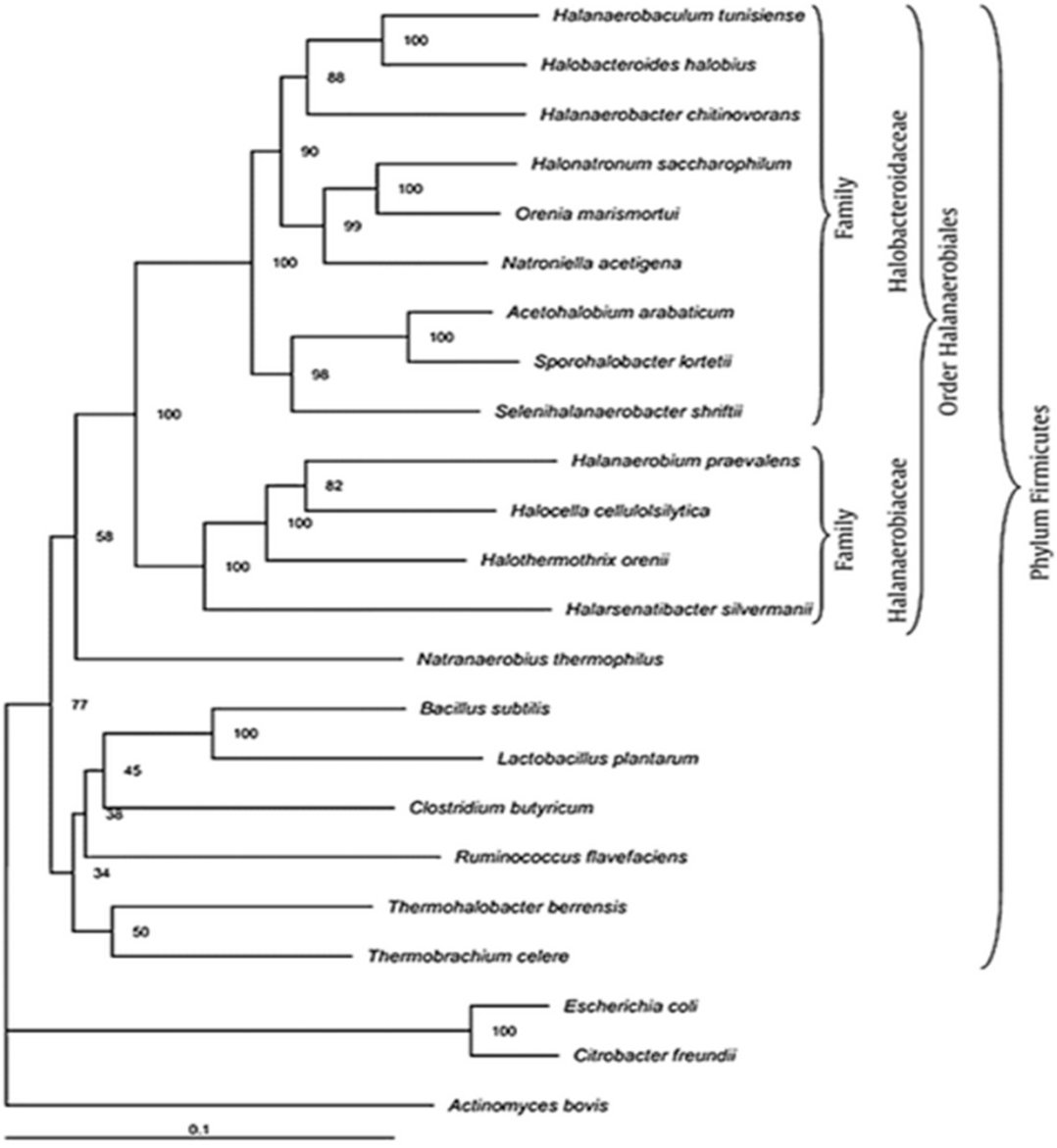

Figure 1. *Phylogeny tree shows types of halophilic fermentative bacteria and also relations with other non- fermentative bacteria. [1]*

2.3 Halophiles

Halophiles grow anaerobically and it has optimal growth at 0.5M to 2.5M of salt concentration. It is a gram negative bacteria, used for fermentation of glycerol and hydrogen production found in sediments of hypersaline lakes. Most of the Halophiles exist in rod, disc, triangular and square shape, but most of them are rod shape, it has maximum growth at pH range 7.3 to 7.4. [4] The proteins of halobacteria requires high salt for activity, negative charge at the surface is important for attraction and dissolution of halophilic proteins and it also prevents denaturation (lysis). The membrane of halobacteria is purple membrane contains two dimensional crystalline pattern of chromoprotein and bacteriorhodopsin. Halorodhopsin contains light driven chloride pump and bacteriorhodopsin contains protein which is light driven proton pump, these membrane proteins used for ATP synthesis, shown in Figure 2. Halobacteria also produces red - orange carotenoids and gas vesicles. Carotenoids responsible for repair of photorepair system and retinal produced from cleavage of carotene is responsible to mediate the phototactic response by using retinal protein bacteriorhodopsin and halorhodopsin. Gas vesicles which have low solubility of oxygen make cells float at surface layer and it also shows purple membrane mediated photophosphorylation. [3]

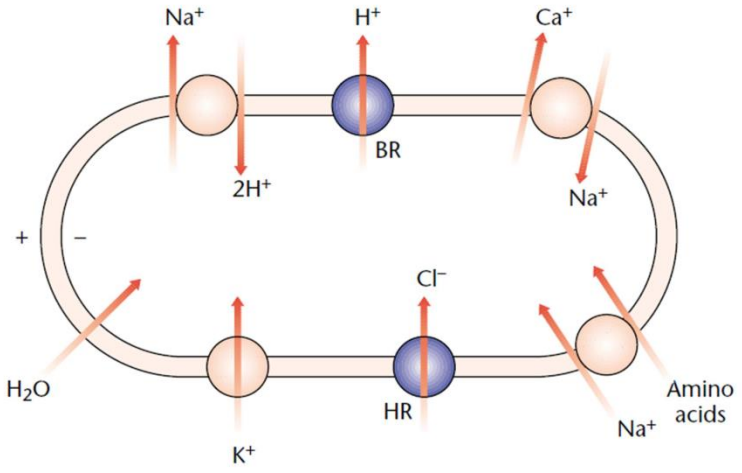

Figure 2. *Membranes of halobacteria, BR (Bacteriorhodopsin) light driven proton pump and HR (Halorhodopsin) light driven chloride pump. The directions of amino acids, water are indicated by arrows. Also charges across the membranes are indicated (+) outer and (-) inside. [3]*

Based on the substrates utilized there are three different fermentative routes of metabolism; 1) usage of carbohydrates (glycerol) produces lactate, 1,3 propanediol and butyrate, but not all strains. 2) utilize amino acids as substrate and produces organic acids, CO_2 and NH_3. 3) Utilizes organic substrates like lactate, ethanol, and glutamate, produce acetate as fermentative products. [1]

2.4 Glucose

Glucose is considered major energy component supporting the life cycle of most beings. In the glycolysis pathway glucose is oxidized to produce pyruvate, ATP and NADH. Industrial production of glucose is mainly done by fermentation of plant materials. Starch rich raw materials like corn, sugarcane are the major raw material for glucose and fructose. Both glucose and fructose are used in food industry as sweetener and aroma. Economically glucose is the best option than other products. In scientific research, glucose has been used for more than a century in studying organisms and its pathways at cellular and molecular level. In the bioenergy research, production of ethanol from hemicellulose raw materials utilizes simple sugars like glucose, xylose and other starch molecules for fermentation. [8]

2.5 Glycerol and 1,3 propanediol

It is a colorless, odorless, viscous polyol compound (Mol. For. $C_3H_8O_6$). However, it had important application in pharmaceutical and food industry application; glycerol has been produced as a waste by product in Bioenergy industry. 1,3 propanediol (Mol for $CH_2(CH_2OH)_2$) application in polymer and pharmaceutical industry. Recently, several studies imply that the conversion of 1,3 propanediol from glycerol through biological fermentation gives better yield. As glycerol is considered as waste in forth coming bioenergy industry, utilization of glycerol for PDA production add on our effort to reduce the use of non-renewable resources. [6]

2.6 High Performance Liquid Chromatography

Chromatography is the method of separating two immiscible phases in order to identify, quantify and purify individual components of the mixture. It was developed by Mikhail Tswett in 1901 when he was working on the isolation of leaf pigments. Progressively, column based chromatography was developed in the 1920s for industrial purpose to separate sucrose and fructose. HPLC is the highly advanced method of column chromatography, which applies pressure to pull the solvent against gravity to make the process faster. The sample is mixed with solvent under certain pressure which moves into the HPLC column. Interaction between stationary phase, analyte and mobile solvent phase facilitates to identify individual compounds. Recent chromatographic advances in transferring analyte with the mobile phase into detector and automated switching functions increases the accuracy and speed of the process. [5]

2.7 Gas Chromatography

In order to study the mixture gaseous substances, gas chromatographic machines are ideally designed with pressurized automated instrument to carry analyzing gas with a carrier gas like helium into the detector. The principles of GC were developed in early 1960s [9], when the tubular column was introduce, after that series of studies done to increase efficiency and productivity [10]. GC is designed to measure the compounds which vaporize even at low temperature; the machine employed perforated temperature through the measure. Other than the gaseous phase, it has similarity with column chromatography where the carrier solvent gas carries analyte gas into the detector. The detection is made through the interaction between stationary phase mainly made up of zeolite, silica gel or activated alumina with mobile phase. Optimization of GC is done in injection methods, temperature control, column length, detector sensitivity etc. [10]

2.8 Antibiotic resistance of Halaneobium saccholuticum subsp. saccholyticum.

Antibiotics are typically natural compounds that either annihilate or inhibit bacterial growth, However, due to wide use and/or misuse of these drugs over prolonged interval, the infectious organism, in this case the bacteria adapts to the antibiotic, thus rendering the drug less effective. This ability of the bacteria to survive, multiply, and/or resist the effects of the drug is termed as antibiotic resistance. This is of great significance considering that antibiotic resistant bacteria require usage were expensive and sometimes toxic, which in certain instances may result in severe disability or death. The antibiotic resistance exhibited by one such bacteria *Halanaerobium saccharolyticum* subsp. *saccharolyticum* is discussed in this chapter. *Halanaerobium saccharolyticum* subsp. *saccharolyticum* exhibits resistance towards at least three antibiotics featuring on the 18th WHO Model List of Essential Medicines (2013), namely, chloramphenicol, kanamycin and ampicillin, as presented below.

2.8.1 Chloramphenicol

Chloramphenicol, or chloromycetin as it was referred, is a broad spectrum antibiotic isolated originally from *Streptomyces venezuelae* in 1947 [11]. The structure of the antibiotic is remarkable, for it was the first naturally occurring substance described to contain a nitro group, the p-nitrophenyl group at C1, besides the N-dichloroacetyl substitute at C2, attached to 1,3 propanediol [12]. Owing to the relative simplicity, chloramphenicol has been exclusively produced and marketed as a product of chemical synthesis since the 1950's. It is highly stable, and can be stored at room temperature for prolonged interval. [13]

Chloramphenicol remains unionised and amphiphilic at physiological pH, and penetrates cell membrane to target intracellular bacteria [12] [14] [15]. It is highly specific, and inhibits bacterial protein biosynthesis by preventing protein chain elongation. This bacteriostatic activity of chloramphenicol happens through reversible binding to the peptidyl transferase centre at the 50S subunit of the 70S ribosomes.

Bacterial resistance to these inhibitory effects of chloramphenicol developed over time, and is most frequently attributed to the enzymatic inactivation through acetylation via several chloramphenicol acetyl transferases [16]. However, several other mechanisms such as, inactivation of phospotransferases, target site mutation, efflux systems, and permeability barriers have also been reported [12] [17].

Despite being a broad spectrum antibiotic, the application of chloramphenicol in human medicine has been restricted to a small number of life threatening infections due to adverse side-effects, such as dose-related bone marrow suppression, or grey syndrome in infants and neonates [12]. Dose-unrelated irreversible plastic anaemia is observed at frequencies 1:10,000 - 1:40,000 or 1:20,000 - 1:600, 000 [14]. Besides, chloramphenicol hypersensitivity ranges from skin rash to anaphylaxis [15].

2.8.2 Kanamycin

Kanamycin is an amino glycoside antibiotic complex isolated around 1957, from *Streptomyces kanamycetius*, and encompasses a family of three antibiotics, kanamycin A, B, and C. However, it is the disulfate salt of kanamycin A, a broad spectrum antibacterial agent that is most widely used and studied [18] [19]. Dose dependant side-effects such as ototoxicity and nephrotoxicity have been reported similar to most amino glycosides. [20]

2.8.3 Ampicillin

Ampicillin, originally referred as penbritin, is a semi-synthetic penicillin, that comprises a thiazolidine ring attached to a β-lactam ring, which is modified by a benzene side chain with an additional amino group [21]. This amine group increases the hydrophilicity, and renders it permeable to the porins in the outer membrane of certain gram-negative bacteria. Unlike the other broad spectrum antibiotics, which are usually limited in their application by the untoward side-effects, ampicillin is generally non-toxic [22].

2.9 Efflux pumps in antibiotic resistance

Efflux pumps are transport proteins drives out the toxic substances, also some antibiotics within cells, these transport proteins available in both gram positive and gram negative bacteria. [23] Gram negative bacteria are more resistant than gram positive towards antibiotics because of intrinsic resistance of gram negative bacteria. Intrinsic resistance of bacteria is due to outer membrane which is responsible for slow penetration of halophilic solutes and reduces the rate of diffusion of solutes.

Multi drug efflux pumps plays important role in intrinsic resistance of bacteria. In gram negative bacteria multi drug efflux pumps helps to pump out toxic substances or antibiotics from cytoplasm into periplasmic space or outer membrane layer. In gram positive bacteria toxic agents are pumped out till cytoplasmic membrane so there are possibilities of agents or antibiotics to diffuse back through lipid bilayer domain in cytoplasmic membrane. [24]

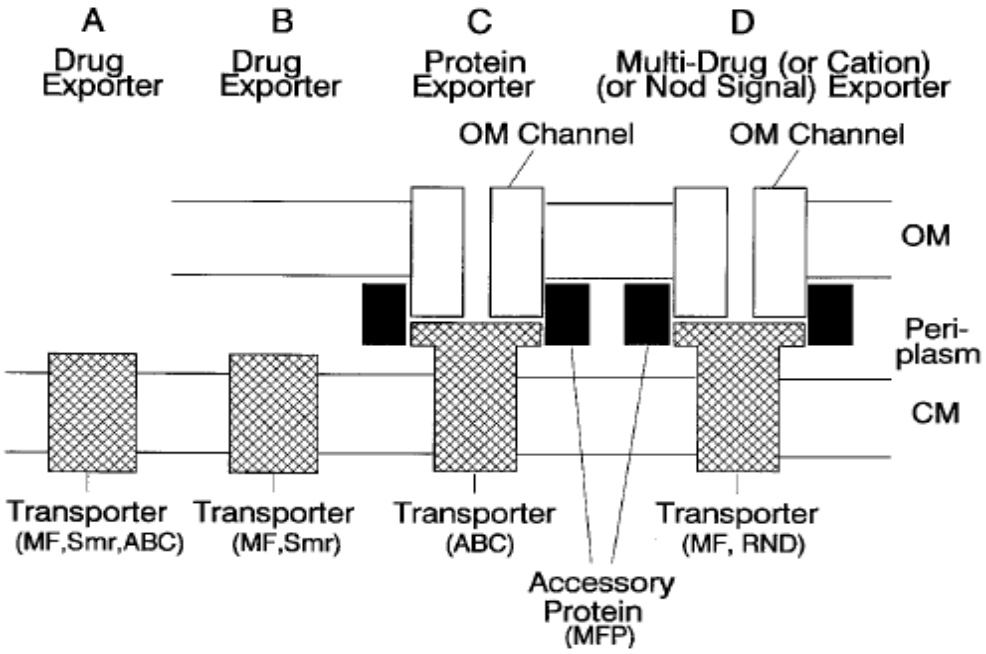

Figure 3. *Efflux Pumps (A) & (B) Single component pump in gram positive bacteria and gram negative bacteria respectively (C) Protein exporter with Transporter, Accessory and outer membrane proteins (D) Multi drug efflux pump with similar system as protein exporter in addition pumps different range of drugs.[24]*

MDR efflux pumps categorized into five types of efflux pumps 1) ATP Binding Cassette (ABC), 2) Major Facilitator Superfamily (MFS), 3) Multi drug and Toxic compound Extrusion (MATE) 4) Small Multi-drug Resistance (SMR) and 5) Resistance Nodulation Division (RND). The above multi drug efflux systems in general consists of transporter protein, membrane fusion protein and outer membrane proteins which plays vital role in excretion of drugs. These categories are based on the transporter proteins, number of components in pump and substrates. Figure 3 shows different types of efflux pumps (A) In Gram positive bacteria; Single component pump catalyzes the drugs through Cytoplasmic Membrane (CM) (B) Single component pump in gram negative bacteria catalyzes the drugs and transport to periplasm (C) Protein exporter consist of transporter protein (ABC) and MFS which links Outer membrane (OM) (D) Complex system of Mutli drug efflux pump in gram negative bacteria, MF and RND are the transporter proteins, different range of drugs are catalyzed and pumped out through this system, otherwise its similar to protein exporter. [24]

Gram negative bacteria are mostly multicomponent multi drug efflux pumps; RND family is such system belongs to MDR, pumped by proton motive force by which hydrogen ion mediates in transporting drugs. Transporter protein in inner membrane of the bacterium binds with drugs (antibiotics) in phospholipid bilayer and drives to outer medium by outer membrane protein, both the proteins are facilitated by membrane fusion protein. [25]

2.10 Mutagenesis

Mutagenesis is the process of breaking or repairing or rearranging of genetic information in gene, It can be either natural or artificially in laboratory by using mutagens. Common mechanisms of mutagenesis are DNA damage, mismatches in DNA bases, DNA repair and chemical damages. In general DNA base pair are A-T (Adenine pairs with thymine) and G-C (Guanine pairs with cytosine), when subject to mutation, mismatches occurs For example A-G and T-C. Three possible ways of mutations are Purine –Purine, Pyridimine -Pyridimine and Purine – Pyridimine, chemical damage like deamination of cytosine methylation of guanine. Mutagenesis processes are commonly two different types, Site specific mutagenesis and random mutagenesis. Mutation that makes changes and modify at specific site of gene based on specific gene product considered as site specific mutagenesis. Random mutagenesis allows to makes changes or modify at random site of gene. [26]

2.10.1 Hydroxylamine: chemical mutagen

Mutation includes changes in DNA base pair, in such hydroxylmine (HA) is an inorganic compound used as a chemical mutagen to repair the DNA bases. HA reacts only with cytosine and 5-hydroxymethyl cytosine but not with thymine. Hydroxylamine causes point mutation for example purine to purine or pyridimine to pyridimine also known as transition. HA in reaction with cytosine breaks the pyridimine ring and affects the resonance form, which eventually damages or repairs the gene sequence. After HA subjected to mutation, Cytosine do not pair with G, because 3° position hydrogen sterically hindered by hydrogen atom in guanine which induces the base pair transition. [27]

2.10.2 UV mutagenesis

Ultraviolet light induces mutation in the cell, by producing toxic effect to the genome and damage in DNA. UV radiation are categorized into three types based on the wavelength UV-A (320-400nm), UV-B (290-320nm) and UV-C (<290nm). UV-A and UV-B are considered as solar UV radiation, it damages DNA by photochemical reaction either at cyclobutane pyrimidine dimers or pyrimidine (6-4) pyrimidone photoproducts. UV-C is germicidal UV radiation affects both cyclobutane pyridimine dimers and pyridimine (6-4) pyrimidine photoproducts. It causes specific types of mutation, by base subsititution of cytosine C→T and CC→TT, these types of mutation known as UV signature. [28] [29]

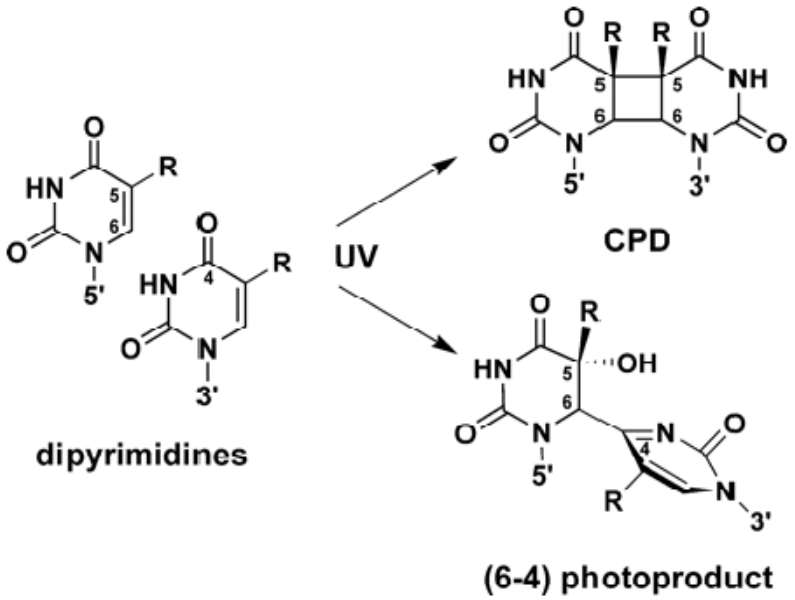

Figure 4. *UV mutation causes damages in DNA by forming dimer between two pyridimine bases. Cyclobutane Pyridimine Dimer (CPD) and (6-4) photoproduct are the UV products formed in general after subject to UV mutation [30]*

2.11 Natural Transformation

Natural transformation encompasses transforming of genetic materials to competent (Bacterial) cells in natural habitats. Compare to conjugation and transduction gene transfer methods, transformation method has minimum criteria. In conjugation gene transfer method, both donor and recipient cells must be in metabolically active to transfer DNA and other genetic information. In transduction both donor and competent cells must be related to transfer DNA. Natural transformation method enables to transfer DNA if competent cells ready to uptake DNA, the only requirement in transformation method is recipient cells must be biologically active to receive plasmid DNA. [31] Plasmid is an extra chromosomal element of circular DNA contains many genes, also used as vector to insert foreign DNA into competent cells. Origin of replication, cloning site and resistance gene (Selectable marker) are the three components of plasmid assists in transformation of Plasmid DNA, it enter the competent bacteria through pores in cell membrane or using chemical. Transformed bacteria can be isolated by agar plating method containing antibiotic, therefore bacteria with transformed plasmid with particular antibiotic resistance grows in agar plate, based on the concentration of plasmid the transformation efficiency differs. [32]

2.12 Sceening of Mutants

Apart from mutagenesis, the most important task is to identify the mutants. There are several methods to screen the mutants PCR, Replica plating method, blue white screening method etc. Specific to this work replica plating method was used to screen the mutants due to most consistent method to screen antibiotic sensitive mutants.

2.12.1 Replica plating method

Replica plating method is a screening method of antibiotic sensitive mutants. [33]. The three stages in this method i) formation of colonies on agar medium, were plates exposed to mutagenic agent considered as Master plate ii) Using pad with sterile velveteen to stamp or make replica from master plate to medium with antibiotic or different medium and iii) comparing master plate with other replica plates, colonies which are missing in replica plates considered to have possible mutants. [34]

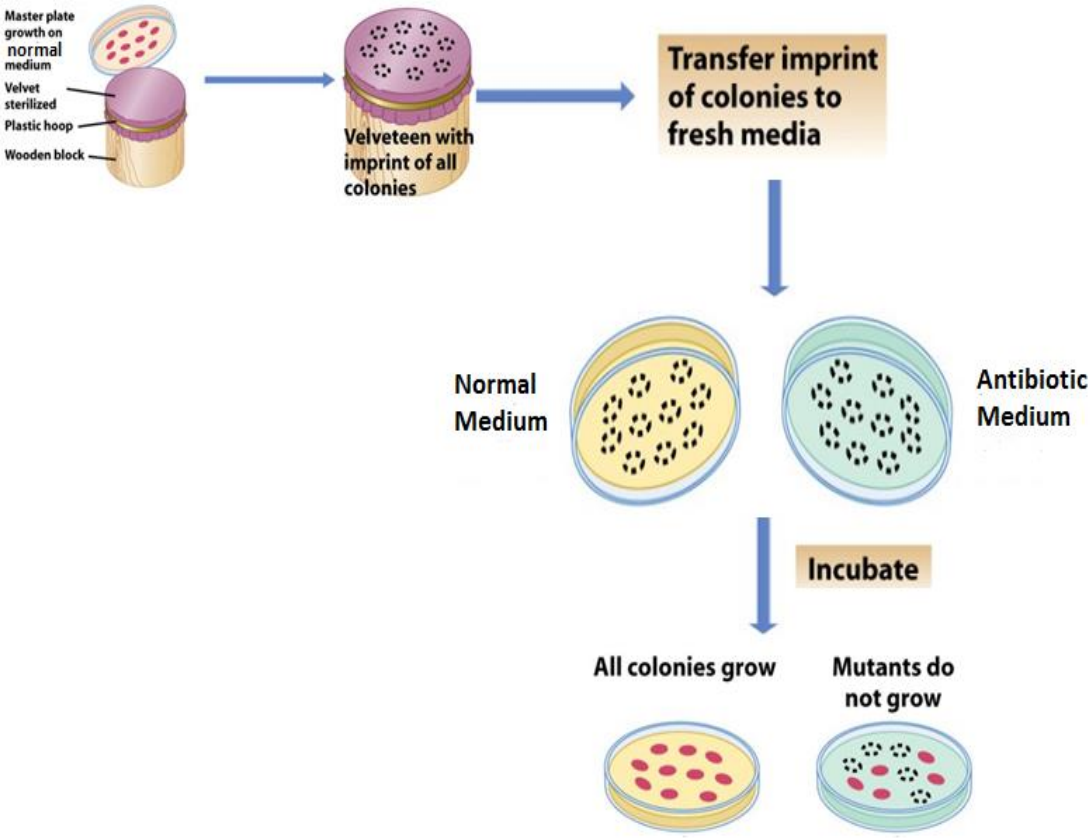

Figure 5. *Replica plate method: Using velveteen stamping master plate, were colonies grown on normal medium (subjected to mutagenesis). Transferring imprints to the normal medium and medium containing antibiotic further incubated at 37 °C until colony formation. Colonies that are not seen in antibiotic medium considered as mutants [35]*

3. Materials and methods

3.1 Strain and chemicals used

Halanaerobium saccharolyticum subsp. Saccharolyticum strain DSM 6643 was from German collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany). E.coli strain KRX was from Promega Corporation USA. Clostridium butyricum strain DSM 2478 from hydrogen producing bioreactor [41].

Ammonium chloride, Potassium chloride, Na-acetate x 3H2O, Bacto Tryptone was purchased from Becton Dickinson & Co (NJ, USA), Sodium Chloride from VWR International (West Chester, PA, USA), Potassium dihydrogen phosphate and dipotassium hydrogen phosphate from Mallinck-rodt Baker (Phillipsburg, NJ, USA), L-Cystine HCl.H2O from Sigma Aldrich, potassium hydroxide, calcium chloride and magnesium chloride from Merck & Co (Whitehouse Station, NJ, USA).

3.2 Preperation of medium and culture conditions

H. saccharolyticum subsp. saccharolyticum was grown in halophilic (HM100) medium consisting of 1g/l NH4Cl, 4g/l KCl, 0.5g/l Na acetate x 3H2O, 2g/l Bacto-Tryptone, 150g/l NaCl, KH2PO(50mM) - 2.6g/l, 5.29g/l K2HPO4, 0.6g/l Cystine HCl monohydrate, 0.002g/l resazurin, vitamins (biotin 20µg/l, folic acid 20 µg/l, pyrisoxine-HCl 0.1 mg/l, thiamine-HCl.2H2O 50µg/l riboflavin 50 µg/l, nicotinic acid 50 µg/l, d-Ca-pantothenate 50µg/l, vitamin B12 5 µg/l, p-aminobenzoic acid 50µg/l, lipoic acid 50 µg/l) and trace elements (HCl 0.077mM, FeCl2 .4H2 O 1.5 mg/l, ZnCl2 0.07 mg/l, MnCl2 . 4H2 O 0.10 mg/l, H3BO3 6 µg/l, COCl2 .6H2O 0.19 mg/l, CuCl2 .2H2O 2 µg/l, NiCl2 .6H2O 24 µg/l, and Na2 MoO4 .2H2 O 36 µg/l) [36]. The rich medium additionally supplemented with 16 g/l bacto tryptone and 10g/l yeast extract.

Using 1M potassium hydroxide, pH was adjusted initially to 7.4 and the medium was boiled for 10 minutes. The medium was purged with N2 and transferred into tubes (10 ml of medium in 25 ml test tube). The tubes were purged with N2 for 10 minutes, sealed with rubber cork and aluminum caps and sterilized at 121°C (autoclaved). Potassium hydroxide (1M & 0.2M), 40% of glucose, CaCl2. MgCl2 and empty tubes were purged with N2 and autoclaved. The final concentration of glucose (substrate) in the medium was 5 g/l. Vitamin, glucose and trace elements are added after autoclavation. All the samples in experiments were in triplicates and negative controls, i.e. without any substrate, were included. Preculture was prepared at 37ºC 150rpm for 72h from pure H. saccharolyticum subsp. saccharolyticum and used 1 % (v/v) for inoculation. Throughout the experiment incubation condition was maintained at 37°C at 150rpm.

3.3 Analysis

3.3.1 Growth rate

Substrates and other stock solutions were added into the medium after autoclavation using sterile and anaerobic techniques. Optical density (OD600) was measured with an Ultraspec 500 pro spectrophotometer (Amersham Biosciences, Munich, Germany) to check the growth rate of bacteria. The experiments were done in triplicates in addition negative controls were used i.e. without any substrates.

3.3.2 Gas concentrations

Hydrogen and carbon dioxide contents were measured using gas chromatography GC-2014 Shimadzu with thermal conductivity as a detector, Nitrogen as carrier gas and the temperature of column and detector were 80°C and 110°C respectively. Gas Chromatography analysis were made by sterilizing the top of bottle before taking gas samples, over pressure and under pressure were checked and purged with N2 for 10-15secs. After analysis samples were stored into incubator at 37°C and 150rpm. Each day the Gas samples, liquid samples and growth rates was continuously analysed. The total volume of each gaseous end product calculated by (mass balance equation),

$$V_{product,t} = V_{product,t-1} + \frac{V_{GC,t} * (V_{total,t} - V_{total,t-1}) + V_{Head}(V_{GC,t} - V_{GC,t-1})}{V_{sample}}$$

$V_{product,\ t}$ is a cumulative volume of gaseous fermentation product (hydrogen or carbon dioxide) produced at time t and $V_{product,t-1}$ at time t-1 (previous measurement); $V_{GC,t}$ is volume of product in sample measured gas chromatographically at time t and $V_{GC,t-1}$ at time t-1; ($V_{total,\ t}$ -$V_{total,t-1}$) is total gas volume produced (measured with needle and syringe) between time points t and t-1; V_{Head} is volume of the gaseous phase in sealed batch bottles or tubes; and V_{sample} is volume of the sample injected into GC analysis. The amount, n [mmol], of gaseous fermentation product can be calculated at each time point using ideal gas law in which the volume is the $V_{product,\ t}$ (calculated from above equation).

The cumulative production (concentration) [mmol/l culture] of gaseous fermentation products at time point t calculated using,

$$c_{product,t} = \frac{n_{product,t}}{V_{liquid}} - \frac{n_{product,control,t}}{V_{liquid}}$$

$C_{\ product,\ t}$ is concentration of gaseous fermentation product at time point t; $n_{\ product,\ t}$ is amount of gaseous substance in moles at time point t; $n_{\ product,\ control,\ t}$ is amount of gaseous product in control (without substrate) at time point t; $V_{\ liquid}$ is volume of the liquid phase. Hydrogen and carbon dioxide yields [mol/mol substrate] are calculated using

$$Yield = \frac{c_{product}}{c_{substrate\ \ used}}$$

3.3.3 Liquid concentrations

Metabolites were analysed using High Performance Liquid Chromatography (HPLC) method through liquid samples. Initially samples were centrifuged at 10000 g for 5 mins and supernatant was used and diluted 10 times (100µl of samples with 900µl of MQ) and then filtered using polycarbonate filter (Chromafil 194 195 PET-45/25, Duren, Germany) and transferred into HPLC bottles. The samples was analysed for glucose, glycerol and 1,3 – propanediol at a column temperature of 54°C, LC-20AC (Shimadzu, Kyoto, Japan) liquid chromatography equipped with an RID-10A refractive index detector, DGU-20A5 degasser, CBM-20A communication bus module and SIL-20AC 200 autosampler and 30cm Shodex SH1011 column. 0.01N H_2SO_4 was used as an eluent at a flow rate of 0.600ml/min.

The metabolites are analysed based on the retention time and concentrations of it and yields are calculated. The concentration of liquid fermentation products were calculated as follows,

$$c_{product} = d_f * (c_{product,HPLC} - c_{product,control,HPLC})$$

$C_{product}$ is concentration of a liquid fermentation product; d_f is dilution factor used in preparation of HPLC sample (10); $C_{product, HPLC}$ is concentration of a liquid fermentation product in HPLC sample; and $C_{product, control, HPLC}$ is concentration of a liquid fermentation product in control HPLC sample. The yields of liquid fermentation products were calculated as follows based on the concentration of product produced ($C_{product}$, [M]) and the concentration of substrate utilized ($C_{substrate\ used}$, [M]).

$$Yield = \frac{c_{product}}{c_{substrate\ \ used}}$$

3.4 Random mutagenesis

Mutagenesis was performed by two different methods. Physical method by subjecting samples to UV radiation and chemical method using chemical mutagen called Hydroxylamine.

3.4.1 Physical method: UV mutagenesis

H. saccharolyticum subsp. *saccharolyticum* were allowed to grow as liquid culture until mid-exponential phase was attained. Different dilution factors (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were used along with controls (without subjecting to UV). Agar plates were prepared of 16 g/l tryptone, 10 g/l yeast extract, 16 g/l agar, 150 g/l NaCl, 0.6 g/l cystine HCl and 1 ml/l resazurin. Prepared agar medium was boiled for 10 minutes and purged with N_2 for few minutes. Further autoclaved at 121ºC and transferred into Nitrogen chamber in anaerobic condition, were 25ml of medium was used in each plate and 0.1 ml of each dilution factors was spread in agar plate.

Before using UV radiation, UV chamber was warmed up for 30 minutes. Agar plates were subjected to UV radiation at a distance of 12.5cm from UV lamp 25 W [37]., Several exposure times 10 seconds, 30 seconds, 1 minute, 3 minutes and 5 minutes were used. In order to avoid photo reactivation, experiments are done in the dark region and immediately transferred to the black box. The plates are incubated at 37ºC for several days until colony formation. [38]

3.4.2 Chemical method: Hydroxylamine

H. saccharolyticum were grown in liquid culture until mid-log phase was attained. Culture of 5 ml was centrifuged at 10000rpm for 15 minutes, were 1 ml of sample was suspended into 4ml of fresh medium. Suspended culture was exposed to the chemical mutagen hydroxylamine, were the final concentration was 0.25 M.

Further samples were incubated at 37 ºC, 150 rpm for different time intervals 30, 60, 90, 120 and 150 minutes. To stop the mutagenic treatment at specific time interval, exposed

samples was centrifuged at 10000rpm for 5 minutes and wash with fresh medium, to prevent further mutagenic reactions wash the treated samples twice with fresh medium [39]. Different dilution factors (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of bacterial suspension were used in agar plates along with control i.e. without exposing to hydroxylamine. Medium prepared for agar plating contains 16 g/l tryptone, 10 g/l yeast extract, 16 g/l agar, 150 g/l NaCl, 0.6 g/l cystine HCl, 1 ml/l resazurin and pH 7.4 were adjusted initially using 1M of KOH. Prepared agar medium was boiled for 10 minutes and purged with N_2 for few minutes. Further autoclaved at 121ºC and transferred into Nitrogen (N_2) chamber in anaerobic condition, were 25ml of medium was used in each plate and 0.1 ml of each dilution factors was spread in agar plate.

3.5 Natural Transformation of pMDS133

Transforming of plasmid into natural habitat of competent cells called as natural transformation. Halanerobium Saccharolyticum subsp. Saccharolyticum (competent cells) were grown until mid-log phase at 37° C at 150rpm. Plasmid pMDS133 contains novobiocin resistance gene was used in transformation. Different concentrations 100ng, 300ng, 500ng and 1000ng of pMDS133 plasmid inoculated into 10 ml of culture tubes, and incubated at 37°C at 150 rpm for 4 hours. Finally 100 µl of cells from each culture tubes were used in agar plating, with different concentrations of novobiocin 0.6 µg/ml, 1 µg/ml and 3 µg/ml and incubated at 37°C for formation of colonies. Control culture will be handled just as the transformed cultures, however without addition of plasmid. Compare the transformed culture to the control plate in order to obtain transformants. To calculate transformation efficiency following formula was used. [42] [43]

$$\text{Transformation Efficiency} = \frac{\text{Total number of colonies (CFU)}}{\text{amount of DNA plated in } \mu\text{g/ml}}$$

3.6 Screening of mutants

Replica plating methods were used for screening of antibiotic sensitive mutants. The plates exposed to mutagenic reaction called as master plates, two types of plates were used, normal medium (without antibiotic) and with 1µg/ml novobiocin antibiotic. The normal and antibiotic plates were inoculated by stamping using master plates as templates. For stamping, marks were made on both the master plates and target plates and stamped location wise using sterile velveteen cloth. Repeat the same step for all the master plates, and incubate at 37 ºC until formation of colonies. Based on the position of master plates and target plates mutant colonies are identified. Further inoculated into liquid medium and checked for novobiocin antibiotic sensitivity. [40]

4. Results

4.1 Growth of H. Saccharolyticum subs. Saccharolyticum with high concentration of novobiocin and chloramphenicol

H.saccharolyticum subs. Saccharolyticum grown in medium (without yeast extract), growth was measured using Ultra spec 500 spectrophotometer. Different concentrations of novobiocin 50 µg/ml, 30 µg/ml, 20 µg/ml and 15 µg/ml and 5µg/ml of chloromphenicol antibiotic are used to check the sensitivity of bacteria. Figure 6 shows that 15 µg/ml is higher concentration, which inhibits the growth. However all the samples have small growth in initial phase, Control without antibiotic has obtained 0.445 maximum OD value compare to other samples. Experiments repeated several times and based on the results, decided to continue with low concentration of novobiocin.

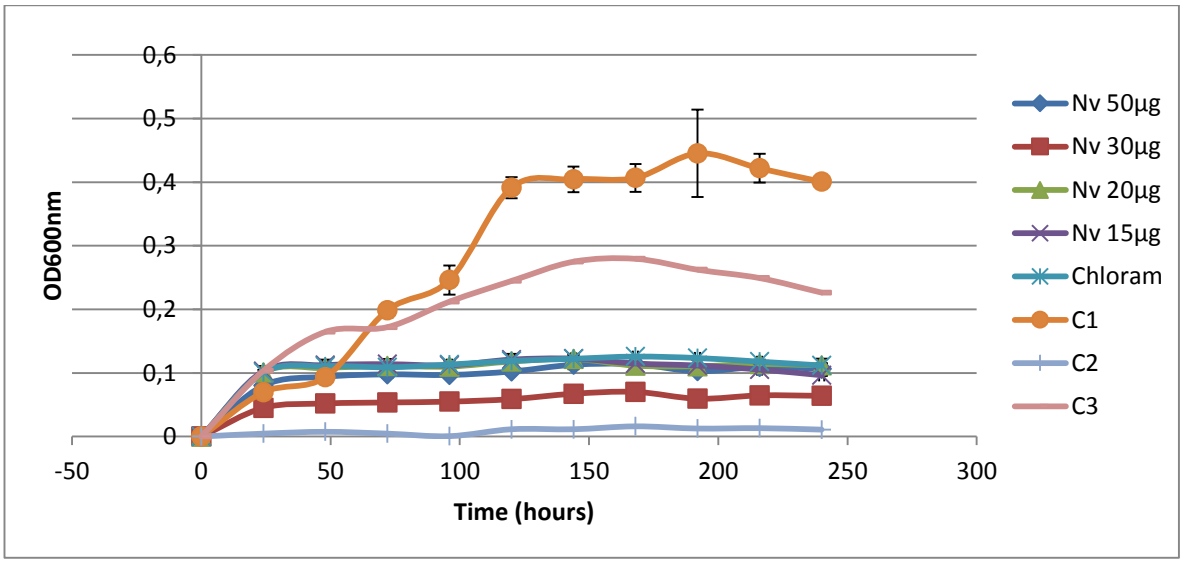

***Figure 6**. Growth curve of Halanerobium Sacchrolyticum subspc. Sacchrolyticum with novobiocin and chloramphenicol. Three different controls were used C1 Control without novobiocin & chloramphenicol, C2 is negative control without bacteria and C3 control without glucose (substrate).*

4.2 Ampicillin and Kanamycin antibiotics resistant to H. Saccharolyticum subsp. Saccharolyticum

H. saccharolyticum resistance to Ampicillin and Kanamycin antibitotics, 100µg/ml of ampicillin and kanamycin antibiotics are used in growth of H. Sacchrolyticum. In figure log phase of kanamycin from 24hours after inoculation were the growth is similar to control 1 (without antibiotic) however growth with Ampicillin initiated after 48 hours of inoculation. H. Saacharolyticum is resistance to Kanamycin and ampicillin, were maximum growth of 0.527 & 0.395 respectively in minimal medium (Without yeast and 2g/l of bacto Tryptone).

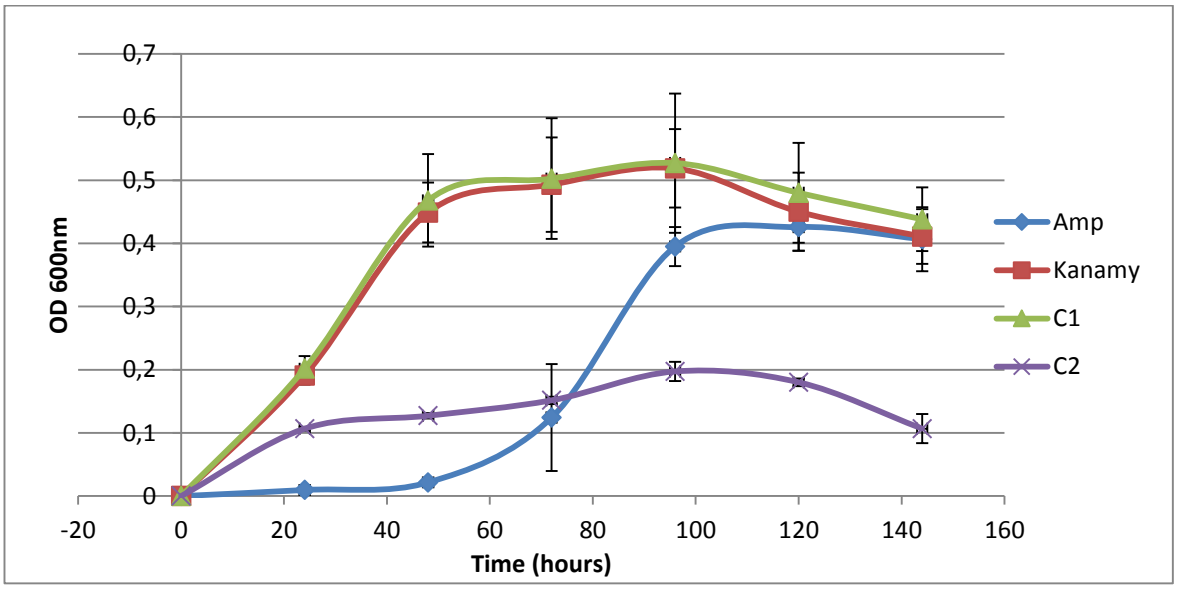

Figure 7*. Gowth curve of H. sacchrolyticum with concentration of 100 µg/ml of ampicillin and kanamycin antibiotics. C1 were control without antibiotics and C2 were control without substrate (glucose).*

4.3 Repetition by including different concentration of novobiocin and chloramphenicol

In the figure 6 the concentration range was high, and since it's not resistance to novobiocin. Experiment repeated with medium, including minimum and maximum concentration of Novobiocin to confirm the growth of H.sacchrolyticum. 10ml of Medium are filled in 25 ml of test tube, throughout the project all the experiments has triplicate and average is calculated. In this experiment range of 10-70 µg/ml of novobiocin and 5µg/ml chloramphenicol included to the previous concentrations. Maximum growth found in h.saccharolyticum with chloramphenicol and in control 1. However the result remains the same, no significant growth in H.sacchrolyticum with novobiocin. 50µg/ml concentration of Novobiocin has growth upto 0.15, perhaps because of flocculation in medium.

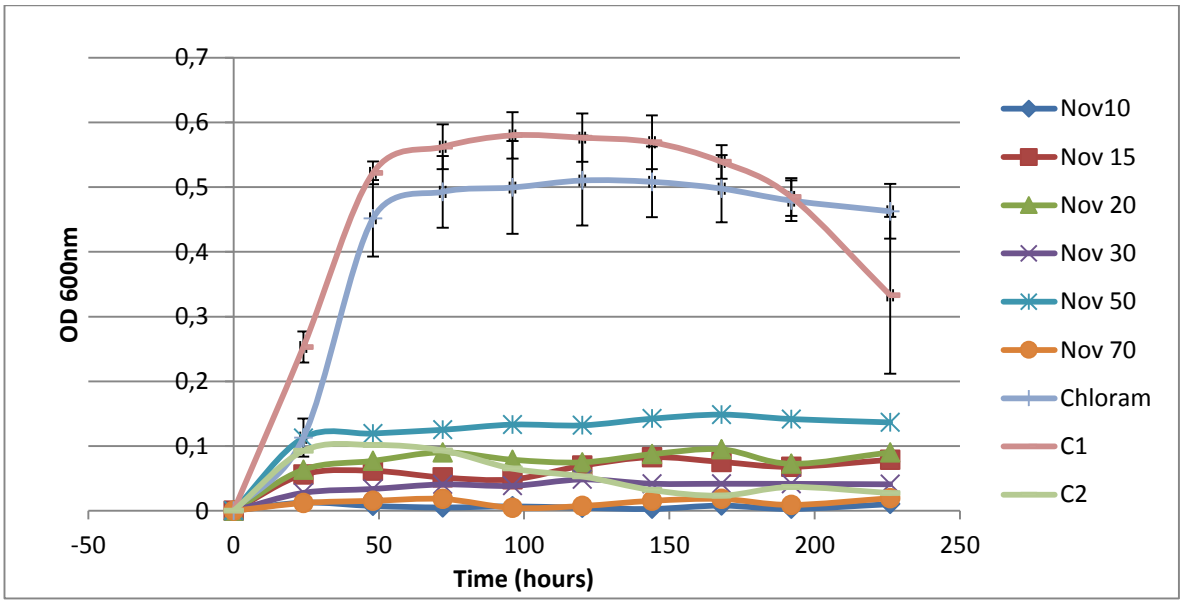

***Figure 8**. Gowth curve of H. sacchrolyticum by including concentration of 10 and 70 µg/ml of novobiocin antibiotics. C1 were control without antibiotics and C2 were control without substrate (glucose) and antibiotics.*

4.4 Rich Medium

Since not significant growth has been found in the previous experiment, used rich medium (Tryptone 16g/l, Yeast extract 10g/l, agar 16g/l, NaCl 150g/l, Glucose10g/l, Vitamin 10ml/l) in addition to old strain (DSM 6643), new halophilic strain used. The figure shows the H.sacchrolyticum is resistance to chloramphenicol in both medium, whereas it is not resistance to novobiocin due to higher concentration, although minimum concentration 15µg/ml of novobiocin is sensitive (non-resistant) which inhibits the growth of H.Sacchrolyticum. Old strain (DSM 6643) with rich medium shows similar results, novobiocin concentration affects the growth of bacteria in both normal and rich medium.

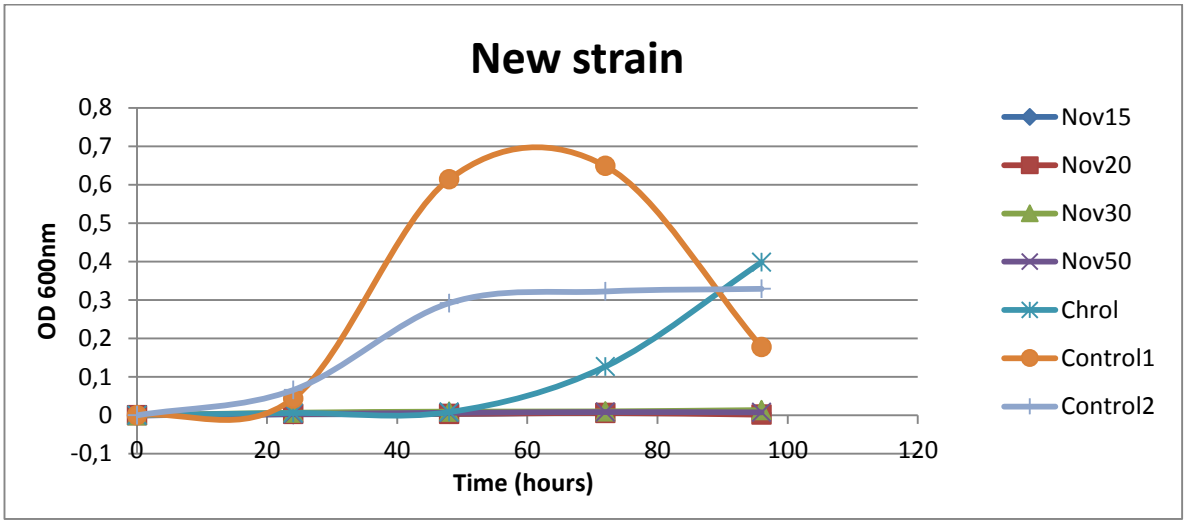

***Figure 9**. Growth curve of (new strain) H. sacchrolyticum with novobiocin antibiotics in rich medium. C1 were control without antibiotics and C2 were control without substrate (glucose).*

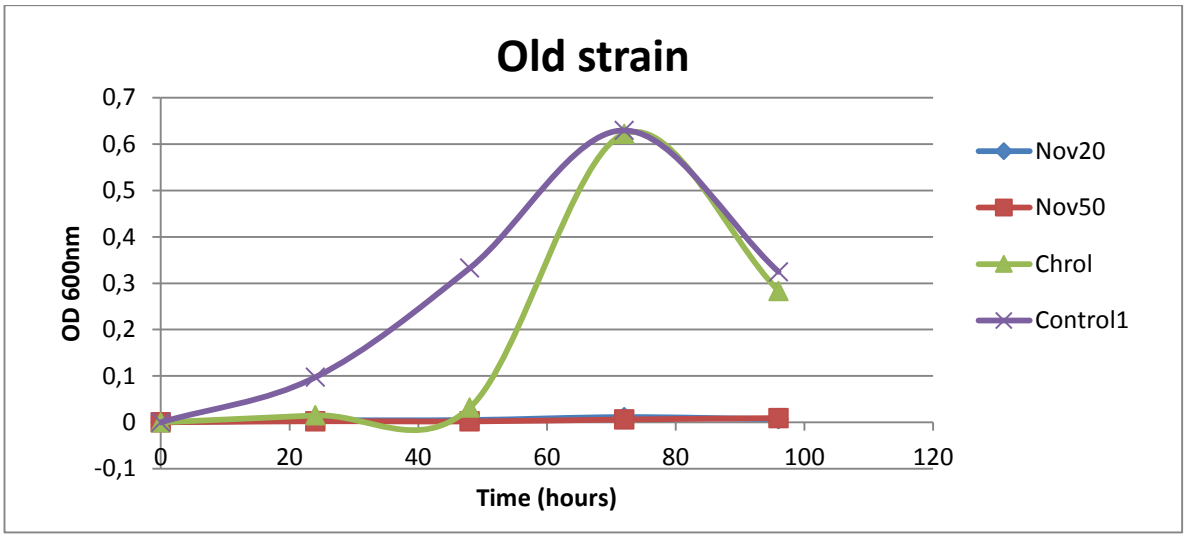

Figure 10 *. Gowth curve of (old strain) H. sacchrolyticum with novobiocin antibiotics in rich medium. C1 were control without antibiotics and C2 were control without substrate (glucose).*

4.5 Comparsion with *Clostridium Butyricum*

Clostridium butyricum (Anerobic , Gram positive) grown in rich medium except Nacl 10g/l to confirm the composition of medium is suitable for the growth of H.sacchaolyticum, since no significant results has been shown in the previous results in favour of novobiocin, its vibrant that either concentration is higher which makes its more sensitive or *H.Saachrolyticum* is not resistance to novobiovin. Figure 11 shows optical density has reached above 0.6 in control without antibitotic, with 15µg/ml of novobiocin maximum OD of 0.116 in rich medium. *Clostridium Butyricum* maximum OD of 0.95 in control, with 15µg/ml of novobiocin 0.76 OD reached, however figure 12 shows exponential growth from log phase and gives essential results to experiment, further able to continue with concentration less than 15µg/ml.

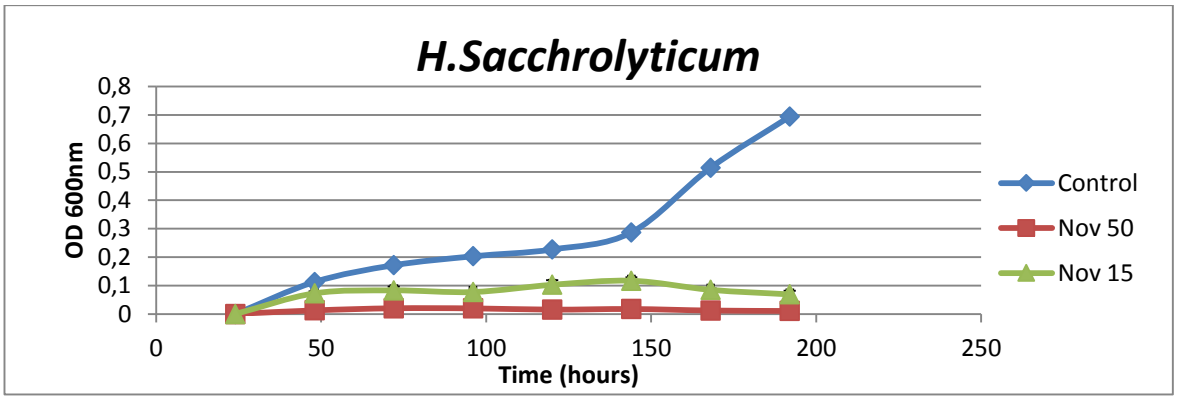

Figure 11. *H. sacchrolyticum with 15 and 50 µg/ml of novobiocin, here control is without novobiocin.*

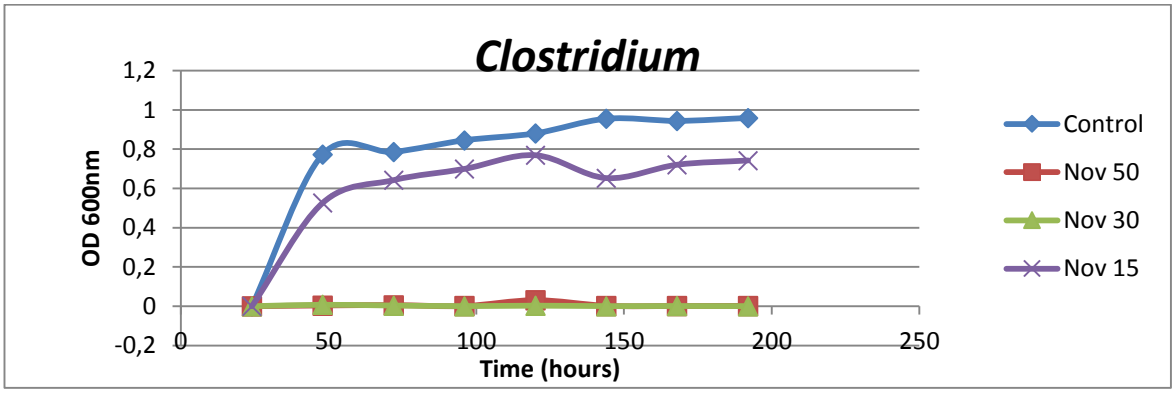

Figure 12. *Clostridium butyricum with 5, 30 and 15 µg/ml of novobiocin, here control is without novobiocin.*

4.6 With low Concentrations of Novobiocin

Concentration of novobiocin in previous experiments are sensitive, inhibits the growth of *H.Sacchrolyticum*. Thus novobiocin concentrations range from 0.3 - 5µg/ml included in this experiment to ensure the growth and resistance of *H.Sacchrolyticum*. Figure 13 shows least concentration of 0.3 µg/ml has OD of 0.563, control without antibiotic has maximum growth of 0.703 also 0.6 and 1 µg/ml concentrations of novobiocin are resistance to H.Sacchrolyticum. Through several repetition of novobiocin concentration range from 1 - 70 µg/ml, this results indicates < 1 µg/ml concentration has more resistance.

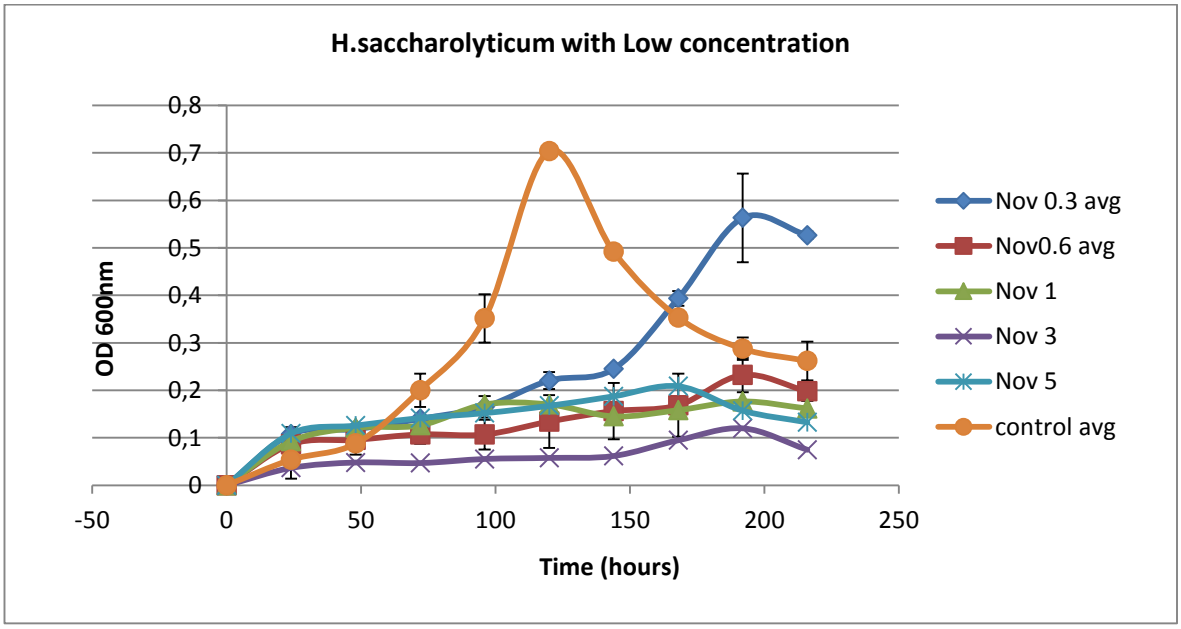

Figure 13. *Gowth curve of H. sacchrolyticum with low concentrations of novobiocin in medium. Control was without antibiotics.*

4.7 UV Mutagenesis

Innoculum of mid-exponential phase of H.Sacchrolyticum with 0.3 µg/ml concentration of novobiocin was diluted (10-1, 10-2, 10-3, 10-4 and 10-5). All the dilution factors were agar plated anaerobically and subjected to UV radiation (25W). Time interval of 10, 30, 60, 180 and 300 seconds agar plates was exposed to UV to obtain prominent colonies. Fig 14 shows that 83 colonies formed in plate exposed for 10 seconds, periodically formation of colonies are reduced to 12, 5, 3, 1 in 30, 60, 180 and 300 seconds respectively. The exposure time of UV affects the formations of colonies and agar plate not exposed to UV considered as control which contains more colonies (112). Further prominent single colonies are picked from each dilution factor using inoculating loop and inoculated anaerobically.

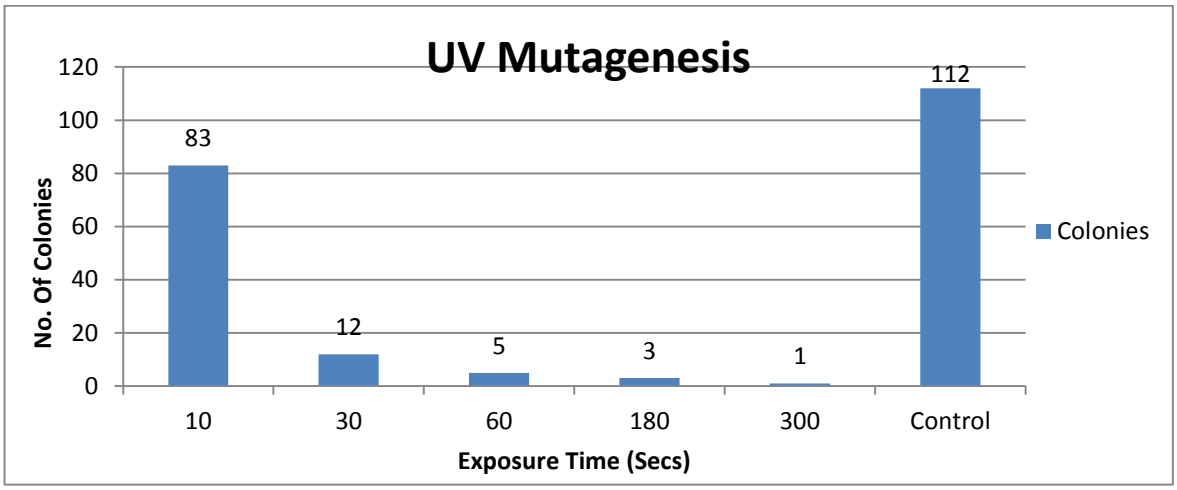

Figure 14. *Colony formations after subjected to UV mutation, here control plate was without exposing to UV.*

4.8 Hydroxylamine

Mid exponential phase of H.Saccharolyticum with 0.3 µg/ml concentration of novobiocin centrifuged and washed using fresh medium. Further incubated for different time intervals (30, 60, 90, 120 and 150 mins), samples from each time interval are exposed to agar plate contains 0.25M Hydroxylamine a chemical mutagen. Anaerobic agar plates are incubated for several days to form colonies. Figure 15 shows that 74 and 31 colonies are formed by sample that exposed by Hydroxylamine for 60 and 90 mins respectively. High exposure time of hydroxylamine (120 and 150 minutes) has suppressed colony formations because of sensitive to moderate halophilic bacteria.

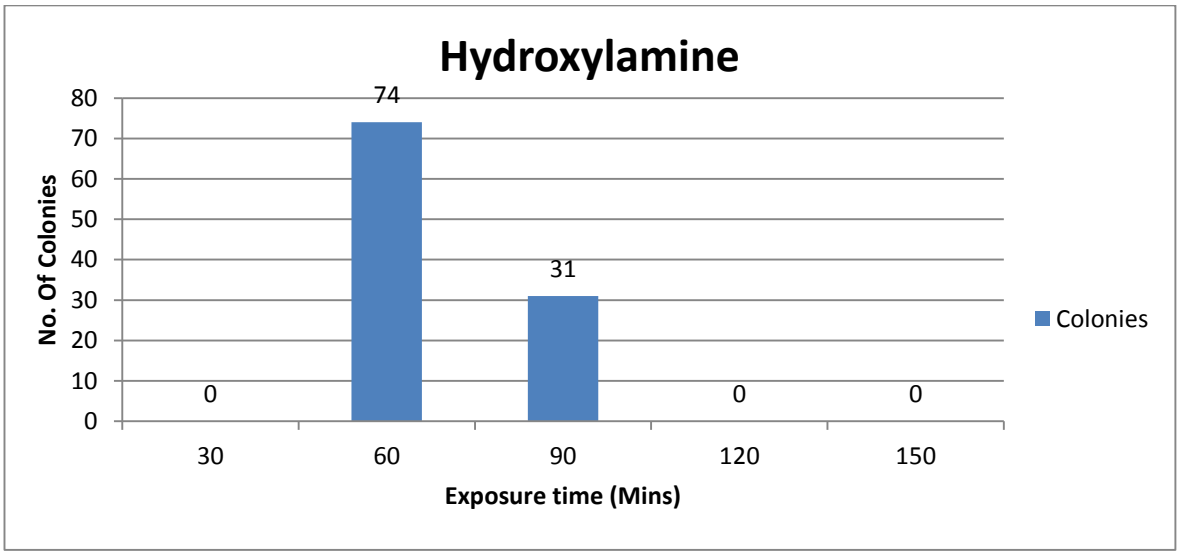

***Figure 15**. Colony formations after subjected to hydroxylamine mutation.*

4.9 33% Hydrogen Tolerance

Colonies from UV and hydroxylamine mutagenesis experiments were inoculated with 33 % of hydrogen. The growth and hydrogen content was calculated. Figure 16 shows that UV 5 minutes sample produces high hydrogen content than the other samples, however UV 3 minutes and hydroxylamine 60 minutes has produced high percentage of hydrogen. Figure 17 shows hydrogen yields by halanerobium sacchrolyticum subsp. Sacchrolyticum with glucose as substrate. The highest hydrogen yields after mutation, obtain by UV 5 minutes sample 1.54 mol/mol glucose. UV 3 minutes and hydroxylamine 60minutes samples able to obtain 1.24 and 1.33 mol/mol glucose respectively with concentration of 5 g/l glucose.

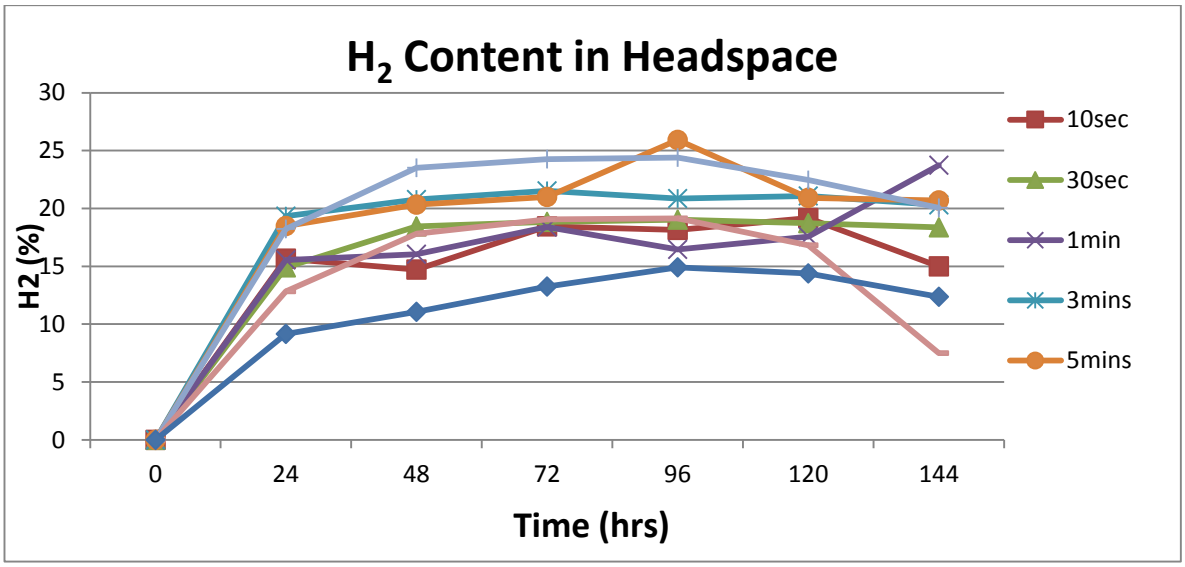

***Figure 16**. Hydrogen content produced by H. sacchrolyticum after mutation with 33 % of H_2 tolerance.*

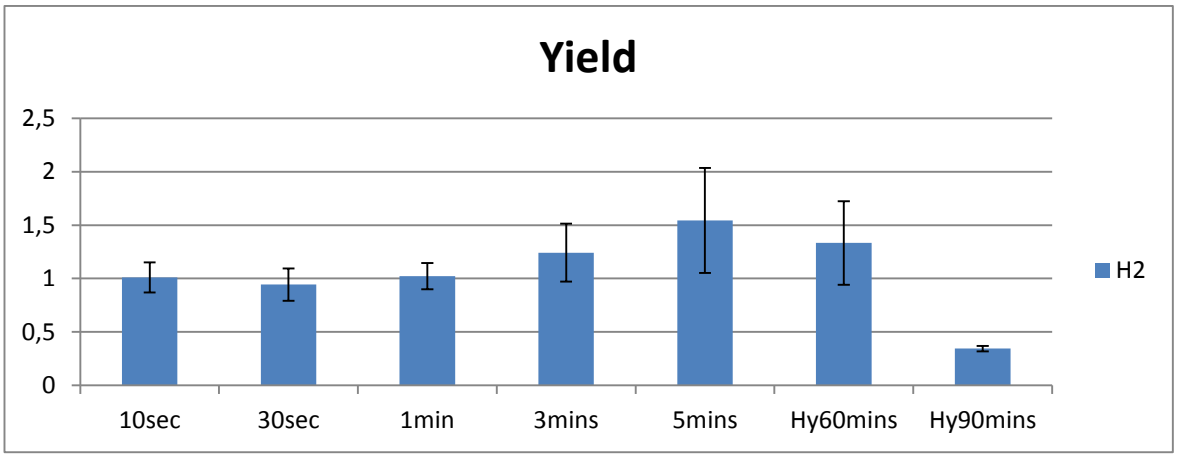

***Figure 17**. H_2 yield (mol/mol glucose) obtained with initial hydrogen tolerance.*

4.10 Hydrogen Production

Further UV 5 minutes, hydroxylamine 60 minutes and unmutated samples was selected for confirmation of hydrogen production. This experiment was carried out without any initial hydrogen. Figure 18 indicates that UV 5 minutes sample has higher H2 concentration than normal (unmutated) H. sacchrolyticum subsp. Sacchrolyticum. This sample has produced high concentration of 6.202 mmol of hydrogen content, followed by hydroxylamine 60 minutes and unmutated samples.

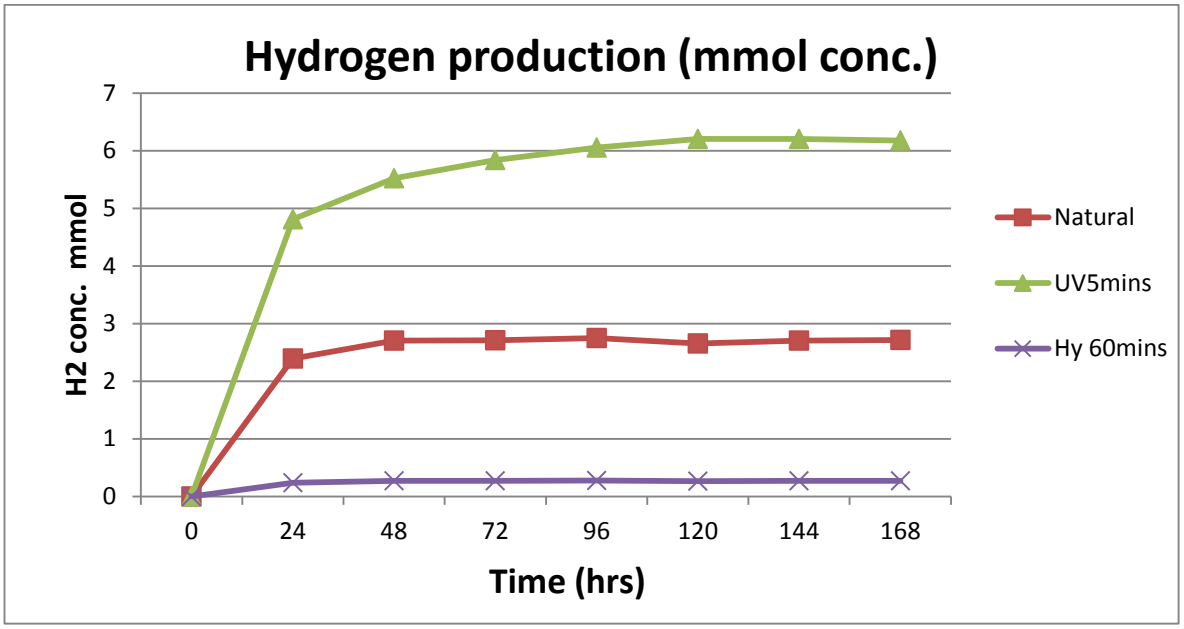

***Figure 18**. Hydrogen production after mutation without initial tolerance.*

Hydrogen yields calculated using concentration of H2 product by concentration of substrate utilized. Figure 19 shows constructive results, were UV 5 minutes and hydroxylamine 60 minutes (mutated) samples obtained higher yields compare to non-mutated sample. This result indicates possibilities of mutations in the samples.

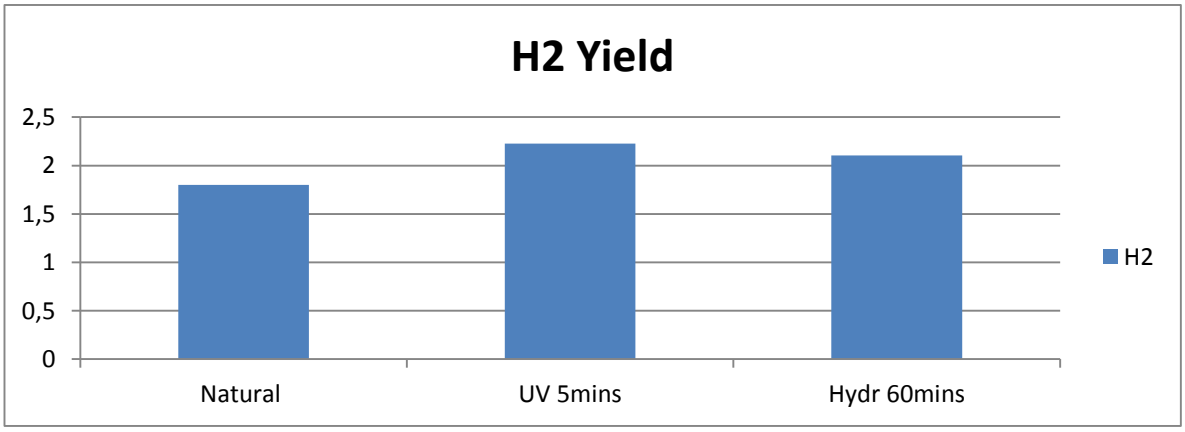

***Figure 19**. H_2 yield (mol/mol glucose) obtained with initial hydrogen tolerance.*

5. Discussion

5.1 Effect of antibiotics in Halanerobium Sacchrolyticum subsp. Sacharolyticum.

Novobiocin, chloramphenicol, ampicillin and kanamycin were the antibiotics used. Results indicate that except novobiocin, other antibiotics were resistant to H. Sacchrolyticum. Since the concentrations of novobiocin 50 µg/ml, 30 µg/ml, 20 µg/ml and 15 µg/ml was high, in next consecutive experiments concentrations of novobiocin reduced to > 5 µg/ml. Significant growth of bacteria has been detected in 0.3 µg/ml of novobiocin (lowest concentration). The study shows that novobiocin antibiotic, affects the growth of bacteria by inhibiting the activity of DNA gyrase [44], however sensitivity of bacteria not only depends on concentration range of novobiocin. ampicillin and kanamycin are the aminoglycoside antibiotics resistance to member of halobacteriaceace [45].

5.1.1 Multi drug efflux pumps in resistance of Halanerobium Sacchrolyticum Subsp. Sacchrolyticum.

Mutli drug efflux pumps play important role in resistance of gram negative bacteria, these pumps drive antibiotic from cytoplasm to outer membrane layer. SMR, MF and RND are the three different types of MDR efflux systems involve in gram negative bacteria. [24]. Moderate halophilic bacteria are armed with complex device that protects from toxic substance, such as RND type efflux system [46]. RND efflux system drives by proton motive force, in this efflux system AcrB is a transporter protein in inner membrane binds with antibiotic (drug) in phospholipid bilayer by (AcrA) linker protein and drives to outer membrane layer using OM protein (TolC) [47]. H. Sacchrolyticum is resistant to novobiocin with concentration of > 1 µg/ml. Thus RND efflux pumps excreting higher concentrations of novobiocin from cytoplasm to OM layer.

5.2 Transformation of pMDS133 into *Halanerobium Sacchrolyticum* subsp. *Sacharolyticum.*

Transforming of pMDS133 plasmid contain novobiocin resistance gene to halanerobium sacchrolyticum subsp. sacchrolyticum by natural transforming method. Different concentrations of pMDS133 added to mid log phase cells (competent cells) and incubated at 37 °C, 150rpm for 4 hours. Further cells are plated on agar plate with different novobiocin concentration, initially experimented with 3 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 50 µg/ml of novobiocin. Since no colonies were formed, based on the results planned to repeat with low concentration of novobiocin < 1 µg/ml. No colonies were formed in control with plasmid and bacteria, therefore no conclusion can be made from this experiment. Competent cells were not ability to uptake the plasmids, concentration of novobiocin were some of the obstacle in this method. Including different methods of transformation would have given appropriate results, for example plasmid mediated transformation which includes chemical transformation, electro-transformation, biolistic and sonic transformation [48]

5.3 Effect of mutagenesis

Random mutagenesis of Halanerobium sacchrolyticum subsp. Sacchrolyticum resistance to novobiocin (0.3 µg/ml) leads to improved hydrogen tolerance. In this study, mutation cause increases in growth of bacteria and production of hydrogen. UV mutagnenesis (Physical method) and hydroxylamine (Chemical method) were the two different methods used in this study. UV radiation creates specific damage to DNA causes base substitution of C→T (mutation) at dipyrimidine sites [28]. Though in [4] halanerobium sacchrolyticum subsp. Sacchrolyticum has obtained higher H_2 yield of 2.4 mol/mol glucose. The highest H_2 yield was obtained, when bacteria subjected to UV mutagenesis and hydroxylamine mutagen 2.2 mol/mol glucose and 2.10 mol/mol glucose respectively. Based on the results, UV mutagenesis and hydroxylamine mutagen has an impact on yield of H_2, before mutation same sample has obtained H_2 yield of 1.5 mol/mol glucose. This mutation has substantially improved hydrogen content with low concentration of novobiocin.

5.4 Hydrogen and 1, 3 propanediol tolerance.

Halanerobium sacchrolyticum subsp. sacchrolyticum is efficient in hydrogen and vitamin B_{12} dependent 1, 3 propanediol production by utilizing glucose and glycerol as substrates [4] [49]. The most sensitive part is to obtain such productions in industrial scale, which arise some of the obstacles like high contamination, complex in fermentation process. In this study after mutation of H.Sacchrolyticum , 33 % of H2 tolerance and 6 % of 1, 3 propanediol tolerance were tested initially by subjecting to H2 and 1, 3-PD respectively. The results indicates that, with 33 % of initial H2, H. Sacchrolyticum has produced yield of 1.54 mol/mol (glucose), thus this bacteria has tolerance of H2, leads to proceed in industrial scale using fermentation technology. The ability to tolerance of h2 possibly resolves the consequences by end metabolites ethanol and acetate which affects production of hydrogen [50]. No significant growth of H. Sacchrolyticum with initial 1, 3 – PD content. Some of the reason were i) 60 g/l (6 %) of 1, 3 –PD was sensitive ii) high initial concentration of 1, 3 – PD affects the final production [8] ii) difficulties in solid medium (agar plating), no colonies were formed iii) error in cultivation of bacteria specifically to 1, 3-PD. Open fermentation process has potential in producing high H2 and 1,3 – PD in large scale, [52] Study describes that naturally evolved mixed culture has produced hydrogen and also converted glycerol into1, 3- PD which has same efficiency of normal culture by open fermentation method.

5.5 Selection of mutants.

After mutation, samples were analysed for H2 production with novobiocin, also tolerance of H2 and 1, 3-PD tolerance. However results indicates potential mutants might available, due to increase in H2 yield after subject to mutations. Further proceeded with replica plating technique, a method to screenthe mutants, this method was used in this study because of most reliable method for identifying antibiotic activity [53]. Initially replica p1ating method was experienced with E.coli KRX stain were efficiency was > 80 %. In the case of H.Saccholyticum replica plating method, formation of colonies was challenging. The master plate was prepared by inoculating UV 5 minutes and hydroxylamine 60 minutes culture (mutagenesis), were samples was diluted to 10^5 in order to get single prominent colonies. Stamped into agar plates containing 0.6 µg/ml and 1 µg/ml of novobiocin resulted in cluster formation (no colonies are formed). Some of the reasons were i) cloth material (velvet) stamping from master plate was difficult ii) hard printing has affected the colony formations iii) unidentified precipitation formed in the agar plates. However few colonies are formed in the plate containing 0.6 µg/ml of novobiocin were collected and inoculated. DGGE, CSGE and DHPLC are the different

methods for screening mutants, by using different methods to identify the mutants improves the possibilities of obtaining mutants. [54]

6. Conclusions

This study done by analyzing growth of Halanaerobium Sacchrolyticum Subsp. Sacchrolyticum with different antibiotics (Ampicillin, kanamycin, chloramphenicol and novobiocin) in hydrogen production, followed by random mutagenesis to construct new strains which improves in high hydrogen and 1, 3-PD tolerance. Replica plating method was performed to screen the mutants.

The UV mutation and hydroxylamine mutation were two different types of mutagenesis gives high yield of H_2. We have shown that Halanaerobium Sacchrolyticum Subsp. Sacchrolyticum after subject to mutation has obtained H_2 yield of 1.54 mol/mol (glucose) with 33% of hydrogen tolerance and 2.4 mol/mol (glucose) without H_2 tolerance. This study shows that 60 g/l of 1, 3 – PD tolerance inhibits the growth, affects the production of hydrogen and 1, 3-PD.

Screening of mutants undergoes many obstacles as discussed in 5.5. Further approach gives different prospective some of them includes 1) Identifying of gene responsible for antibiotic resistance produces sensitive stains by mutations 2) to produce large scale, open and continuous fermentation can be used 3) using mixed cultures improves hydrogen production and 4) controlling cell metabolic pathway to enhance the production of hydrogen.

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