rijksuniversiteit groningen

SELECTION OF MICOBIAL CELLS WITH INCREASED PRODUCT YIELD FOR INDUSTRIAL FERMENTATIONS

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1 ABSTRACT

Lactic acid bacteria (LAB) are commonly used organisms in the fermentation of food to prevent its spoilage. *Lactococcus lactis* among the LAB are known to improve the organoleptic properties of the food products such as flavor, texture and aroma. Thus gaining much importance in food industry. *L.lactis MG1363* has a relatively simple carbohydrate metabolic pathway that can be rerouted easily, thus used as a model of choice in metabolic engineering. This allows for the isolation of strains producing desired metabolite. Identification and isolation of the strain could be possible by usage of indicator strains responding to metabolites being produced by the desired strain.

Engineering of Lactoccocal metabolism can result in the introduction of heterologous genes, derive energy from glycolysis metabolic pathway, and producvariety of compounds during the fermentation such as diacetyl, ethanol, acetoin, acetaldehyde etc. Diacetyl contributes to the buttery aroma of the dairy products such as butter, acid cream, cottage cheese, yogurt etc. Acetaldehyde contributes to a fruity flavor in yogurt and ethanol is present in alcoholic beverages.

Few promoters that are capable of responding to organic compounds such as diacetyl, acetaldehyde, and ethanol were identified by transcriptomic analysis prior to this research study. In this study such promoters were introduced into the pSEUDO – gfp plasmid and later transformed in *Escherichia coli* DH5α bacterial cells to obtain multiple copies of the bacterial cells. Such copies were further transfrmed into *Lactococcus lactis* MG1363 thus allowing the plasmid to be integrated into the chromosomal genome of *Lactococcus lactis* MG1363. Thereby, constructing indicator cells for sensing the desired compounds.

The indicator cells were there checked for the fluorescence intensity in the presence and absence of the desired compounds. Indicator cells with promoters *butA/B, llmg_1772* were found to show some fluorescence intensity in the presence of 2% acetaldehyde. Promoters *llmg_0317, llmg_0941, llmg_1677,* butA/B also showed the presence of fluorescence signal in the presence of 4% ethanol and the promoters *llmg_1019* and *dhaM* that were found to be responsive to diacetyl also showed the presence of fluorescence intensity. However, the results obtained with acetaldehyde is not promising as the bacterial cells showed negligible growth pattern even after 10 hours of incubation.

2 INTRODUCTION

2.1 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are gram positive, non sporulating anaerobic microbes producing lactic acid as the end product during fermentation process *(A Halasz,2009).* LAB occurs in nutrient rich habitat such as decomposing plant material and fruits, in dairy products such as cheese, yogurt, milk, fermented meat and fish, sauerkraut, pickles, beer, wine, cider, kimchi, as well as animal feeds such as silage *(König, H.,2009).* They also exhibit symbiotic relation by inhabiting human gastrointestinal tract *(De Vuyst, L.,2007).*

Fermentation is considered as one of the major food processing technology since ancient times along with salting, drying etc. These food processing techniques prevent spoilage of food *(Axelsson, L.,2000).* Physiologically, fermentation is one of the metabolic pathways to derive energy in the absence of oxygen. In general, fermentation is a process where microorganisms such as LAB are capable of initiating biochemical changes in the food containing carbohydrates as the organic substrates in order to produce acid, alcohol and gases as the end products *(Leroy, F.,2004).* In addition to food preservation, LAB are also capable of modifying or improving the organoleptic properties of food such as the texture, flavor and aroma thus has gained much importance in food industry. They also produce variety of functional components such as vitamins, exopolysaccharides, enzymes, bacteriocins etc, thus playing a vital role in food industry, chemical industry and pharmaceuticals *(Florou-Paneri, P., 2013). (Figure1).*

LAB are widely used in fermentation process mainly because they can withstand high temperature as high as 70 ºC *(Cavanagh, D. et.al.,2005).*They are categorized into homo-fermentative producing chiefly lactic acid from glucose, lactose and hetero-fermentative producing acetic acid, ethanol, and various other organic compounds. *(A Halasz.,2009).* Hetero-fermentation occurs due to shortage of carbohydrate supply and draws energy from glycolysis or pentose phosphate pathway *(König, H.,2009; Thomas, T. D. et.al.,1979).*

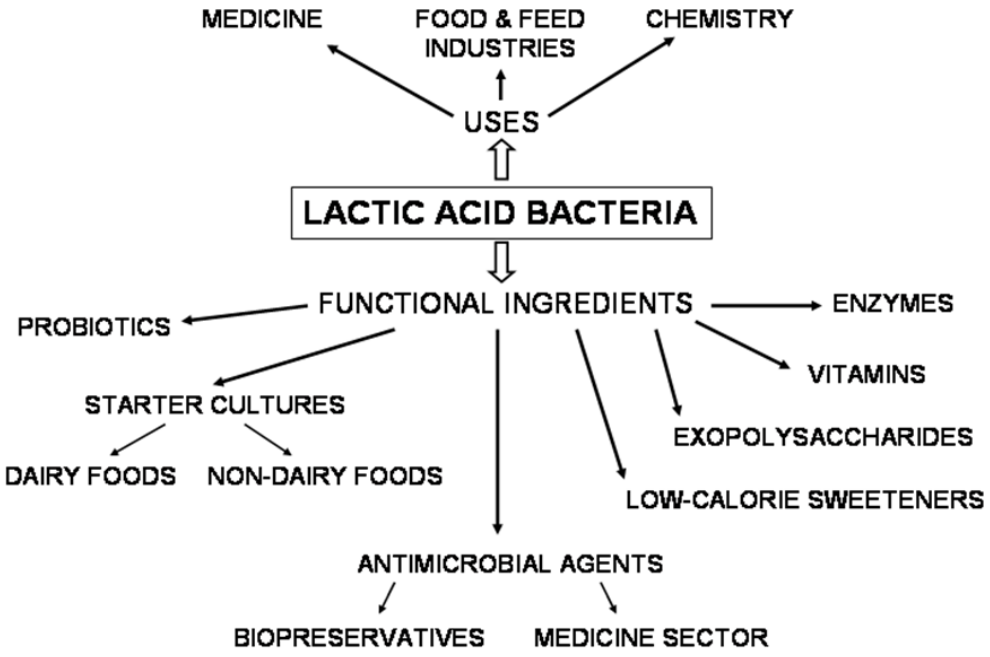


Figure1: Uses and functional ingredients of Lactic Acid Bacteria (Florou-Paneri, P.et.al. 2013)

2.2 TAXONOMIC CLASSIFICATION OF LAB

LAB belongs to the order Lactobacillales that includes the following genera: *Lactobacillus, Carnobacterium, Lactococcus, Streptococcus, Enterococcus, Vagococcus, Leuconostoc, Oenococcus, Pediococcus, Tetragonococcus, Aerococcus and Weissella (Florou-Paneri, P.et.al., 2013).* Many species are included with the nine genera. However, complete genome sequence of eight commensal species are published and include: *Lactococcus lactis, Lactobacillus plantarum, Lactobacillus johnsonii, Lactobacillus acidophilus, Lactobacillus sakei, Lactobacillus bulgaricus, Lactobacillus salivarius, and Streptococcus thermophiles (Makarova, K.,2006)(Figure2)*

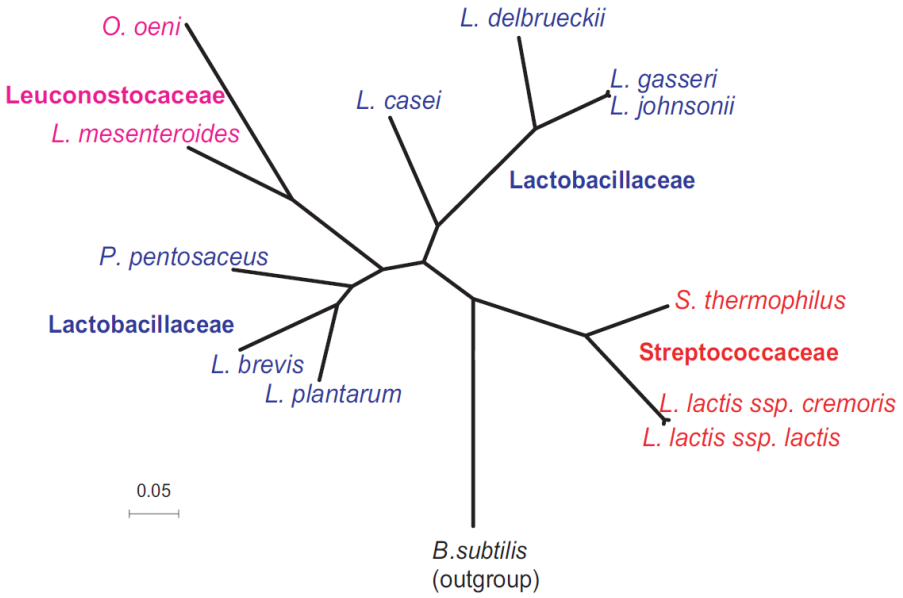


Figure2: Phylogentic tree of order Lactobacillales (Makarova,K.,2006)

2.3 *LACTOCOCCUS LACTIS*

Lactococcus lactis among all the species is known to be the most widely studied species due its significance in dairy industry *(Kleerebezemab, M.et.al.,2000).* Typical morphological characteristics of *L. lactis* are spherical or ovoid shape and size measuring 1.5µm in diameter and are non-motile. They do not form spores. Studies show that *Lactococcus lactis* differ from other lactic acid bacteria in pH, salt and temperature tolerances, which are important for their growth. Therefore serves as an significant characteristics to be used in starter cultures for cheese making, yogurt, alcoholic beverages etc. *(Todar, K.,2012).*

Human tissues are not a natural habitat of *L. lactis*. However, they become activated and colonize gastrointestinal tract of humans when they consume plants which are the natural habitat for *L. lactis (Todar, K., 2012; Song, A. A. L. et al.,2017).*

2.4 L. LACTIS SUBSPECIES DESIGNATION

Before 1985, dairy *streptococci* was the broadly used term to refer the members of the genus *lactococcus.* However, other dairy strains were pathogenic such as *streptococci sensu stricto, streptococcus lactis,* and *streptococcus cremoris*. In order to differentiate between the non-pathogenic and pathogenic dairy strains, *streptococci genus* was reclassified into *Lactococcus genus* in 1985. It is further divided into four subspecies namely: *lactis, cremoris, hordniae and tructae* and phenotypically it is classified into biovariant *diacetylactis.* The biovariant is responsible for citrate fermentation and is known to yield diacetyl and acetoin compounds *(Perez, T.et.al.,201; Song, A. A. L. et al.,2017).* Designation of subspecies is established based on the phenotypic characteristics that were identified by their metabolic traits *(figure 3).* However, genotypically, the subspecies *lactis* and *cremoris* exhibit 85% identity and were identified by comparative genome analysis *(Cavanagh, D, et.al., 2005).*

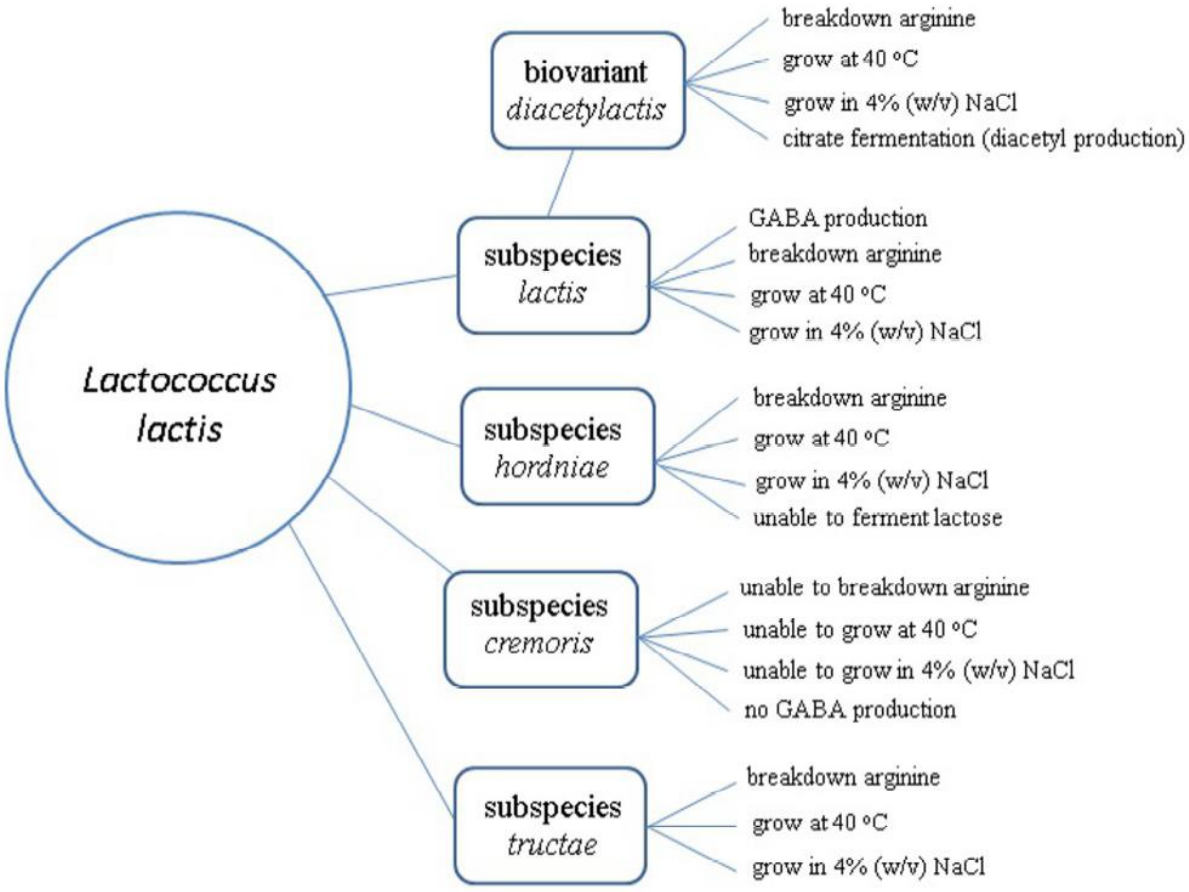


Figure3: Phenotypic traits for Lactococcus lactis subspecies identification (*Cavanagh, D, et.al., 2005).*

2.5 USES IN FOOD AND FEED INDUSTRIES

The inventory of microbial food cultures updated the inventory of microorganisms in 2012 and reported two species of *lactococcus* namely *Lactococcus raffinolactis* and *Lactococcus lactis*

mainly used in food and dairy industry. These bacterial strains are recognized as GRAS (Genetically Regarded As Safe) by the Food and Drug Administration (FDA) in 2010, *(Ross, R.P.et.al.,2000).* Some strains produces bacteriocins that aids in food preservation. They are also known to be the principle organism in dairy starter culture systems producing fermented end products such as cheese, milk, buttermilk, sour cream etc. *Lactococcus lactis* is also used in mixed starter culture along with other lactic acid bacteria such as lactobacillus and streptococcus species, thus exhibiting a symbiotic relation. Therefore, Lactococcus with such benefits marks its significant role in the dairy industry *(Song, A. A. L. et al.,2017).*

Acidification activity and resistance to bacteriophage infection are known to be the significant factors to be considered while selecting bacterial strains to use in food products to achieve a desirable characteristic *(Cavanagh, D, et.al., 2005).* One such strain widely used in the food industry and in our study is *Lactococcus lactis* cremoris MG1363 [NC_002662.1/ accession number: AM406671]. The *Lactococcus lactis* MG1363 has a circular chromosome and is devoid of a plasmid. The chromosome contains a total of 2,529,478 bases, making up the DNA. The total amount of guanine to cytosine in the DNA in relative to adenine and thymine is 35.8%. Capable of encoding 2436 proteins with 530 being distinctive in nature *(Carlos, C.et.al., 2007)* (Table1).

The strain MG1363, phenotypically is characterized of unique sex factor, insertion elements and the integration hotspot region. These are grouped as mobile genetic elements. The integration hotspot region in particular has a significant role in determining how the genome of *L. lactis* MG1363 and related strains evolve by facilitating stable integration of the genetic material acquired by any other organism through horizontal gene transfer. This region also enabled to conserve an operon that is important to maintain the bacterial growth in milk.

L.lactis is also known to maintain COG (clusters of orthologous groups), which is the largest functional category of novel proteins. COG contains 46 proteins out of 530 proteins. This functional group of proteins is known to carry out the carbohydrate metabolism and transportation. The robust nature and significant genetic response have opened the doors to analyze the introduced lactococcal and heterologous DNA. Flavor of the fermented food products is remarkably improved using *L.lactis* subsp. cremoris strains, thus are selected over *L. lactis* subsp. lactis strains *(Carlos, C.et.al., 2007).*

Feature	**Value in *L. lactis* MG1363 strain:**
Size (bp) of chromosome	2,529.478
GC %	35.8
No. of predicted CDSs	2517
No. of IS elements	71
No. of phage genes	178

Table1: General Genome Features

The small sized genome (2.3Mbp) with the development of multiple inducible expression and cloning systems has made *Lactococcus lactis* a desirable model system to be used over other gram positive bacteria such as *Bacillus subtilis* and *Lactobacillus plantarum* also against the alternative gram negative bacteria *Escherichia coli (figure4)*

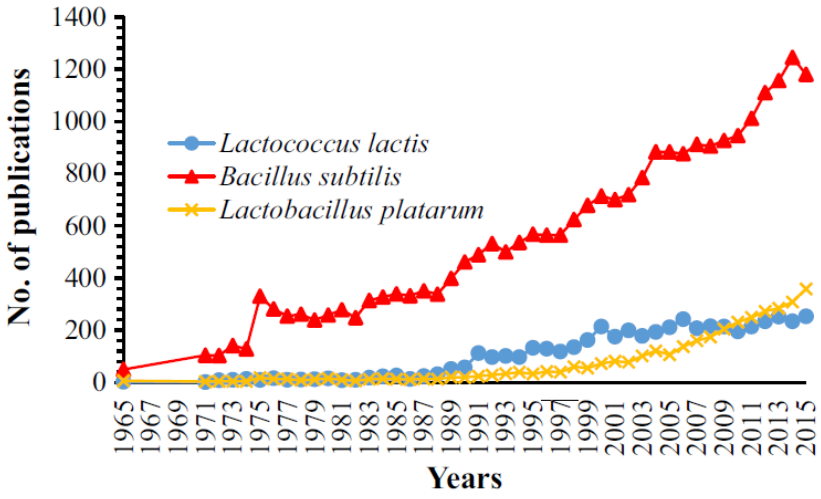


Figure4: Comparison of publications between Gram positive model organisms over the past 50 years (Song, A. A. L. et al.,2017).

2.6 METABOLISM

During fermentation process, the bacteria utilizes lactose and other six carbon sugars present in the dairy starter cultures. Lactose is broken down to lactic acid thus lowering the pH making it favorable for the bacteria to release the enzyme proteinases to break down the milk proteins such as casein. Therefore, protect the food from spoilage *(El‐Zahar, K., 2003;Cavanagh, D, et.al., 2005; Kleerebezemab, M.et.al.,2000).* The final end products produced during the process will possess significant organoleptic properties and microbial quality, thereby being beneficial to the dairy industry *(Ross, R.P.et.al.,2000).* Such fermentation process occurs in the presence of lactate dehydrogenase (LDH) and produces intermediate product pyruvate, which later gets converted to the single compound l-lactate, thus homofermentation. However, *L. lactis* is capable of producing mixed compounds such as ethanol, acetate, lactate, acetaldehyde and compounds with aromatic properties such as diacetyl, acetoin and butanediol and the process is called heterofermentation. Such fermentation occurs when the physiological conditions such as pH and pyruvate concentrations vary and there is availability of three enzymes, which gets activated in low pH (≤6.0) and high pyruvate concentrations. Enzymes include α-acetolactate synthase (ALS), pyruvate-formate-lyase (PFL), and pyruvate dehydrogenase (PDH) *(Song, A. A. L. et al.,2017).*

Diacetyl contributes to the buttery aroma of the dairy products such as butter, acid cream, cottage cheese, and yogurt etc *(Hugenholtz, J.et.al., 2000; Quintans, N.G.et.al., 2008).* The biovariant (strain that differs biochemically and physiologically from other strains) *L. lactis subsp. lactis biovar. Diacetylactis* naturally produces diacetyl by utilizing citrate in the presence of enzyme citrate permease *(figure5).* However, other subspecies of *L. lactis* lack the enzyme citrate permease thus cannot utilize citrate *(Cavanagh, D, et.al., 2005).* In addition, citrate is not the main source of carbon in dairy fermentation, thus the strains used in dairy fermentation such as *L. lactis subsp lactis, L. lactis subsp cremoris* are genetically engineered to efficiently reroute the metabolic fluxes. This can be achieved by constructing promoters based on the consensus promoter elements -35 and -10 hexamers of *L.lactis,* thereby developing a set of constitutive promoters that exhibit range of expressions *(Kleerebezemab, M.et.al,2000).*

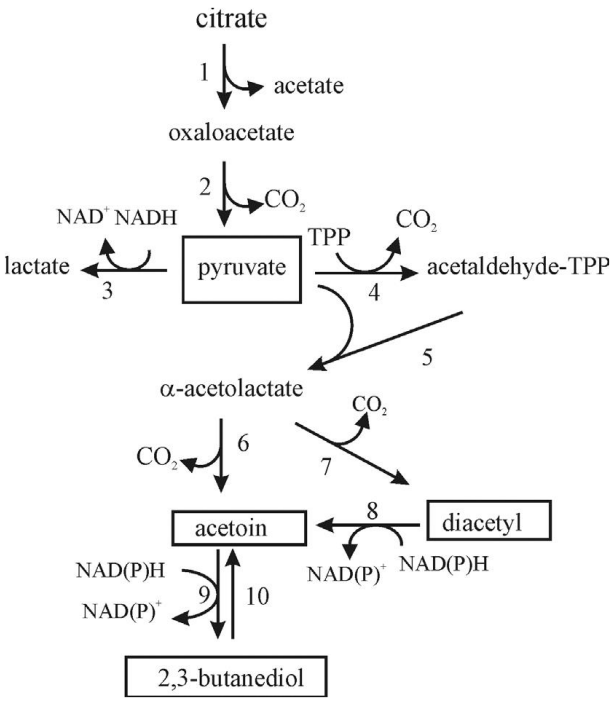


Figure5: Citrate utilization pathways in bacteria and its end products. (Quintans,N.G.et.al.,2008)

As previously mentioned, glycolysis is the main energy driving pathway in mixed acid fermentation, produces pyruvate as the intermediate product. Pyruvate produced is rerouted to produce aromatic compounds by oxidative decarboxylation in the presence of the enzyme a-AL synthase (ALS) and is rerouted to produce other compounds such as acetate in the presence of pyruvate dehydrogenase, acetaldehyde and ethanol in the presence of alcohol dehydrogenase *(figure6)*

Acetaldehyde is another aromatic compound produced by rerouting pyruvate metabolism by *L. lactis.* This compound has a fruity flavor and usually found in yogurt. In carbon metabolism carried out by *lacctococcus,* acetyl coenzyme A is produced as the intermediate product in the presence

of pyruvate dehydrogenase (PDH). The acetyl CoA is rerouted to produce acetaldehyde by the enzyme aldehyde dehydrogenase (ADH) (figure5) *(Bongers, R. S.et.al.,2005).*

Furthermore, acetaldehyde can potentially be converted to ethanol in the presence of alcohol dehydrogenase (ADH) *(figure5) (Kleerebezemab, M.et.al,2000)* and it is the main compound produced in alcoholic beverages. Due to its robust nature and adaptability to grow on cheap industrial media, yeast has been and is the prominent organisms used in the production of ethanol. However, it has few disadvantageous such as slow fermentation rate, less tolerance towards high temperature, and can ferment limited sugars naturally. Therefore, *Lactococcus lactis* is being selected over yeast *(Solem, C.et.al.,2013)*

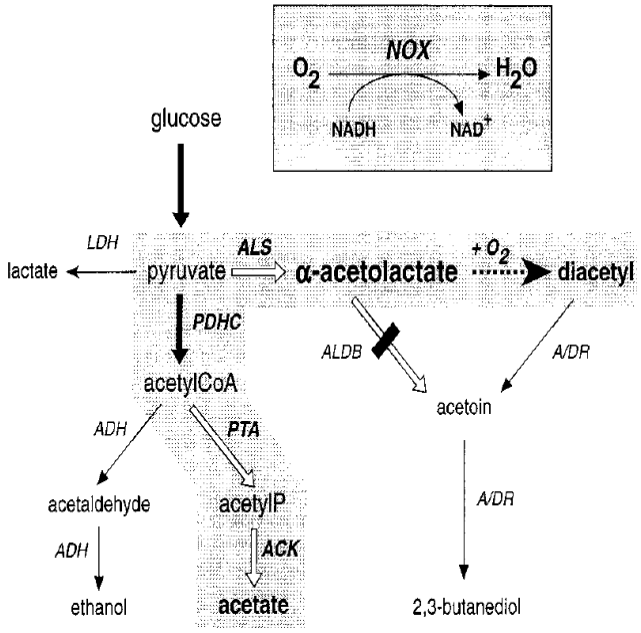


Figure6: Rerouting of Pyruvate Metabolism *(Kleerebezemab, M.et.al,2000)*

3 Challenges to overcome in food industry

Market value of the food products estimates their demand in the society and by enhancing the quality of the dairy products in terms of flavor, texture and their shelf life directly influences the market value. The value of the food products also increases by manufacturing products with heterogeneity and also taking into consideration the specific health benefits each dairy product offers *(Pedersen, M. B.et.al.,2005; van Hylckama Vlieg, J. E.et.al.,2006).* A constant upgrading of the starter culture with the desired trait capable of producing products with desired characteristics has to be maintained by the industries. The potential approach to achieve this is the metabolic engineering strategies where an organism (whose genetic material is manipulated through unnatural gene transfer methods such as mutations, insertion, and deletion of genes) is introduced into the food chain. However, due to insufficient public acceptance and the government regulations towards GMO's, this approach is difficult to incorporate into the food industries. This subjects the

industries to look into alternative approaches to improve the starter cultures. *(Cavanagh, D. et.al.,2005).* One such approach is examination and identification of the existing species in the LAB group inside and outside dairy environment. This results in development of novel starter cultures from which novel industrial strains can be derived with desired traits to increase the flavor, texture and nutrient value of the product *(Ayad, E. H.et.al., 2000).* However, to characterize which among these industrial strains is the best producer of the desired product with favorable traits is challenging. To address this problem, bacterial biosensors has been developed that is capable of detecting the natural strains in the biodiversity that produce desirable product in sufficiently large amount.

4 Biosensors

A bacterial biosensor is a device used as an analyzing or diagnostic tool by fusing a biological sensing element such as a reporter gene with a promoter of the bacteria *(Su, L.et.al., 2011).* Reporter genes are transcriptionally controlled and include the genes encoding for β-galactosidase (lacZ), or alkaline phosphatase (AP, phoA) or firefly luciferase (lux or luc) or green fluorescent protein (GFP) *(Sekhon, S. S.et.al., 2014).* We have identified several metabolite responsive promoters that initiates transcription of the reporter gene when activated by compounds such as diacetyl, ethanol, and acetaldehyde. Thus, chemical compounds behaves as an analyte *(Mahr. R. et.al., 2016)* In this study, the reporter gene encodes a fluorescent protein known as Green fluorescent protein (GFP) *(figure 7).*When the bacterial cells are exposed to light of specific wavelength (600nm), the GFP emits light that is shifted towards the red end of the spectrum. Electrochemical or optical instruments such as flow cytometer can be used to detect and measure the emitted light. The intensity of the emitted light is directly proportional to amount of reporter protein present in the sample *(De Jong, H. et.al., 2014).* Therefore, this approach helps in characterizing the strains as to which enhances the product yield and flavor. It is observed that the organisms themselves have closely regulated the transcriptional machinery. *(Mahr R. et.al., 2016).* Due to this, the activity of the promoters is modified according to the varied purpose of organisms. This renders a limitation in the sensitivity and dynamic range of the biosensors that are functional based on the native transcription factors and promoters *(Zhang, F. et.al., 2012; Blazeck, J. et.al.,2013)*

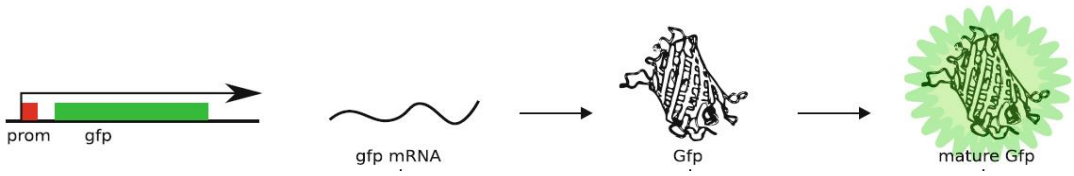


Figure7: When chemical compounds bind to the metabolic responsive promoter (red), it initiates the transcription of reporter gene (green) to produce a mRNA. mRNA is then translated to produce GFP that later on undergo autocatalytic process to form a mature fluorescent protein that is easily detected (De Jong, H. et.al., 2014).

5 HOMOLOGOUS RECOMBINATION

According to the study conducted by Joao P. C. Pinto and colleagues, plasmid pSEUDO-gfp was constructed by inserting to superfolded gfp gene at *llmg_pseudo_10* locus of *Escherichia coli* that exhibited homology with *Lactococcus lactis.* The pSEUDO-gfp plasmid contains antibiotic erythromycin resistant gene that helps in selection of bacterial cells when grown in selective media containing arythromycin. The oroP gene is a transporter gene that allows for the transportation of 5 –fluoroorotate inside the cell. However, when the plasmid is integrated into the genome of *L.lactis*, the bacterial cell undergoes homologous recombination and can occur in two ways (1) single recombination where one part of the plasmid is integrated to the genome. In such condition, L.lactis posses erythromycin and oroP gene along with the superfolded gene and the promoter and can lead to decreased stability with multiple replication of the bacterial cell. The second way of undergoing homologous recombination is double recombination where the plasmid is completely excised from the genome leaving behind the superfolded gene and the promoter as they are present within the homologous region. In such condition, the bacteria doesn't loses its stability with multiple replication. Screening of bacterial cells with single and double recombination is done by growing them in a selective media with 5-fluoroorotate. 5-fluoroorotate is a pyrmidine analog and is known to be lethal to *L.lactis* when transported inside the bacterial cell. Thereby, the bacterial cell that has undergone successful double recombination can survive in the selective media. *(Casey. J.et.al.1991; 37.Pinto, J. P. et. al., 2011).*

6. RESEARCH STRATEGY

The aim of the project is to select the potential microbial cells that increases the yield of organic compounds such as diacetyl, acetaldehyde and ethanol in dairy food products, thus, enhancing the texture flavor and aroma of the products.

- **Goals**
    1. Construction of indicator cells having the promoter of interest fused to gfp gene
    2. Measuring the fluorescence signal of the indicator cells using Flow Cytometry thus enabling their characterization

7 MATERIALS AND METHODS

7.1 BACTERIAL STRAINS AND CULTURES

Bacterial strains used in this study include *Escherichia coli* and *Lactococcus lactis* strains listed in table 2. *E.coli* strains are grown in Luria Bertani broth containing erythromycin (250µg/ml). *L. lactis* strains are grown in M17 media supplemented with 0.5%(w/v) glucose (GM17) containing 5µg/ml erythromycin. The transformation of *E. coli* plasmid into L. *lactis* genome needs M17 media suplemented with 0.5M sucrose (SM17), 0.5%(w/v) glucose, 20 mM $MgCl_2$, 2mM $CaCl_2$.

Reference strains	Strain No	Accession No
Escherichia coli	DH5α pSEUDO - GFP	NA
Lactococcus lactis cremoris	MG1363	AM406671

Table2: Bacterial strains used in the study

7.2 TRANSCRIPTOMIC ANALYSIS

Transcriptomic analysis was previously performed on *L.lactis* MG1363 to identify the promoter regions, responsive to the compounds diacetyl, acetaldehyde and ethanol followed by data analysis using Genome 2D software and array pro-analyzer.

7.3 CONSTRUCTION OF INDICATOR CELLS

Indicator cells are the desired constructs with the responsive promoter and the reporter gene GFP. This was achieved with two sets of transformation process into two different host cells. First transformation is the insertion of responsive promoter region into *E. coli* pSEUDO10-GFP plasmid using various restriction enzymes described in the following sections. The second transformation is the chromosomal integration into *L.lactis* genome *(figure 8)*

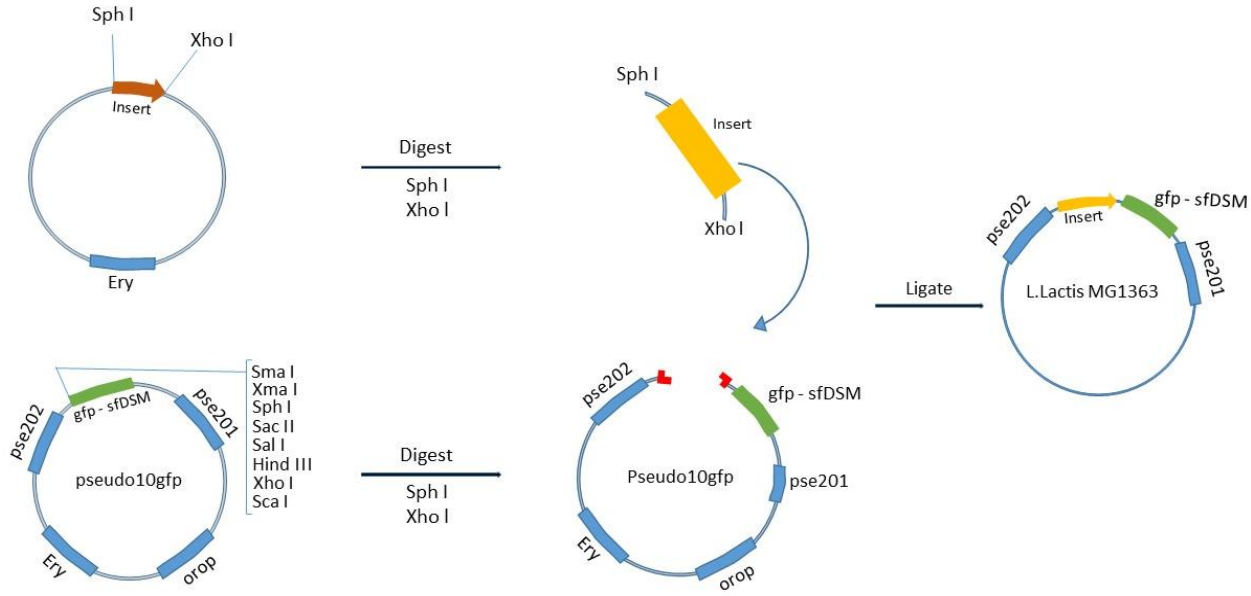


Figure8: Outline of construction of indicator cells

7.4 PRIMER DESIGN FOR PCR BASED CLONING:

Primers are short oligonucleotide sequences that are required for DNA synthesis. The ideal primer sequence should have three main features such as

1. A restriction site that is also present within the desired region of the recipient plasmid vector(usually 8-10bps)
2. A extra base pairs (usually 6-8bps) inserted upstream of the restriction endonucleases sites to avoid inefficiency of the digestion
3. The hybridization sequence, region of the primer that amplifies the sequence of interest.(usually 25-30bps)

Primers	Sequence (5'-3')
butA/B_Fw	GTAATAGGATTTGGATGTTCTGCTCGAGGACAAA
butA/B_Rv	GAAATAGCATGCAAAAAATTCTTAGCTTTTTATA
copR_Fw	TATTCGCTCGAGTAAAATAAGTTTACACGTG
copr_Rv	GTGACCGCATGCCTGGTCTGGTCGAAATTCCC
malF_Fw	ATAATACTCGAGTTTATAAATTAAGAGGTCAG
malF_Rv	TTTGCAGCATGCTCAGCAACTAAAGACCAAGC
dhaM_Fw	TAAAATCAAGCTCTTTTTGTATCTGTGCGCATGCTTGATTAT
dhaM_Rv	ATTAACCTCGAGGATAATAACAAAAACACCTTT
llmg_0317_Fw	GAAATTAGCATGCGTCCTCGCTTTGTTGAACC
llmg_0317_Rv	AAGGACCTCGAGCTTTCGCTGTCAGGTAGGGAC
llmg_1993_Fw	TTGATTCTCGAGTTTATCAACCAATTTATTTTTA
llmg_1993_Rv	GTACAAGCATGCGAAAAAGCAGTAGATACTTTGC
llmg_1019_Fw	GTTTAGCTCGAGTATTTCTCCTTAAGAGTATTTTTTTG
llmg_1019_Rv	AGTTGCGCATGTGGGTTTCCATGAAGTCTAAACGTTC
llmg_0941_Fw	CTTTTAGCATGCCTAGTCCTTTGGGTTATATG
llmg_0941_Rv	TCACTCCTCGAGCTTTAATTTTATTCAAAATC
llmg_1772_Fw	AATTGTCTCGAGTTTCACCTCTTGATTATTTG
llmg_1772_Rv	CTTTAAGCATGCACTTGATGCAACAAAAGATA
llmg_1677_Fw	CTGTGACTCGAGAGTTTGACTTCTACCGATG
llmg_1677_Rv	GCTCGCGCATGCGAAGGTTTGACAGCTAAACA

Table 3: Oligonucleotide primers used in the study

In the study, oligonucleotide primers are designed with the incorporation of restriction endonucleases sites such as *XhoI* (CTCGAG) and *SphI* (GCATGC) for the amplification of the promoter region. This allows the both the Polymerase Chain Reaction product and the recipient plasmid to produce compatible sticky ends when digested. Therefore allowing the two products to be ligated.

Different primer pairs with their positions and other relevant features in *L. lactis* MG1363 to amplify the corresponding promoters are listed in *Table 3.*

7.4.1 Polymerase Chain Reaction.

PCR amplification was performed using PfuX7 enzyme with the help of Mcycler1 PCR machine programmed for 35 cycles and the conditions are listed in *Table 4*. Annealing temperature is considered the most important parameter. It is the temperature at which the primers bind to the specific sequences that have to be amplified. The annealing temperature is determined using the melting temperature of the nitrogen base pairs of the primer that binds to the sequence to be amplified.

The PCR product from the PCR reaction mixture was isolated using Macherey – Nagel NucleoSpin Gel and PCR Clean-up kit, and quantified theamount of the recovered DNA by determining A260/A280 ratio using Nanodrop. The PCR products were analyzed on 1% agarose gel to check the size of the product. The PCR product of desired band size is obtained and it can be used for the digestion.

Steps	Temperature	Time
Initial denaturation	98 °C	30s for plasmid 8min for colony PCR
Denaturation	98 °C	10s
Annealing	50 °C	30s
Extension	72 °C	30s/1 Kb
Final extension	72 °C	7min

Table 4: PCR reaction conditions

7.4.2 Digestion and ligation of the DNA: The PCR products are simultaneously digested using restriction enzymes XhoI and SphI. These enzymes recognizes the CTCGAG and GCATGC sequences and cleave after C-1 and G-2 recognition sites respectively, producing sticky ends. Based on the concentration of the PCR product isolated, 1.5µg - 2 µg of the PCR product and 1µg of the recipient plasmid were added together and incubated at 37°C for two hours. After the digestion, the products were cleaned using Macherey – Nagel NucleoSpin Gel and PCR Clean-up kit and were quantified using Nanodrop to determine the concentration of the recovered DNA. The products were analyzed on 1% agarose get to check if the products with desired band size has been obtained. Empty uncut plasmid as control and plasmid cleaved with single digestion were also analyzed to ensure that the enzymes are active.

The digested insert (PCR product) and the plasmid vector were ligated using T4 DNA Ligase. DNA ligase enzyme catalyses the reaction between 3′-OH to the 5′-phosphate groups produced during the digestion process. Usually during the purification of the products, chances of losing the DNA material is high and therefore to increase the efficiency of the ligation process, 100ng/µl of the plasmid vector was used having the insert to vector ratio of approximately 3:1, also 100mM ATP were added to increase the activity of ligase. To check if there has occurred any self-ligation during the ligation process or if the vector or insert is not cut properly, negative control such as plasmid without any insert was also used. It is observed that ligase are active and have high performance at 25°C. However, the DNA fragments prefer binding at 1°C. Therefore, the process is performed by incubating overnight at a temperature ranging from 4°C to 25°C. It increases the efficiency of DNA binding also gives sufficient time for the enzyme to work.

7.5 TRANSFORMATION

7.5.*1 E. coli:* Transformation process was performed using *E.coli* pSEUDO – gfp DH5α competent cells. Approximately 10µl of the ligation mixture was added to the competent cells and stored on ice for 10 minutes. The cells were exposed at 42°C for 1 minute and immediately 800µl of LB media containing 250µg/ml erythromycin antibiotic was added and were incubated at 37°C for 1 hour at 220rpmPM to provide constant aeration. 100µl of the cells were transferred onto LB plates containing 250µg/ml erythromycin and the plates were incubated at 37°C overnight.

Screening: The colonies were picked up to check for successfultransformatiionand colony PCR was performed using following primers pse101B and pseudo gfpsdm_Rv. The products were analyzed on 1% agarose gel and were sent for sequencing to confirm if the promoter (insert) has been inserted at right position in *E.coli* plasmid using the following primers pse101B and pse201A. By analyzing the results of gel electrophoresis and sequencing, the most promising construct were selected and further integrated into *L.lactis* genome by electro transformation.

7.5.2 *L. Lactis*: Approximately 1.5-3µl of the isolated E.coli plasmid with the responsive promoter was transferred to 60 µl of the *L.lactis* MG1363 competent cells in an ice cold electroporation cuvettes. The cell suspension was then exposed to electrical pulse (2.5 kV, 25 µF, 200 Ohm). Immediately 1ml of SMG17MC media(M17 + 0.5M Sucrose + 0.5% (w/v) Glucose + 20mM MgCl2 + 2mM CaCl2) was added to the cuvette and transferred to a new 1.5 ml Eppendorf tube and incubated for 2 hours at 30°C and were plated on M17 plates supplemented with 0.5% (w/v) glucose 0.5M sucrose (SM17), and 1µg/ml erythromycin. Plates were incubated at 37°C for 48h

Screening: The colonies were picked up to check for successful ligation with the help of colony PCR using following primer combination *Table 5.* The PCR products were analyzed on 1% agarose gel and were also sent for sequencing using the following primers pse101B and pse201A to check if the primers amplified the required sequence therefore giving a desired band size.

1	pse101B	pcsoroP
2	pse201A	pcsrev
3	pse100R	pcsoroP

Table 5: Combination of primers used for sequencing the isolated DNA

7.6 HOMOLOGOUS RECOMBINATION

The most promising constructs were grown in chemically defined media M17 supplemented with 0.5% (w/v) glucose for overnight. Wash the overnight culture with SA media (Attachment). The pellet was then diluted in 1:10 ratio in fresh SA media and incubated for 4 hours at 30°C. 10 µl of the culture was streaked onto SA media supplemented with 20 µg/mL of 5-Fluoroorotic acid and

incubated at 30°C overnight. The excision of the vector backbone was checked by performing a colony PCR with the primers pse101B and pse 201A.

7.7 Characterization of the strain

Strain characterization is done based on the intensity of fluorescence produced by different bacterial constructs. Two possible methods to quantify the fluorescence intensity are assays in plate readers and Fluorescence-activated cell sorting (FACS). In plate reader, the cells were grown in M17 media supplemented with 0.5%(w/v) glucose (GM17) containing 5µg/ml erythromycin overnight. The pre culture cells were then diluted in 1:10 ratio in fresh M17 media. On reaching the exponential phase, the cells were pelleted down and washed with Phosphate Buffer Saline (PBS). Compound of interest such as ethanol, diacetyl and acetaldehyde were diluted with CDMPC media supplemented with 0.5%(w/v) glucose. The cells were then added to the media-containing compound in a microtiter plate. An automated plate reader then reads the microplate. All the wells containing the culture were measured at regular intervals of time (usually 24hours) to record the absorbance and fluorescence signals. The absorbance is measured at a wavelength of 600nm on a logarithmic scale while the fluorescence is measured at 535 nm on a linear scale. In plate reader, a whole population of cells at regular intervals of time is measured, whereas in fluorescence, the single cells are measured at different time intervals. For fluorescence measurement, the cells were grown in M17 media supplemented with 0.5%(w/v) glucose (GM17) containing 5µg/ml erythromycin overnight. The pre culture cells were then diluted in 1:10 ratio in CDMPC media supplemented with 0.5%(w/v) glucose. At regular intervals of time (from 5th hour to 24th hour) both absorbance and fluorescence signals were measured. For absorbance measurements, 250 µl of cells were diluted with 750 ml of M17 media and signal was measured at 600nm. For Fluorescence measurements, 80µl the cells were diluted with 3ml of 1X PBS solution and signal was measured at 535nm

8.0 RESULTS AND DISCUSSION

8.1 TRANSCRIPTOMIC ANALYSIS

Transcriptomic analysis helps in identification of promoters that respond differently under the influence of various compounds such as ethanol, diacetyl and acetaldehyde. 47 promoters including 16 upregulated in response to diacetyl, 25 upregulated in acetaldehyde and 6 promoters upregulated in ethanol were identified. Based on the fold change and the p-value of each promoter, certain promoters were chosen to construct the indicator cells.

8.2 BIOINFORMATIC ANALYSIS

Before developing a bacterial model, all the experiments were first checked computationally to make sure the designed primers amplify the correct regions of the genome. The amplified products

were then digested using *XhoI* and *PaeI* and ligated using ligase enzyme to the restriction-digested backbone of *Escherichia coli*. The plasmid was digested using the same set of restriction enzymes used to cleave the PCR products. *In silico* pre analysis was performed using Clone manager software to design primers, identify the enzyme site, their operations, restriction digestion analysis and cloning simulations and map the enzyme sites *(figure 9)* and primer sites that are used to confirm the presence of the promoter and the GFP within the plasmid *(figure 10).*

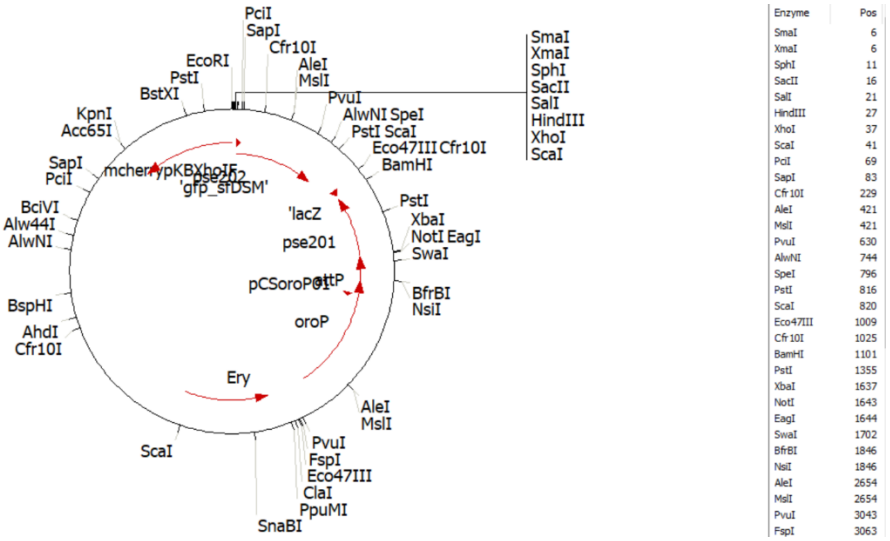
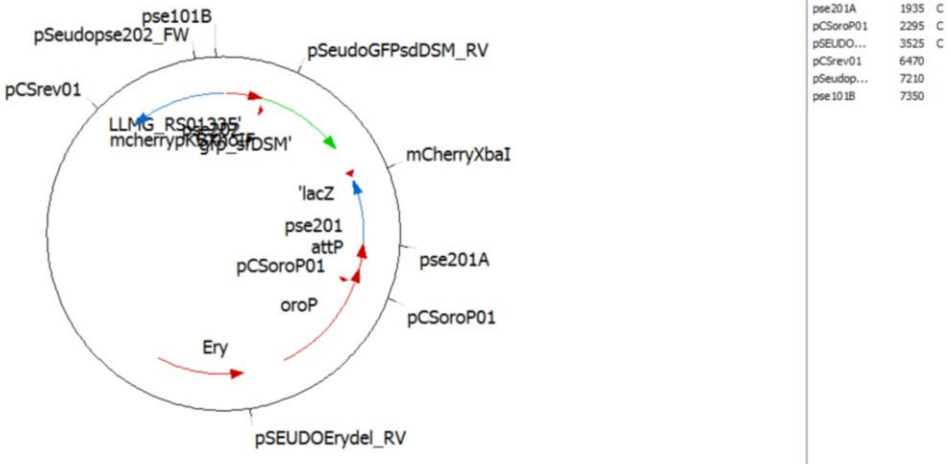


Figure9: List of enzymes mapped on Escherichia coli pSEUDO_GFP_sfDSM plasmid



Homologour recombination sites

Green fluorescent protein gene

Figure10: List of Primer sites mapped on Escherichia coli pSEUDO_GFP_sfDSM plasmid

8.3 COMPARING THE BENCH LAB PCR RESULTS WITH THE *INSILICO* ANALYSIS RESULTS

First, the promoters were amplified and isolated using the set of designed primers. The yield of the isolated DNA is very important to determine the purity of the DNA is usually obtained by measuring the absorbance at 260/280 nm. Generally a ratio of ~ 1.8 is considered to be the pure DNA. In our case all the samples absorbance ratio were averaged to 1.9 attaining a yield ranged from 200 – 450ng/mL. The size of amplified PCR products were analyzed on 1% agarose gel *(figure 11)*. On comparison with the product size obtained by the clone manager software, we found that most of the selected upregulated promoters such as ButA/B, and llmg_*llmg_1772* that are responsive to acetaldehyde were corresponding to the desired band size *(figure 12)*. Promoters such as llmg_*llmg_1019*, and DhaM that are responsive to diacetyl *(figure 13)* and llmg_*llmg_0317*, llmg_*llmg_0941*, llmg_*llmg_1677* promoters that are responsive to ethanol were also corresponding to the desired band size *(figure 14)*. However, for the promoters that failed to get amplified, annealing temperature was varied by ± 1°C and performed again. Even then, few promoters failed to get amplified such as *llmg_1993*, MalF, CopR, 0146, for such promoters, new set of primers has to be designed. Table represents all the promoters that were amplified

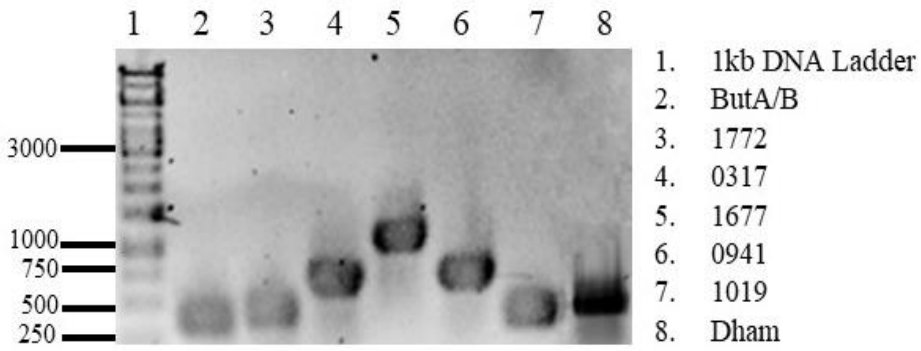


Figure11: Representative image of an agarose gel electrophoresis of amplified and undigested promoter sequences isolated from the genomic DNA where 3-4 µg of the sample was loaded

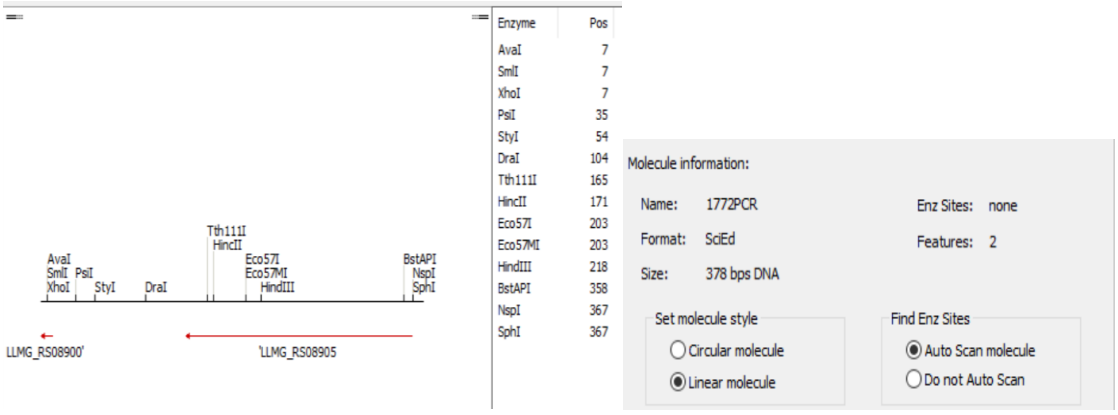


Figure12: Representative image of amplified llmg__1772 promoter gene responsive to Acetaldehyde

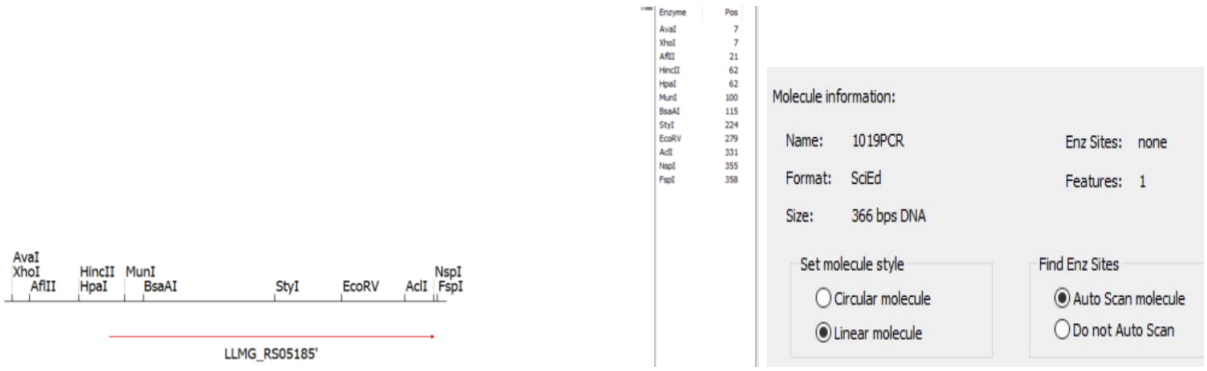


Figure13: Representative image of amplified llmg_llmg_1019 promoter gene responsive to Diacetyl

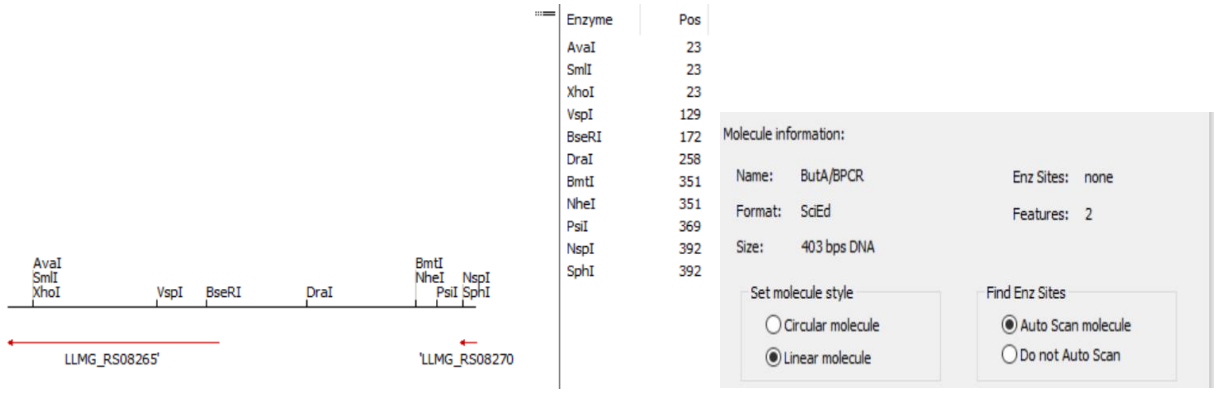


Figure14: Representative image of amplified ButA/B promoter gene responsive to Ethanol

Responsive Promoter	Compound	Desired Band Size
dhaM	Diacetyl	357bp
llmg_1019	Diacetyl	366bp
butA/B	Acetaldehyde	403bp
llmg_1772	Acetaldehyde	378bp
llmg_0317	Ethanol	600bp
llmg_1677	Ethanol	999bp
llmg_0941	Ethanol	663bp
llmg_0146	Ethanol	412bp
copR	Acetaldehyde	351bp
malF	Acetaldehyde	419bp

llmg_1993	Acetaldehyde	336bp

Table6: List of responsive promoters with their corresponding band size

The amplified promoters were subsequently digested using restriction enzymes *XhoI and SphI* to obtain the fragmented DNA. The plasmid was digested with one set of the enzymes such as *XhoI, SphI*, and *PstI* at one time. The plasmid was also digested with both enzymes *XhoI and* were analyzed on 1% agarose gel performed to check the activity of the enzymes *(figure 15). PstI* was used as it has three restriction sites therefore can be used as the control for the other two enzymes. The gel electrophoresis shows that the enzymes are functionally active. However, the size of the band of plasmid digested by both enzymes has high difference between the single digested plasmids. Since restriction digestion results in removal of few base pairs, the difference between single digestion and double digestion should not be much. It is also observed, the difference between the band size of the uncut plasmid and single digested plasmid is negligible. From this observation we can establish that enzymes are functional.

The same agarose gel *(figure 16)* also demonstrates the band size of the amplified and digested promoters such as dhaM, butA/B, copR, malF. All corresponds to the desired band size.

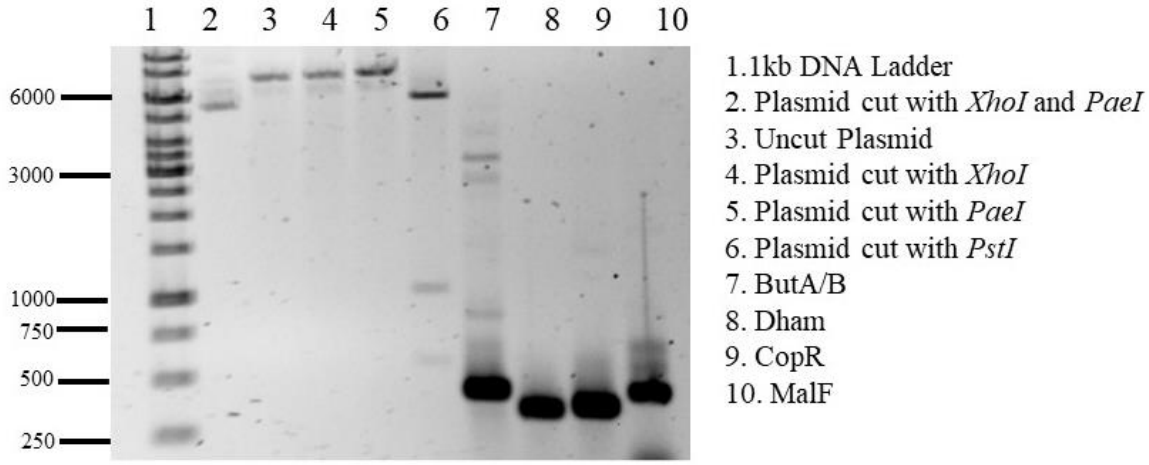


Figure15: 1% Agarose gel electrophoresis of plasmid single digested and double digested (2-6) and amplified restricted digested promoter sequences (7-10) isolated from the genomic DNA where 5-6 µg of the sample was loaded.

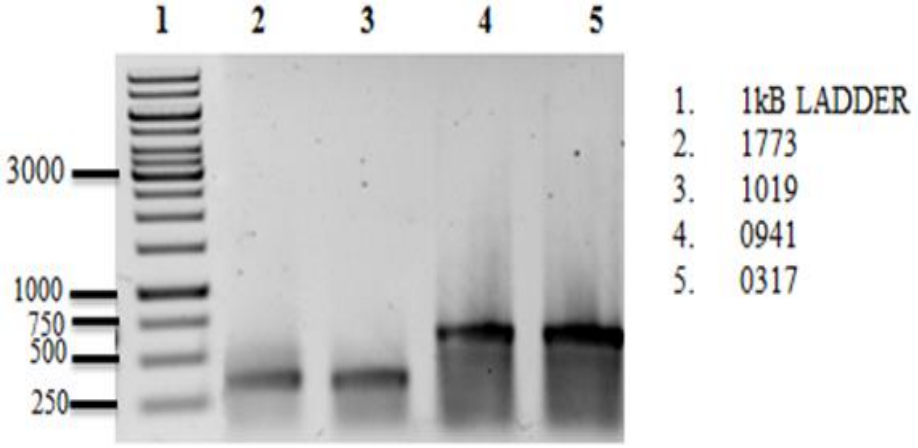
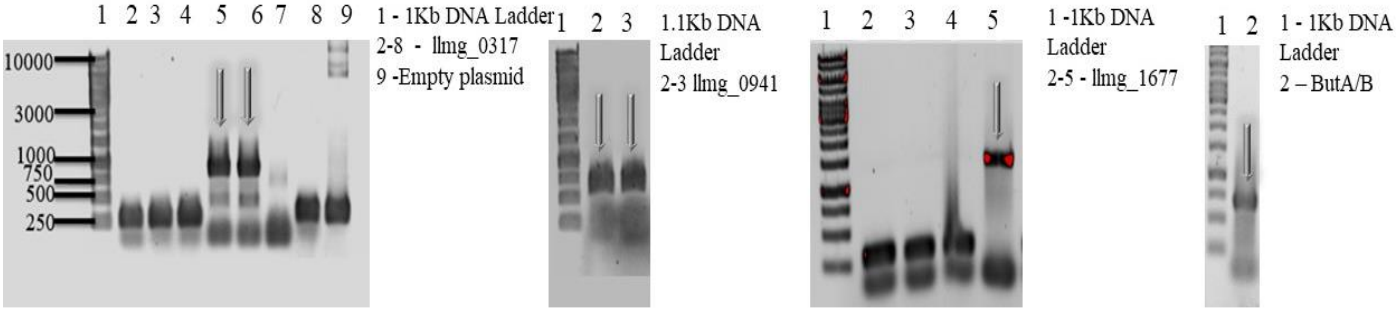


Figure16: Representative image of an agarose gel electrophoresis of amplified and digested promoter sequences isolated from the genomic DNA where 5-6 µg of the sample was loaded.

8.4 TRANSFORMATION

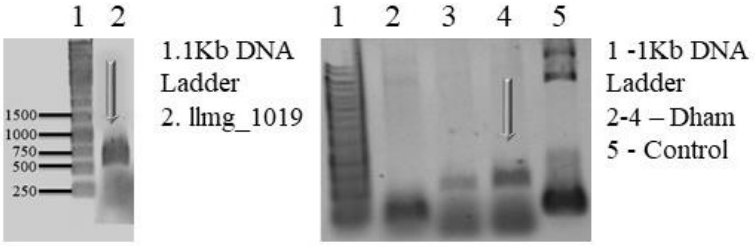
To be able to check the response of the promoter to the compound of interest, the promoter should be integrated into *Escherichia coli* pSEUDO-gfp plasmid followed by chromosomal integration into *Lactococcus lactis* MG1363. Since the integration in the chromosomal genome gives a copy number of 1 and in order to obtain as many copies of the required construct with less false positive constructs, the desired promoter is incorporated first into pSEUDO-gfp plasmid. This transformation gives high copy number and are the colonies are selected against erythromycin resistant plasmid. In our study, the gel electrophoresis revealed that the colony PCR gave desired product, and also an empty E.coli pSEUDO-gfp plasmid as control. Promoters responsive to ethanol, *llmg_0317, llmg_0941, llmg_1677 and butA/B* showed a desired band size on 1% agarose

gel *(Figure 17).* Promoters responsive to diacetyl, *llmg_1019 and dhaM (Figure18)* along with *butA/B and llmg_1776 (Figure19)* that are responsive to acetaldehyde also showed the desired band size when ran on 1% agarose gel. The arrows indicate the presence of desired band size of the promoters with the gfp. These results were then compared with the data generated using clone manager and were observed to be the same. However, the plasmid DNA were isolated and sent to Macrogen for sequencing analysis.



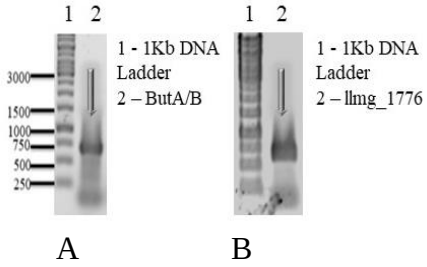
A B C D

Figure17: Representative image of Gel electrophoresis of colony PCR of the Escherichia coli transformants responsive to ethanol (A) Promote llmg_0317 (B)promoter llmg_0941 (C)Promoter llmg_1677 (D) Promoter butA/B



A B

Figure18: Representative image of Gel electrophoresis of colony PCR of the Escherichia coli transformant responsive to diacetyl. (A) Promote llmg_1019 (B)promoter dhaM



A B

Figure19: Representative image of Gel electrophoresis of colony PCR of the Escherichia coli transformant responsive to diacetyl. (A) Promote butA/B (B) Promoter llmg_1776

The sequence of the promoter was found in most of the sequenced samples, confirming the presence of desired promoter in the plasmid. Those promoters such as, malF, copR, *llmg_1993*, llmg*_0416, llmg_2369, llmg_0295* whose sequences were not observed in the sequencing results, yet showed the presence product on the gel and also the colonies appeared fluorescent under ultraviolet light were repeated. Attempts were however still made to transform the plasmid with few changes such as varying the annealing temperature between 47°C - 50°C, increasing the quantity of restriction buffer during digestion, using 100mM ATP to increase the activity of ligase enzyme during ligation (Attachment), but no transformants were obtained. Further attempts were still made in designing new primers for the promoters such as *llmg_0295,* however no analyses were performed to check the functionality of the newly designed primers.

After confirming the promoter has been successfully integrated into the plasmid, plasmid DNA was isolated, purified and transformed into the genome of *L.lactis* MG1363. The transfer of extracellular DNA into the genome is known as horizontal gene transfer in prokaryotes. As visualized in the agarose gel *(figure 20),* all the *E.coli* transformants has been successfully integrated into *L.lactis* MG1363. This type of transferring the extracellular DNA into the chromosomal genome is known as horizontal gene transfer. The DNA was isolated and was sent to Macrogen for sequence analysis. The sequence of the promoter with gfp was observed in the sequenced samples confirming successful genomic integration.

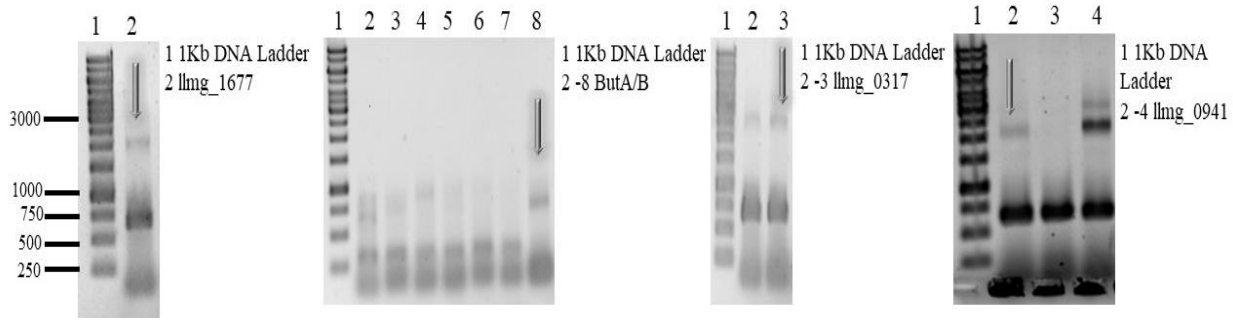
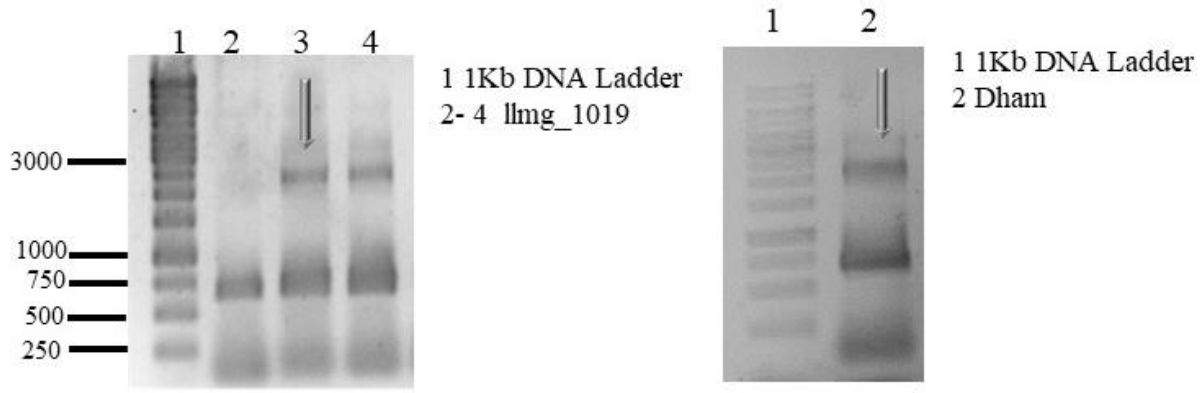


Figure20: Representative image of Gel electrophoresis of colony PCR of the Lacctococcus lactis MG1363 transformant responsive to ethanol. (A) Promoter llmg _1677 (B) Promoter butA/B (C)Promoter llmg_0317 (D) Promoter llmg_0941



A B

Figure21: Representative image of Gel electrophoresis of colony PCR of the Lacctococcus lactis MG1363 transformant responsive to diacetyl. (A) Promoter llmg _1019 (B) Promoter dhaM

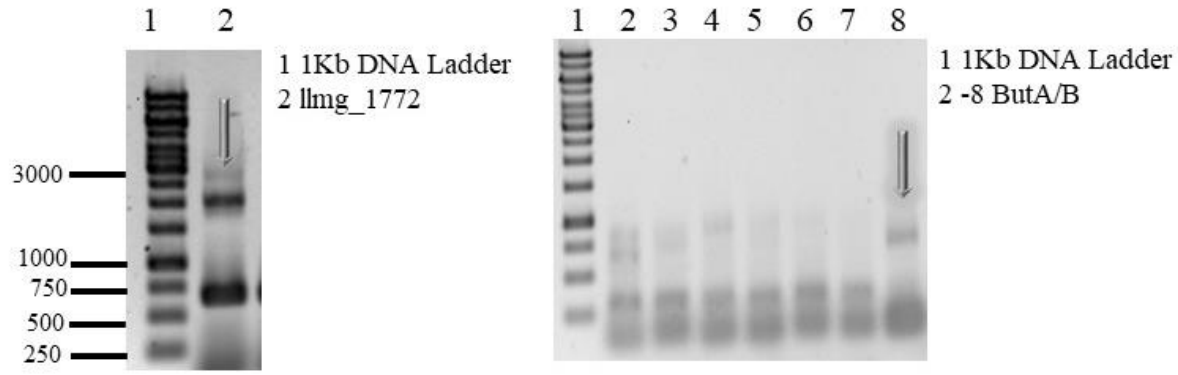


Figure22: Representative image of Gel electrophoresis of colony PCR of the Lacctococcus lactis MG1363 transformant responsive to acetaldehyde (A) Promoter llmg _1772 (B) Promoter butA/B

Chromosomal integration of DNA within the species is possible due to homologous recombination, in which the plasmid DNA has sequence homology with the genomic sequence. However in our study, there are possibilities that the plasmid gets integrated at one end of the homologous region and the promoter is capable of initiating transcription and expression of the fluorescent gene in response to the compound of choice. This explains why two bands of different sizes are observed in the gel picture. In such cases the plasmid are still attached to the genome of *L.lactis*, and the primer is capable of amplifying the homologous region present on the plasmid thus giving a band of smaller size. Larger sized band corresponds to the size of the promoter, reporter gene gfp and the other end of the homologous region. Presence of overhanging plasmid decreases the stability of the bacteria as the genome lack erythromycin resistance gene. Therefore, its preferred to have an indicator cell in which the plasmid has been completely removed. However, to select the cells in which the plasmid vector backbone is fully removed is again a challenge.

L.lactis MG1363 lack oroP transporter gene because of which 5 – Fluoro-orotate is not transported inside the cell. 5 – Fluoro-orotate when present inside lactis is lethal to the cells and kills the

organisms. Hence, the indicator cell that is devoid of the plasmid survives when treated with 5 – fluoro- orotate and the cells that has the plasmid dies to the uptake of 5 – fluoro – orotate. The gel electrophoresis revealed that colony PCR gave desired band size. Although not all the constructs that were observed to be positive from sequencing analysis showed the desired band size *(figure23).* Table 7 differentiates between the construct that has been integrated at one end and the contruct that has been integrated at both ends of homologous regions. However, the DNA was not isolated and sent for sequence analysis, therefore it not confirmed if the sequenced samples has the desired promoter sequence and gfp. The gel picture shows the presence of single band for few indicator cells and two bands for other indicator cells. The presence of single band indicates that the plasmid has been excised from the genome thus ensuring the recombination at both the ends of the genome.

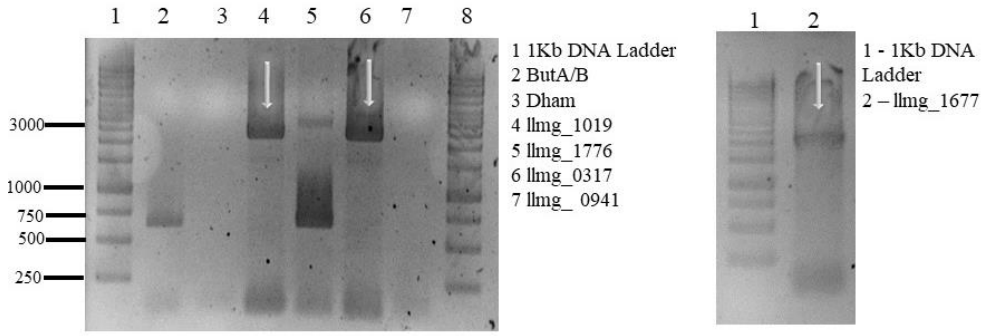


Figure22: Selection of indicator cells devoid of plasmid vector by selecting against 5-Fluoroorotate

Construct	Single Recombination	Double Recombination
L.Lactis MG163 pSEUDO::PbutA/B-sf gfp	✓	×
L.Lactis MG163 pSEUDO:: PdhaM-sf gfp	✓	×
L.Lactis MG163 pSEUDO:: Pllmg_1019-sf gfp	✓	✓
L.Lactis MG163 pSEUDO:: Pllmg_1772-sf gfp	✓	×
L.Lactis MG163 pSEUDO:: Pllmg_0317-sf gfp	✓	✓
L.Lactis MG163 pSEUDO:: Pllmg_0941-sf gfp	✓	×
L.Lactis MG163 pSEUDO:: Pllmg_1677-sf gfp	✓	✓

Table7: Integration of E.coli plasmid containing desired promoter into the genome of L.lactis MG1363 by homologous recombination

8.5 CHARACTERIZATION OF STRAINS:

The expression of the reporter gene gfp facilitated by the promoter in response to organic compounds such as ethanol, acetaldehyde, diacetyl is quantified by measuring its fluorescence intensity. We measured the fluorescence intensity of the indicator cells integrated with the promoter responsive to ethanol using plate reader and found there were lot of variations in the fluorescence intensity *(Figure23 -25).* This is probably due to the background noise. However, we still tried to interpret the absorbance data. We analyzed the growth pattern of bacteria when exposed to ethanol and found that it grows at an exponential rate from 5th hour of the incubation with CDMPC media supplemented with 0.5% glucose.

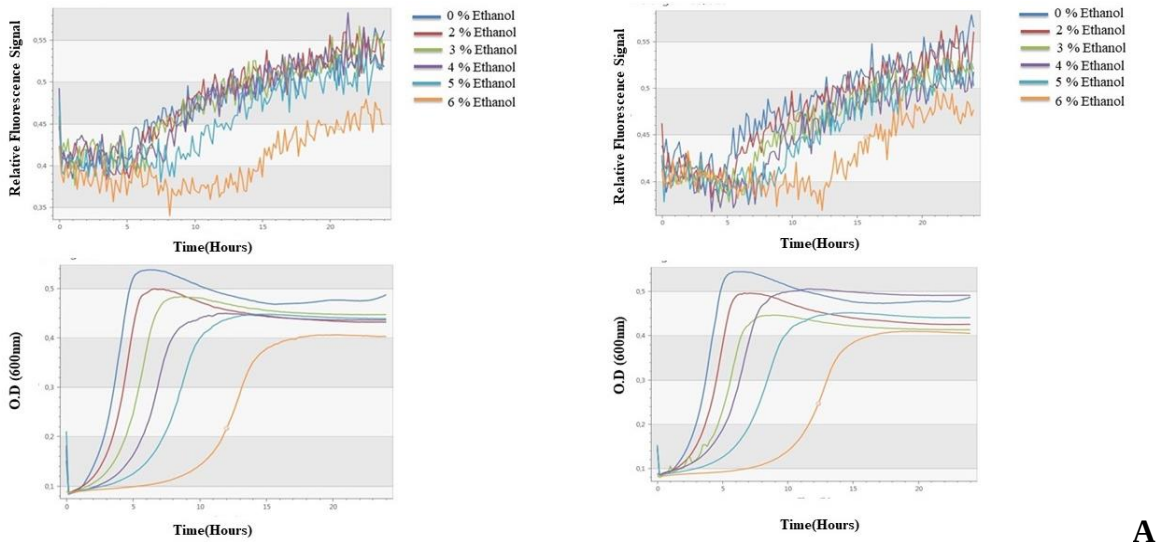
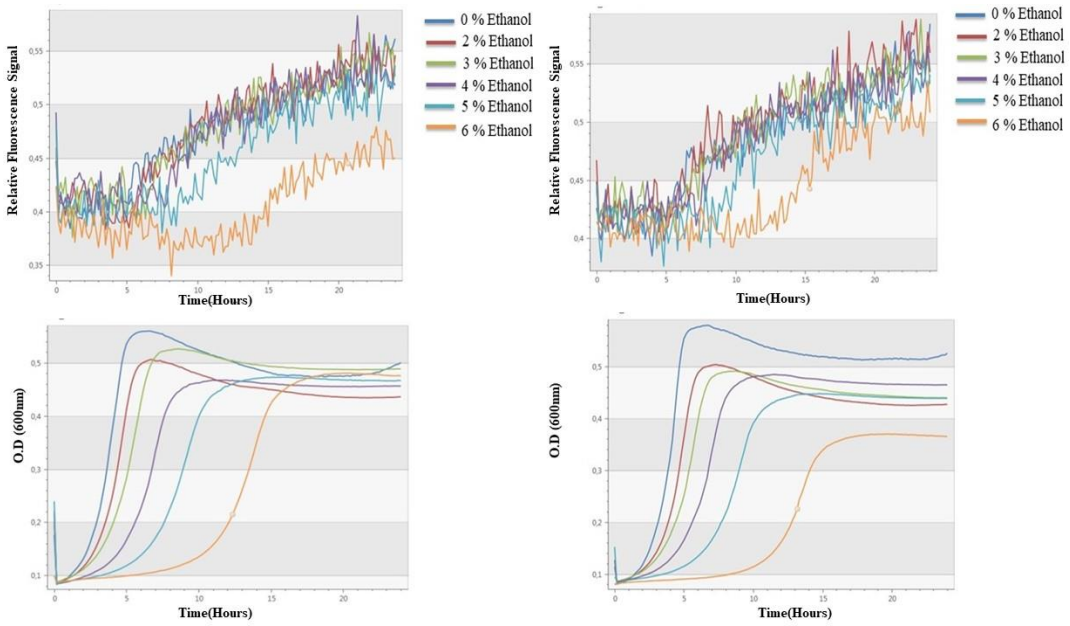


Figure23: Fluorescence intensity and Growth pattern of indicator cell consisting of (A) ButA/B and (B) llmg_0941 promoter when exposed to different concentration of ethanol



A **B**

Figure24: Fluorescence intensity and Growth pattern of indicator cell consisting of (A) llmg_0317 and (B) 1677 promoter when exposed to different concentration of ethanol

Similar growth pattern of bacteria was observed when exposed to acetaldehyde and diacetyl. *(Attachment)*. Therefore, with this interpretation we measured the fluorescence intensity of the indicator cell by treating with different organic compounds such as acetaldehyde, diacetyl and ethanol by FlowCytometry. It was observed that butA/B is responsive to both acetaldehyde and ethanol, therefore the promoter was exposed to both the compounds.

Based on the transcriptomic analysis carried out before the start of this study, it was found that *llmg_1019* and dhaM promoters respond to diacetyl compound and showed a tolerance level of 0.03%. However, to be sure that the fluorescence measured is due to the expression of gfp solely under the influence of the promoter and not because of the compound, other promoters such as Pusp45, Pdar1 and P32 present in *L.lactis* were used as control and were also exposed to 0.03% diacetyl. P32 is known to participate in the glycolysis metabolism. The aforementioned control promoters are known to emit fluorescence without any analyte (organic compound) required to stimulate the promoter to initiate transcription. As observed from the graphs below *llmg_1019 (figure 25)* and dhaM *(figure 26)* exhibit fluorescence signal in the presence of the compound whilst the signal is minimum or negligible in the absence of diacetyl. The fluorescence signal was compared against the control promoter Dar1 and P32. *(Figure 27)* shows the mean fluorescence intensity of the promoter *llmg_1019* and dhaM

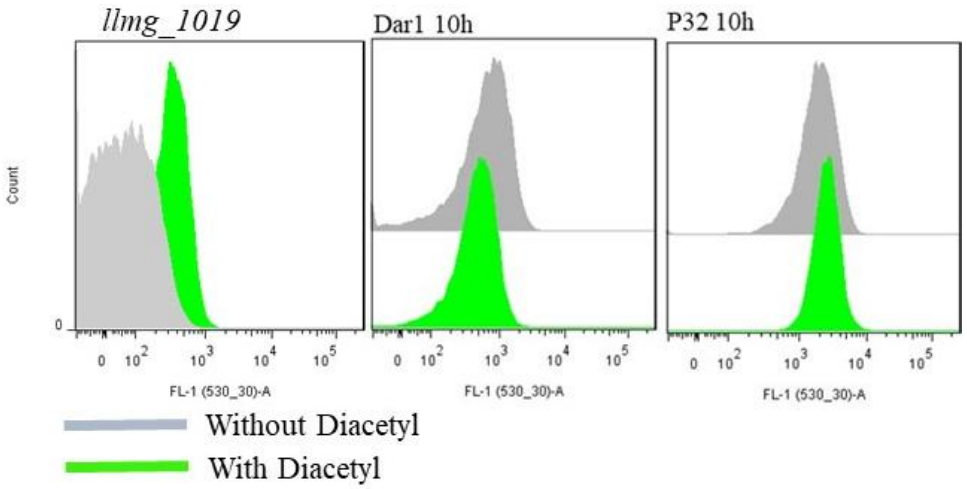


Figure25:Fluorescence intensity of indicator cell infused with llmg_1019 promoter in the presence and absence of diacetyl

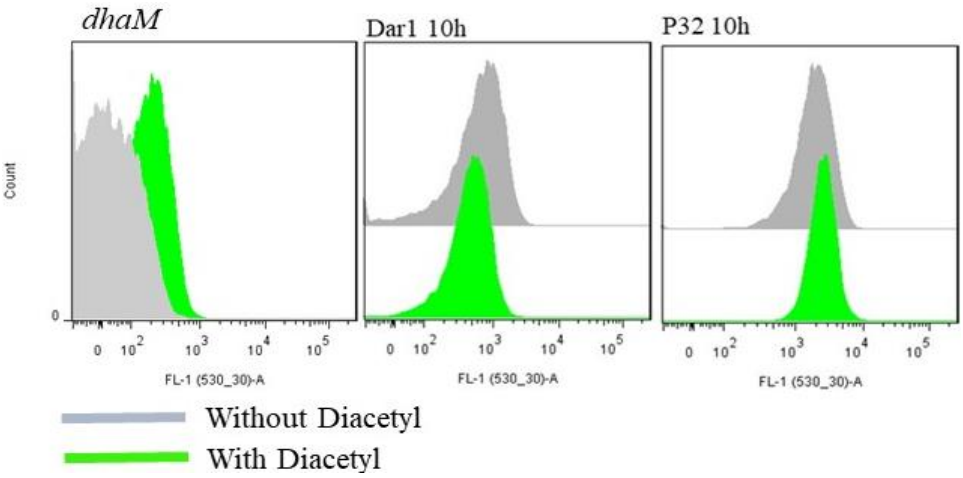


Figure26:Fluorescence intensity of indicator cell infused with dhaM promoter in the presence and absence of diacetyl

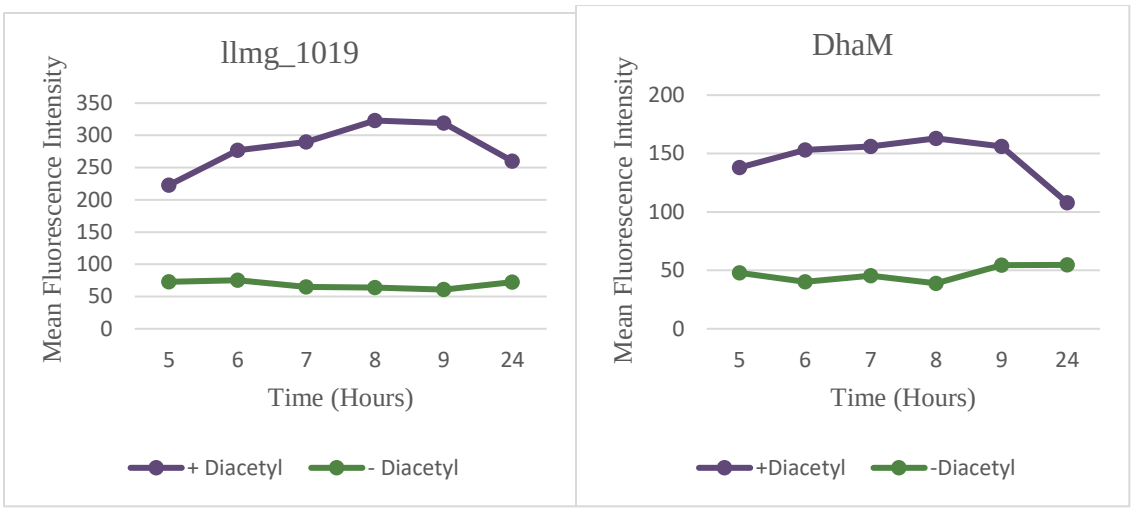


Figure 27: Graphical Representation of mean fluorescence intensity of llmg_1019 and dhaM promoter in the presence and absence of 0.03% diacetyl

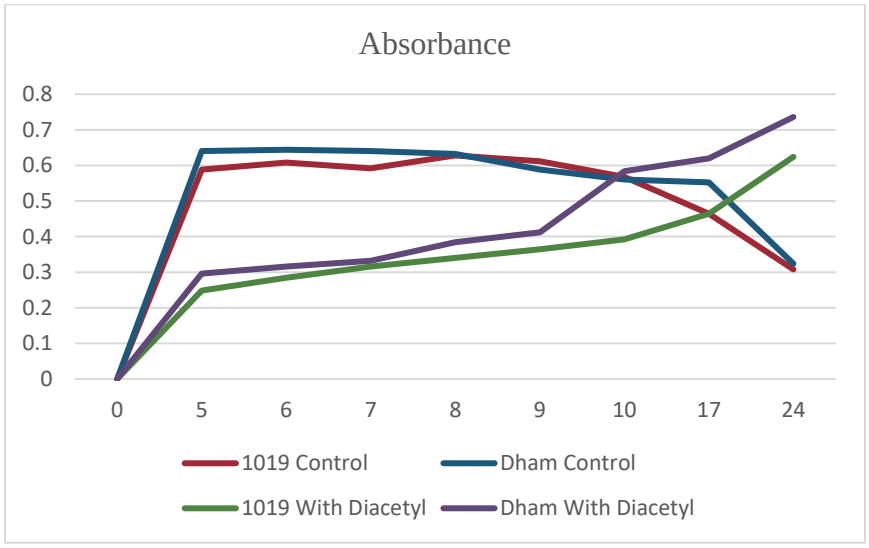


Figure 28: Graphical Representation of absorbance (600nm) of llmg_1019 and DhaM promoter in the presence and absence of diacetyl, thereby indicating the growth pattern of the bacteria

From the *Figure 28* it is observed that the cells continue to grow in the presence of 0.03% diacetyl and the fluorescence is due to the bacterial cells and not due the cell debris, formed in the case of bacterial cell death.

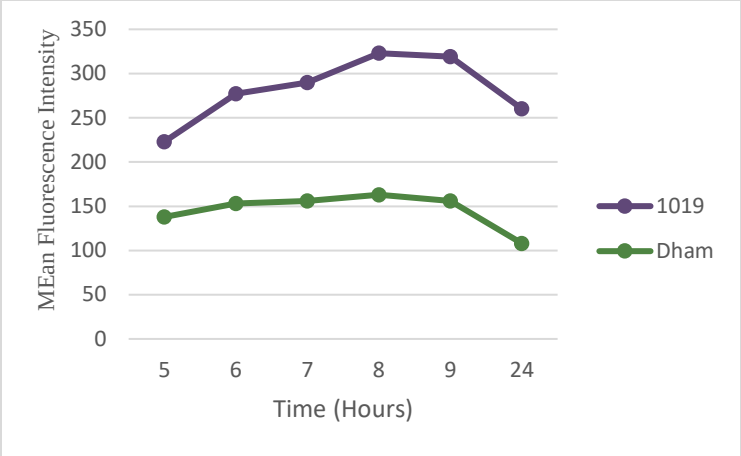


Figure29: Comparison between the mean fluorescence intensity of llmg_1019 and DhaM promoter in the presence of diacetyl.

The graph *(Figure 29)* reveals that *llmg_1019* exhibits significantly high fluorescence signal compared to DhaM. This result can be further supported by comparing the signal with the control promoter. The control promoter shows negligible difference in signal in the presence and absence of the compound. Earlier it was mentioned that plasmid backbone has to be excised from the genome of *L. lactis* while integration and it was found that *llmg_1019* construct has successful excision of the plasmid backbone and the promoter and gfp has been integrated in genome of *L. lactis* within their homologous regions. This explains why *llmg_1019* has better signal intensity and dhaM failed to exhibit high signal intensity. However, cannot conclude that dhaM construct failure to undergo double recombination within the homologous regions of *L. lactis* has resulted in lower fluorescence signal intensity. It can also be that dhaM might require higher or lower diacetyl concentration in order to exhibit higher fluorescence signal or it does not respond to diacetyl as *llmg*_1019 does.

The same experimental procedure was followed for the promoters, responsive to ethanol. Transcriptomic analysis reveals the promoters have a tolerance limit of up to 4% towards ethanol.

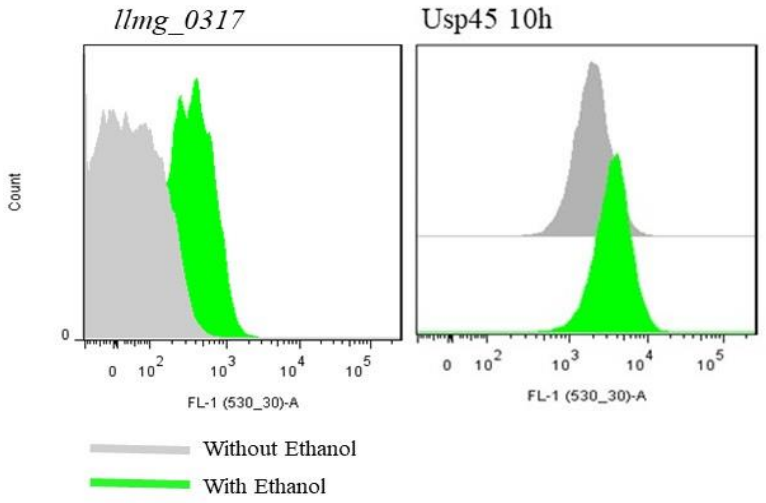


Figure 30: Fluorescence intensity of indicator cell infused with llmg_0341 promoter in the presence and absence of ethanol

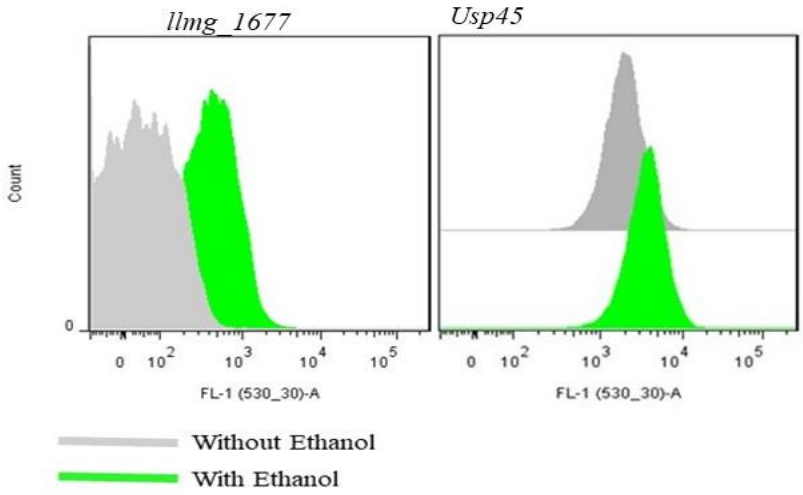


Figure31: Fluorescence intensity of indicator cell infused with llmg_1677 promoter in the presence and absence of ethanol

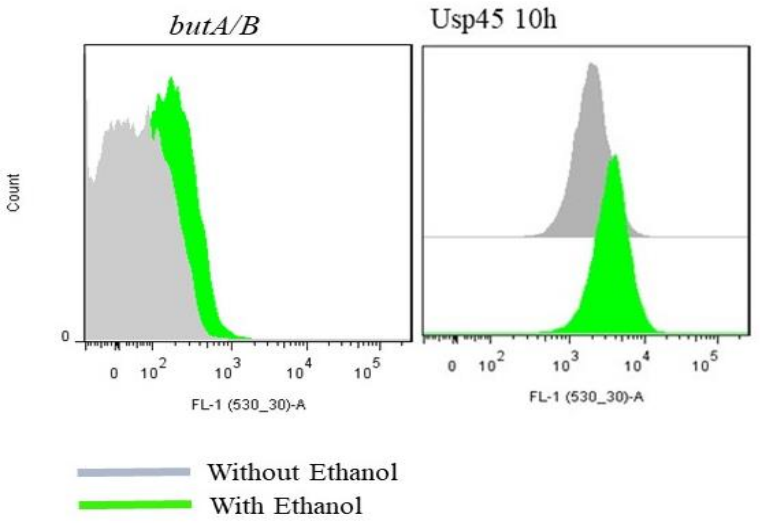


Figure32: Fluorescence intensity of indicator cell infused with butA/B promoter in the presence and absence of ethanol

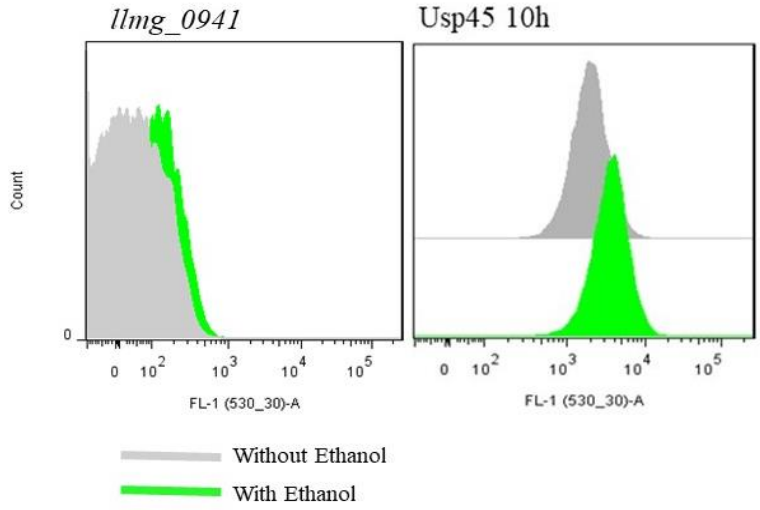
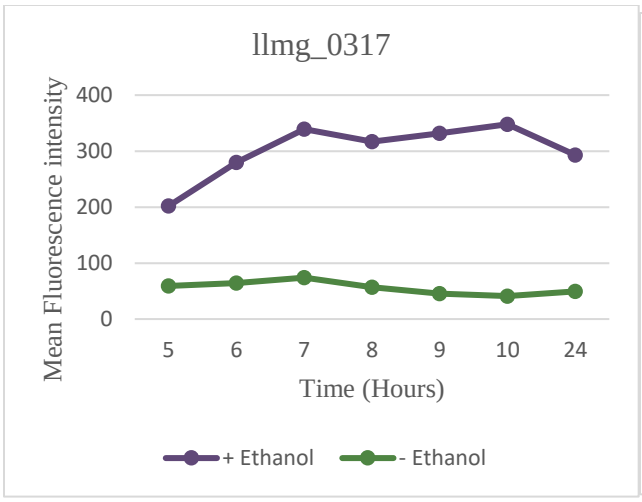
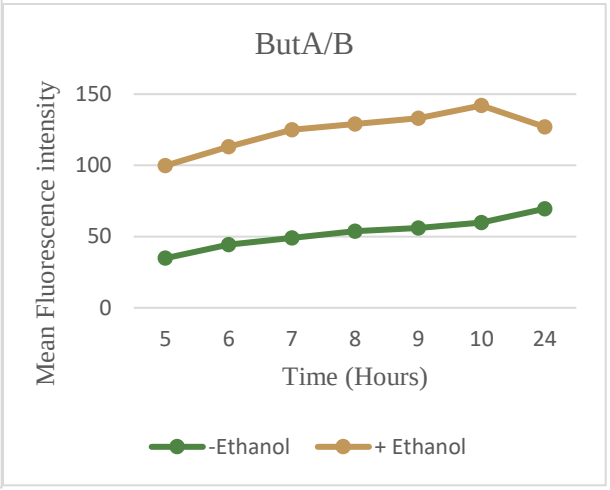


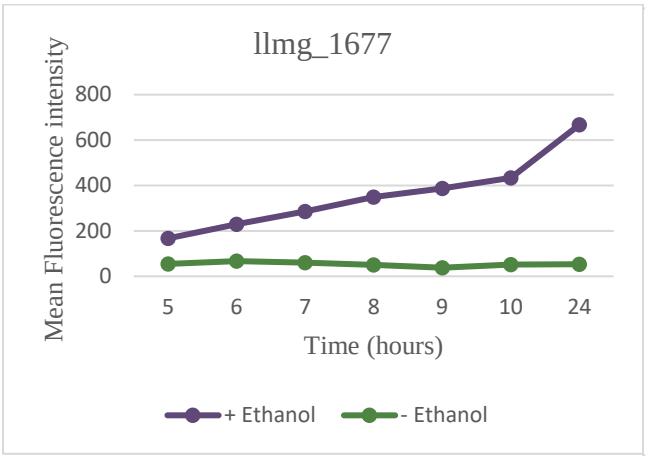
Figure33: Fluorescence intensity of indicator cell infused with llmg_0941 promoter in the presence and absence of ethanol



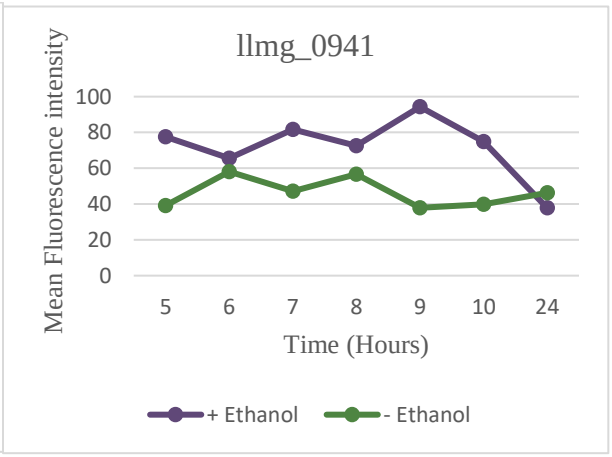
A



B



C



D

Figure 34: Graphical Representation of mean fluorescence intensity of (A)llmg_0317,(B) butA/B (C)llmg_1677, and (D) llmg_0941 promoter in the presence and absence of 4% ethanol

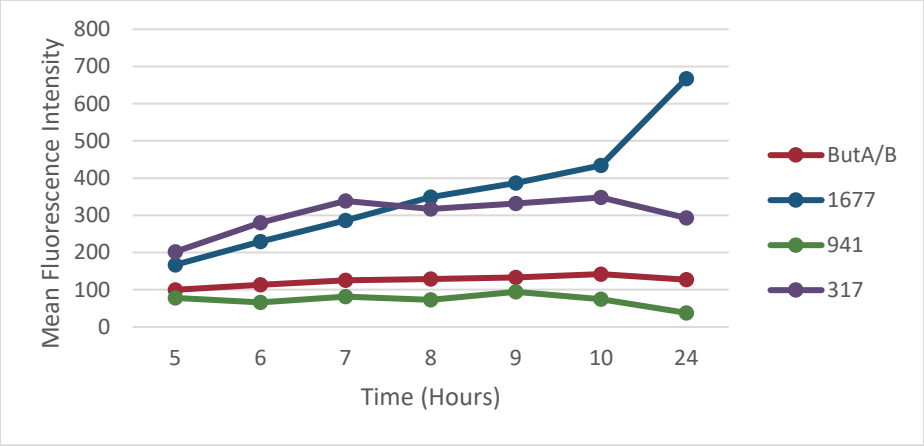


Figure 35: Comparison between the mean fluorescence intensity of different promoters that are responsive to Ethanol

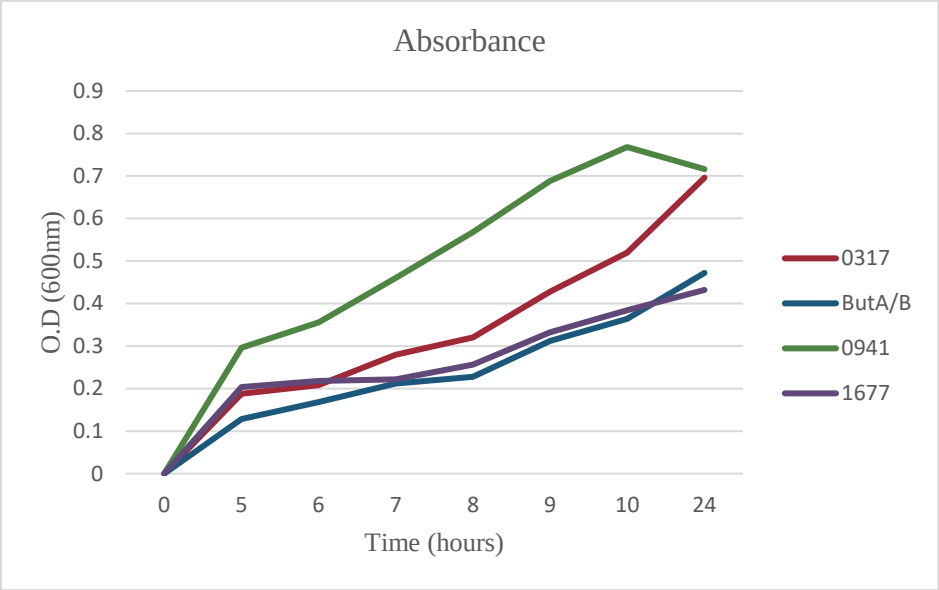


Figure 36: Graphical Representation of absorbance (600nm) of llmg_1677, llmg_0941, llmg_0941 and butA/B promoter in the presence and absence of diacetyl, thereby indicating the growth pattern of the bacteria

Unlike the response of the control promoters with diacetyl, their response with ethanol appears to be varying a lot, due to which Pusp45 was selected as control to be compared against the fluorescence signal of the indicator cells. *(Attachment).* Among the constructs that were identified to be responsive to ethanol, *llmg_0317 (figure 30)* and *llmg_1677 (figure 31)* promoter shows a substantial amount of fluorescence signal in comparison with butA/B *(figure 32)* and *llmg_0941* promoter *(Figure 33).* However, *llmg_1677* and *llmg_0317* exhibit a similar pattern and also the shift in the fluorescence peak of the control promoters in the presence of ethanol makes it difficult

to draw a conclusion *(Figure 36).* We know that *llmg_1677* and *llmg_0317* constructs has successfully integrated within the homologous regions of *L.lactis,* and supports the increased fluorescence signal from these promoters. From the *Figure 35*, it is clear that the cells are continuously growing.

In the case of indicator cells with promoters responsive to acetaldehyde whose tolerance limit was identified to be 2%, the growth of the bacteria remained minimum and failed to reach the exponential phase of their growth *(figure 41).* Acetaldehyde is volatile at ambient temperature and is a reducing agent thus oxidizing glucose present in the media. The availability of the carbon source to the bacterial cell decreases thus hindering their growth. However, there was significant amount of fluorescence signal produced by the cells even there was minimal growth. One possible explanation to this could be that the free radicals produced during the oxidation might interfere with fluorescence signal emission. However, both the promoters produce higher fluorescence signal in the presence of acetaldehyde than in the absence of acetaldehyde *(figure 37-39)*

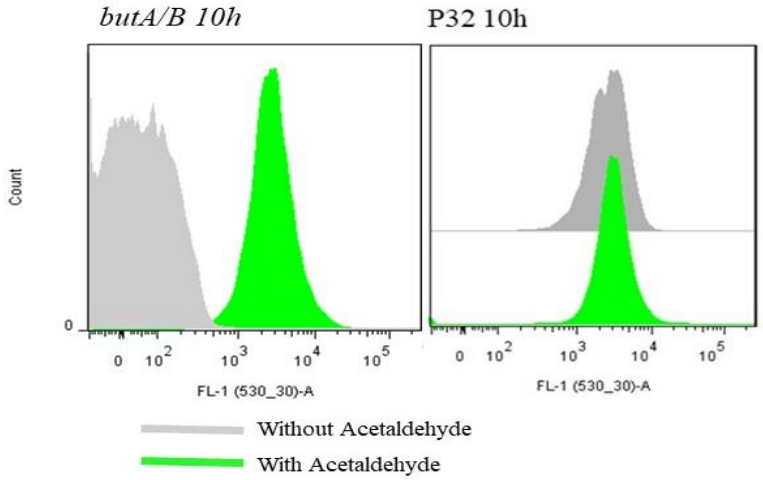


Figure37: Fluorescence intensity of indicator cell infused with butA/B promoter in the presence and absence of acetaldehyde

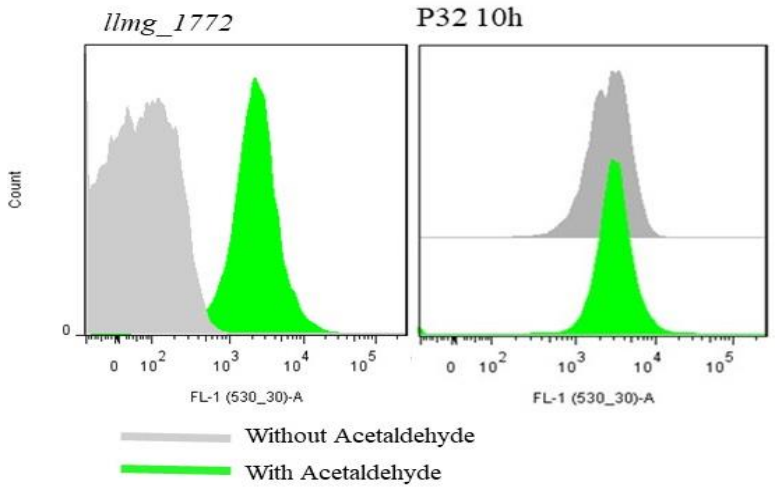


Figure38: Fluorescence intensity of indicator cell infused with llmg_1019 promoter in the presence and absence of acetaldehyde

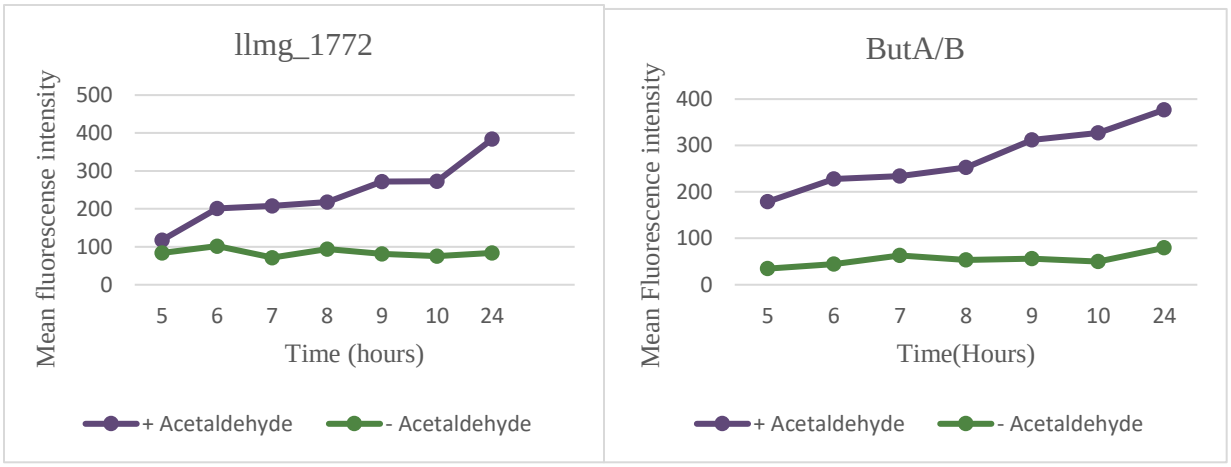


Figure 49: Graphical Representation of mean fluorescence intensity of llmg_1772 and ButA/B promoter in the presence and absence of 2% acetaldehyde

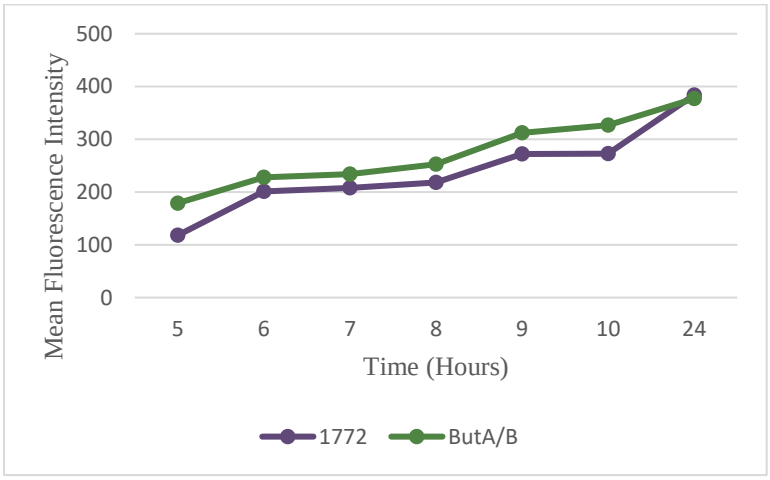


Figure 40: Comparison between the mean fluorescence intensity of llmg_1772 and But A/B promoters that are responsive to Acetaldehyde

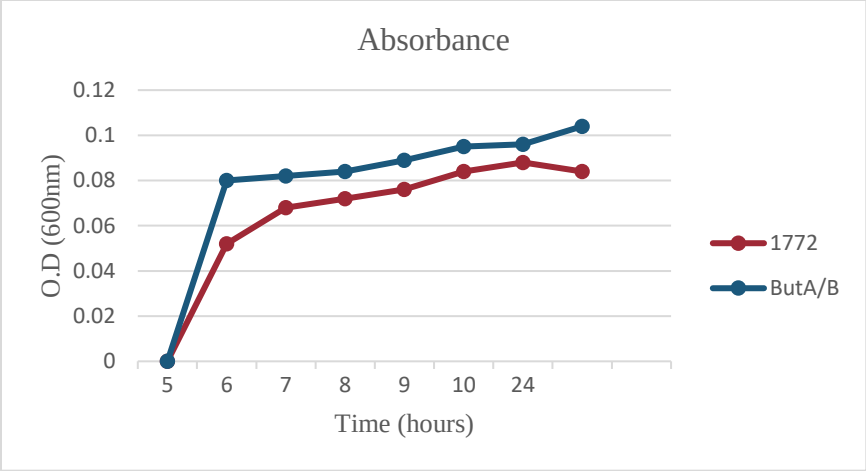


Figure 41: Graphical Representation of absorbance (600nm) of llmg_1019 and DhaM promoter in the presence and absence of diacetyl, thereby indicating the growth pattern of the bacteria

9 Conclusion

The efficient production of diacetyl, ethanol and acetaldehyde from glucose by *L.lactis* reported here is due to the development of an indicator cell with the insertion of a promter sequence and green fluroscent gene (gfp). Various promoters have been identified to be responsive to such compounds and in our study few of the promoters such as *butA/B, llmg_0317,llmg_0941, llmg_1667* responsive to ethanol, *llmg_1019, dhaM* responsive to diacetyl and *llmg_1772 and butA/B* responsive to acetaldehyde were successfully fused with the gfp gene to construct an indicator cell. The indicator cells when tested with the compound showed a lot of variations in the amount of signal generated. Promoters such as *llmg_1019, llmg_0317* and *llmg_1677* showed promising results while the rest of the promoters failed to show significant response to the compounds at their permissible concentrations. The other promoters that were identified by transcriptomic analysis to be responsive to compounds but yet failed to construct an indicator cell requires optimization of the primer to amplify these promoter sequences efficiently.

The development of such indicator cells in conjuction with microdroplet technology facilitate in identifying the most potient bacterial strains from environment and these strains in turn be used in the food industries to manufacture various dairy products and could have many applications for the development for tailored fermented products in the future.

Although few of the constructs measured significant fluorescence signal it is advisable to repeat the experiments to obtain more promising results. In addition, the glycerol stocks of the double recombination are not preserved therefore requires the repetition of the experiment and sequence analysis should be done to confirm the removal of the plasmid. However, indicator cells with promoter *llmg_1019, llmg_0317* and *llmg_1677* can be further processed by microdroplet technology for characterizing the strains.

10 References

1. Axelsson, L., & Ahrné, S. (2000). Lactic acid bacteria. In Applied microbial systematics (pp. 367-388). Springer Netherlands.
2. Ayad, E. H., Verheul, A., Wouters, J. T., & Smit, G. (2000). Application of wild starter cultures for flavour development in pilot plant cheese making. International Dairy Journal, 10(3), 169-179.
3. Blazeck, J., & Alper, H. S. (2013). Promoter engineering: recent advances in controlling transcription at the most fundamental level. Biotechnology journal, 8(1), 46-58.
4. Bongers, R. S., Hoefnagel, M. H., & Kleerebezem, M. (2005). High-level acetaldehyde production in Lactococcus lactis by metabolic engineering. Applied and environmental microbiology, 71(2), 1109-1113.
5. Carlos, C., Marco, V., Alexander, G., Michael, J., Oscar, P. and Jan, K., 2007. Complete Genome Sequence of the Prototype Lactic Acid Bacterium *Lactococcus lactis* subsp. cremoris MG1363.
6. Casey, J. U. D. Y., Daly, C. H. A. R. L. E. S., & Fitzgerald, G. F. (1991). Chromosomal integration of plasmid DNA by homologous recombination in Enterococcus faecalis and Lactococcus lactis subsp. lactis hosts harboring Tn919. Applied and environmental microbiology, 57(9), 2677-2682.
7. Cavanagh, D., Fitzgerald, G.F. and McAuliffe, O., 2015. From field to fermentation: the origins of *Lactococcus lactis* and its domestication to the dairy environment. Food microbiology, 47, pp.45-6.

8. De Jong, H., & Geiselmann, J. (2014). Fluorescent Reporter Genes and the Analysis of Bacterial Regulatory Networks. In International Workshop on Hybrid Systems Biology (pp. 27-50). Springer, Cham.
9. De Vuyst, L., & Leroy, F. (2007). Bacteriocins from lactic acid bacteria: production, purification, and food applications. Journal of molecular microbiology and biotechnology, 13(4), 194-199.
10. El‐Zahar, K., Chobert, J.M., Sitohy, M., Dalgalarrondo, M. and Haertlé, T., 2003. Proteolytic degradation of ewe milk proteins during fermentation of yoghurts and storage. Molecular Nutrition & Food Research, 47(3), pp.199-206.
11. Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic acid bacteria as source of functional ingredients. In Lactic Acid Bacteria-R & D for Food, Health and Livestock Purposes. InTech.
12. Gasson, M. J. (1983). Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. Journal of bacteriology, 154(1), 1-9.
13. Higa, T., & Kinjo, S. (1989, October). Effect of lactic acid fermentation bacteria on plant growth and soil humus formation. In Proc. 1st. Intl. Conf. on Kysuei Nature Fanning (pp. 140-147).
14. Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., de Vos, W. and Hols, P., 2000. *Lactococcus lactis* as a cell factory for high-level diacetyl production. Applied and environmental microbiology, 66(9), pp.4112-4114.
15. Kleerebezemab, M., Hols, P. and Hugenholtz, J., 2000. Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. Enzyme and microbial technology, 26(9), pp.840-848.
16. König, H., & Fröhlich, J. (2009). Lactic acid bacteria. Biology of Microorganisms on Grapes, in Must and in Wine, 3-29.
17. Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology, 15(2), 67-78.
18. Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., ... & Shakhova, V. (2006). Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Sciences, 103(42), 15611-15616.
19. National Research Council, 1992. Applications of biotechnology in traditional fermented foods. National Academies Press.
20. Pedersen, M. B., Iversen, S. L., Sørensen, K. I., & Johansen, E. (2005). The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS microbiology reviews, 29(3), 611-624.
21. Pinto, J. P., Zeyniyev, A., Karsens, H., Trip, H., Lolkema, J. S., Kuipers, O. P., & Kok, J. (2011). pSEUDO, a genetic integration standard for Lactococcus lactis. Applied and environmental microbiology, 77(18), 6687-6690.

22. Quintans, N.G., Blancato, V., Repizo, G., Magni, C. and López, P., 2008. Citrate metabolism and aroma compound production in lactic acid bacteria. Mol. Aspects Lactic Acid Bacteria Tradit. New Appl, pp.65-88.
23. Ross, R.P., Stanton, C., Hill, C., Fitzgerald, G.F. and Coffey, A., 2000. Novel cultures for cheese improvement. Trends in Food Science & Technology, 11(3), pp.96-104.
24. Sekhon, S. S., Ahn, J. Y., Ahn, J. M., Park, J. M., Min, J., & Kim, Y. H. (2014). Stress specific *Escherichia coli* biosensors based on gene promoters for toxicity monitoring. Molecular & Cellular Toxicology, 10(4), 369-377.
25. Solem, C., Dehli, T., & Jensen, P. R. (2013). Rewiring Lactococcus lactis for ethanol production. Applied and environmental microbiology, 79(8), 2512-2518.
26. Song, A. A. L., In, L. L., Lim, S. H. E., & Rahim, R. A. (2017). A review on Lactococcus lactis: from food to factory. Microbial cell factories, 16(1), 55.
27. Su, L., Jia, W., Hou, C., & Lei, Y. (2011). Microbial biosensors: a review. Biosensors and Bioelectronics, 26(5), 1788-1799.
28. Thomas, T. D., Ellwood, D. C., & Longyear, V. M. C. (1979). Change from homo-to heterolactic fermentation by Streptococcus lactis resulting from glucose limitation in anaerobic chemostat cultures. Journal of bacteriology, 138(1), 109-117.
29. Todar, K. (2012). Lactococcus lactis: nominated as the Wisconsin State Microbe. nd (ca. Dec. 2009) http://www. textbookofbacteriology. net/featured_microbe. html.
30. Turner, A., Karube, I., & Wilson, G. S. (1987). Biosensors: fundamentals and applications. Oxford university press.
31. van Hylckama Vlieg, J. E., Rademaker, J. L., Bachmann, H., Molenaar, D., Kelly, W. J., & Siezen, R. J. (2006). Natural diversity and adaptive responses of *Lactococcus lactis*. Current Opinion in Biotechnology, 17(2), 183-190.
32. Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C. & van Sinderen, D. (2007). Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. cremoris MG1363. *Journal of bacteriology*, *189*(8), 3256-3270.
33. Zhang, F., Carothers, J. M., & Keasling, J. D. (2012). Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nature biotechnology, 30(4), 354-359.
34. Zhang, J., Jensen, M. K., & Keasling, J. D. (2015). Development of biosensors and their application in metabolic engineering. Current opinion in chemical biology, 28, 1-8.
35. A Halász - Food Quality and Standards, 2009
36. "Anaerobic respiration." Wikipedia. September 9, 2015. Accessed September 16, 2015. https://en.wikipedia.org/wiki/Anaerobic_respiration.
37. "Metabolism without oxygen," in Principles of Biology, by Robert Bear, David Rintoul, Bruce Snyder, Martha Smith-Caldas, Christopher Herren, and Eva Horne, CC BY 4.0.

11. APPENDIX

- Fluorescence signal of indicator cells with desired promoters was measured using plate reader

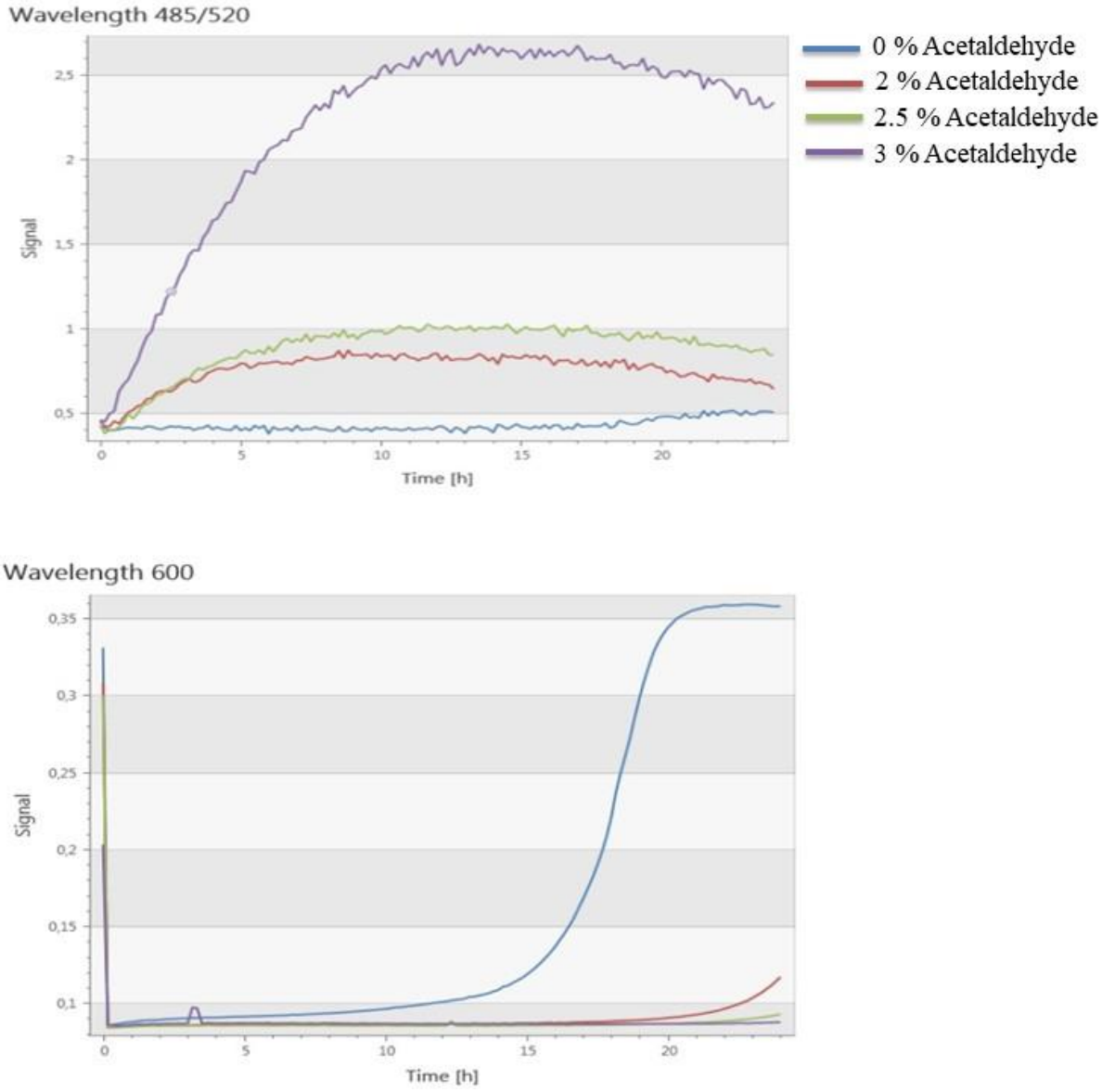


Figure43: Fluorescence intensity and Growth pattern of indicator cell consisting of llmg_1776 promoter when exposed to different concentration of acetaldehyde

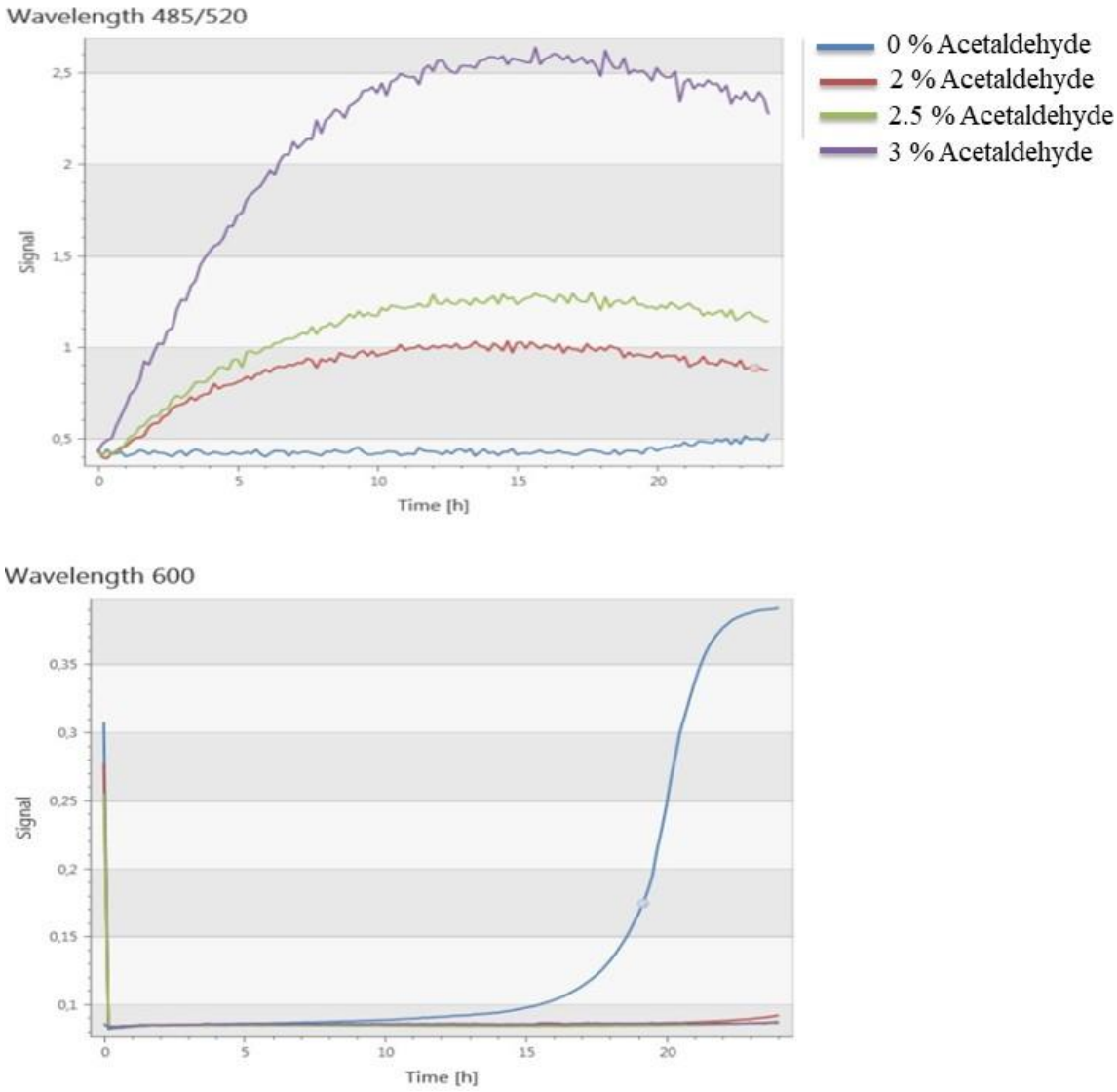


Figure44: Fluorescence intensity and Growth pattern of indicator cell consisting of ButA/B promoter when exposed to different concentration of acetaldehyde

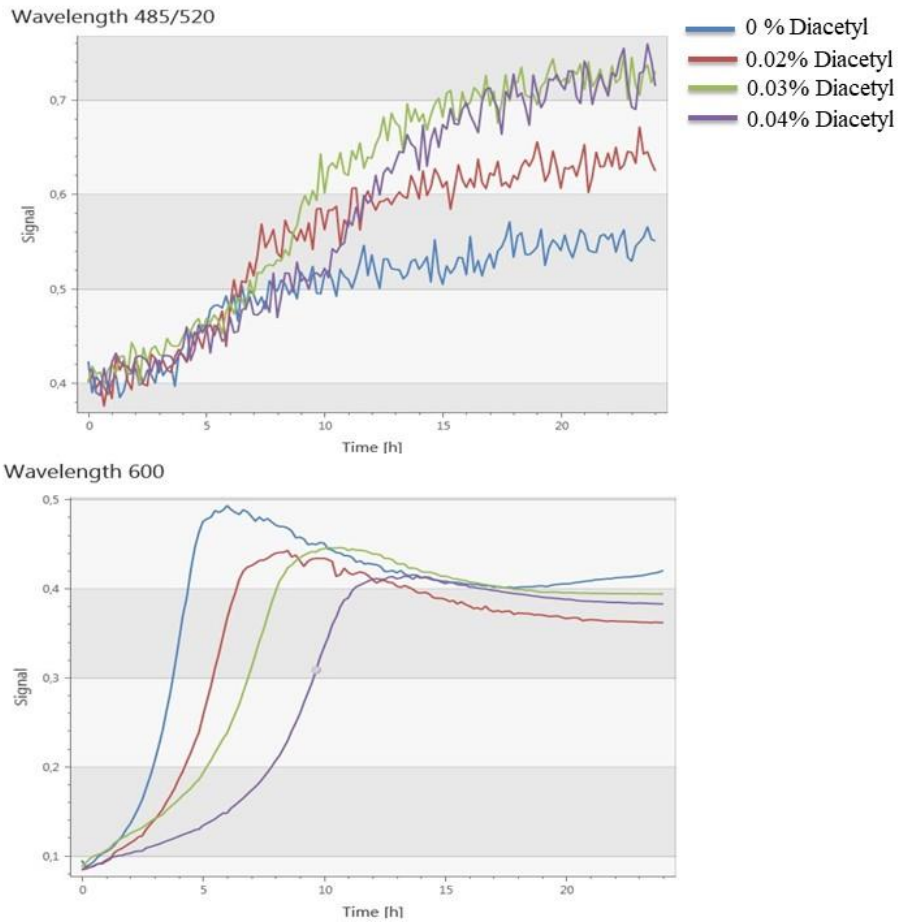


Figure45: Fluorescence intensity and Growth pattern of indicator cell consisting of DhaM promoter when exposed to different concentration of 0.03% diacetyl

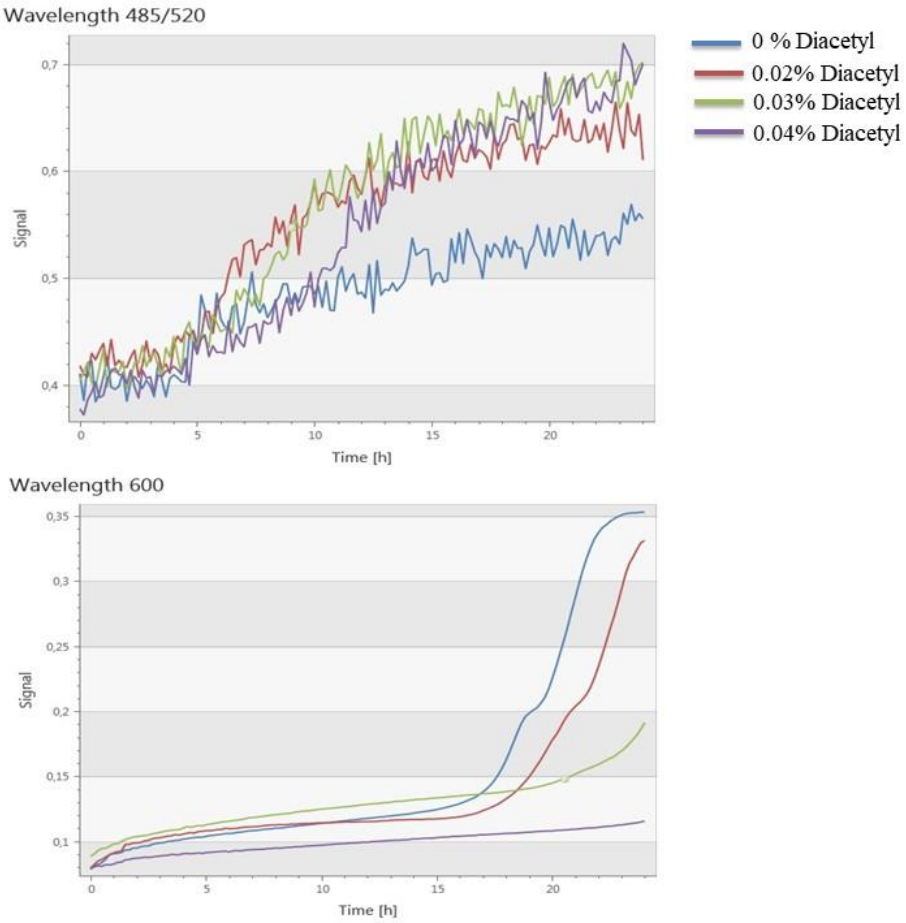


Figure46: Fluorescence intensity and Growth pattern of indicator cell consisting of llmg_1019 promoter when exposed to different concentration of 0.03% diacetyl

Transformation process was also performed with *E.coli* plasmid containing a Dasher GFP gene, whose GFP expression is higher when compared to pSEUDO- GFP gene. Desired promoters such as 2369, Rib3, 0295 which were responsive to diacetyl were incorporated into such plasmid followed by its chromosomal integration into *L.lactis.* Fluorescence was measure using plate reader (figure)

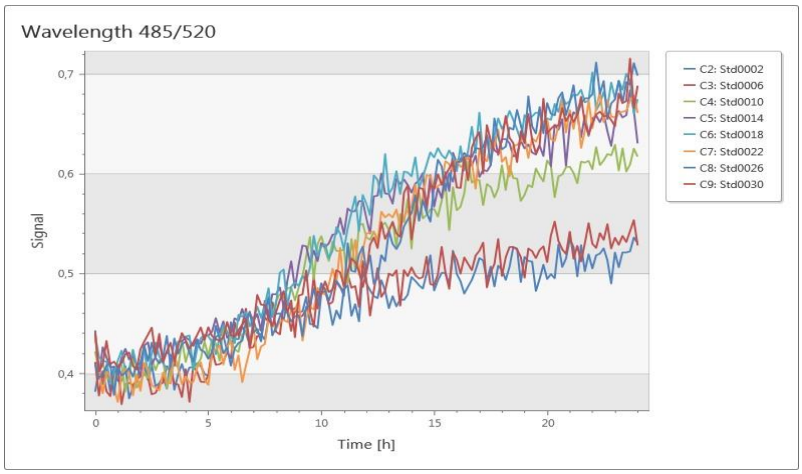


Figure 34: Graphical Representation of Fluorescence measurement of Rib3 promoter inserted into the plasmid containing Dasher GFP.

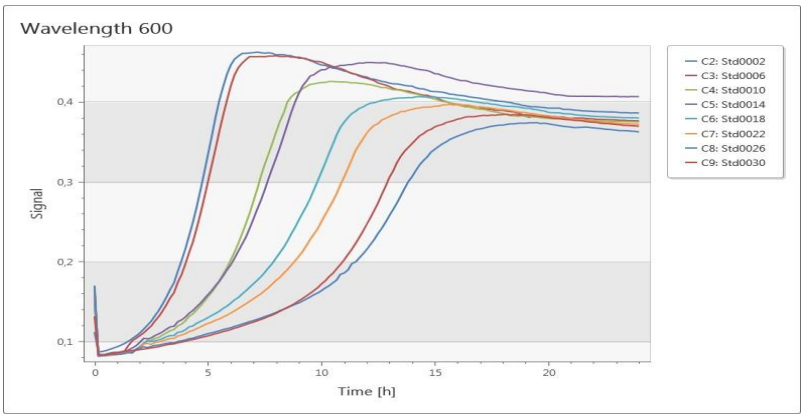


Figure 35: *Graphical Representation of absorbance (600nm) of Rib3 promoter integrated into Dasher GFP in the presence of diacetyl, thereby indicating the growth pattern of the bacteria*

From the above graph, it is observed that there are lot of variations in the measurements. On further analysis of the sequencing results it was observed that the promoter sequence has been incorporated into the plasmid but there was no dasher GFP sequence shown. The same is with the case of 0295 and 2369 promoter.

- Measurement of Fluorescence signal of control promoters Dar1, P32, Usp45 and also *L.lactis MG1363*

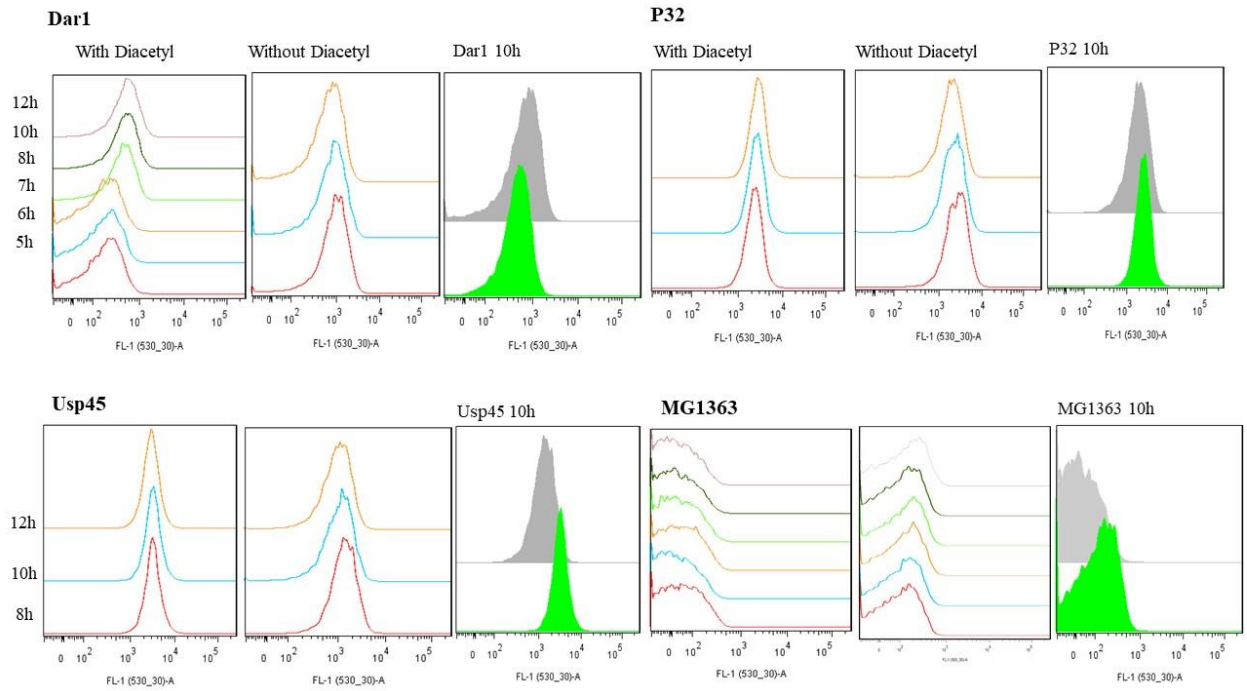


Figure 46: Fluorescence signal of promoters in presence and absence of diacetyl measured at different time points.

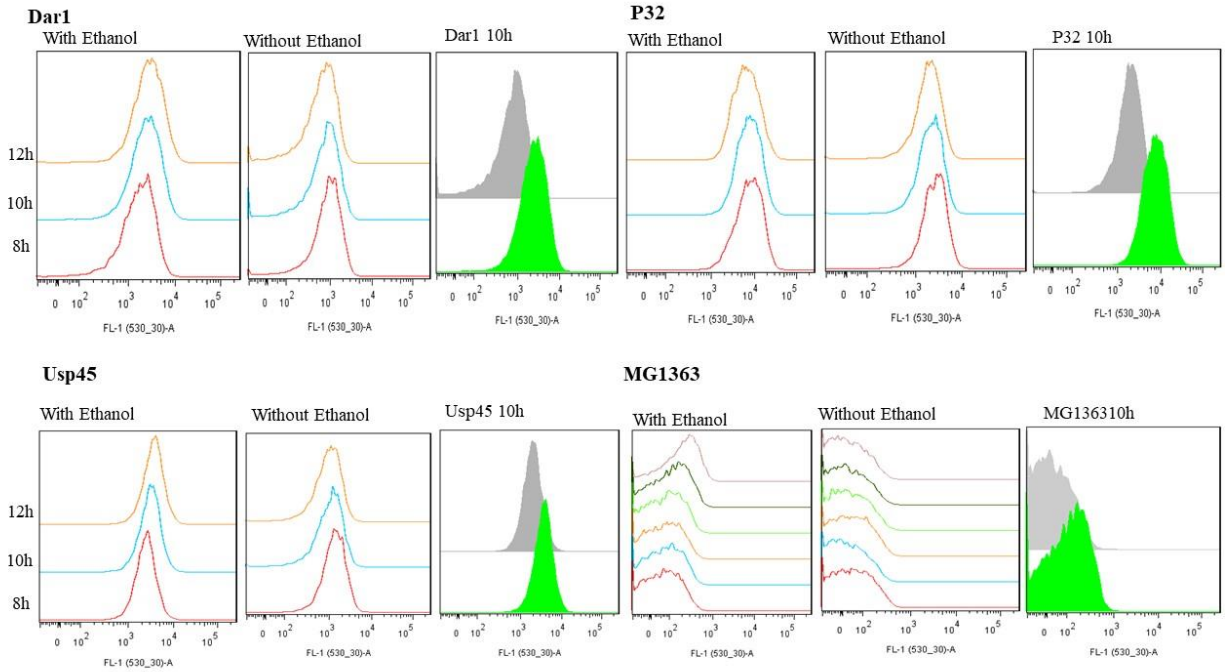


Figure 47: Fluorescence signal of promoters in presence and absence of ethanol measured at different time points.

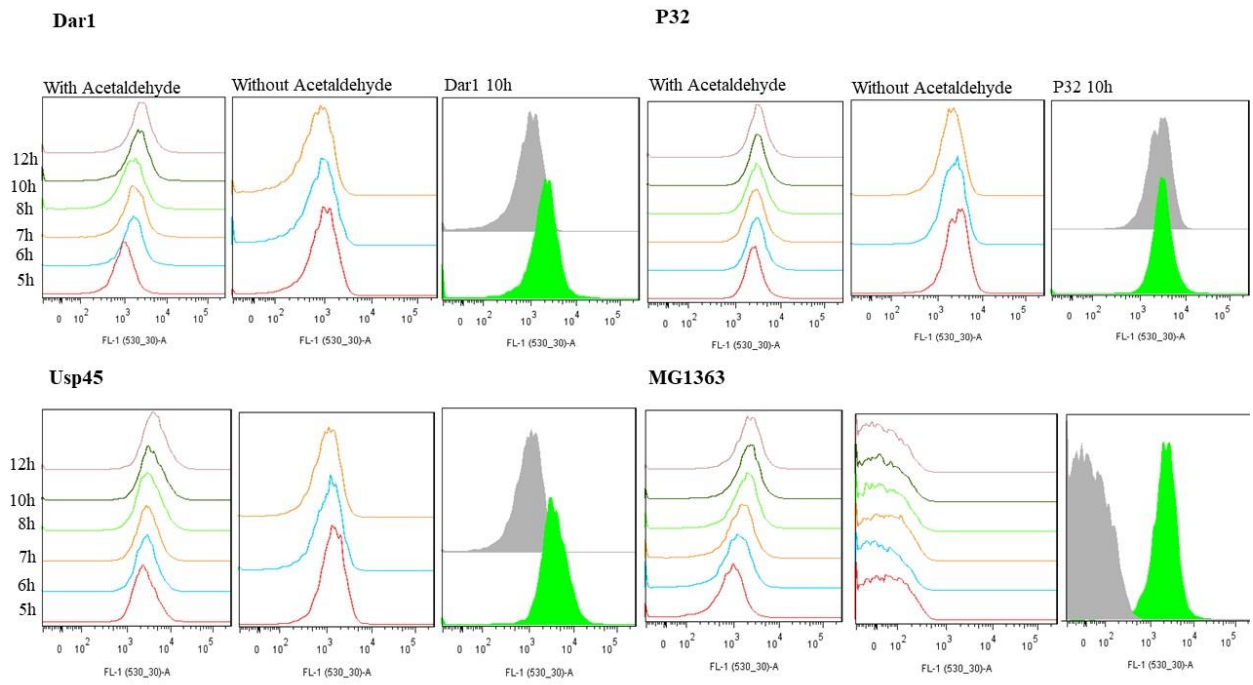


Figure 48: Fluorescence signal of promoters in presence and absence of acetaldehyde measured at different time points.

From the figure above it is observed that MG1363 produces significantly higher fluorescence signal in the presence of acetaldehyde. The experiment with L.lactis MG1363 has to be repeated to obtain a promising results and draw a conclusion.