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Optimization Of Growth Conditions Of "eubacterium Hallii" As A Potential Probiotics

Sampurna Guhathakurta
Wayne State University,

OPTIMIZATION OF GROWTH CONDITIONS OF "*Eubacterium hallii*" AS A POTENTIAL PROBIOTICS

by

SAMPURNA GUHATHAKURTA

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DEDICATION

I dedicate my thesis work to my parents Shri Jyotirmoy Guhathakurta and Smt Mita Guhathakurta for constantly encouraging me and being the only source of my strength throughout this entire program.

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CHAPTER 1: INTRODUCTION

1. General view of probiotics

The concept of probiotics comes from the substances which are secreted from one microorganism and stimulates the growth of another microorganism. In spite of the fact that a lot of research has been done on probiotics, there remains much portions to be learned. Probiotics are generally the live bacteria and yeasts which are beneficial for human health, specifically digestive system. This term became common after 1980s. As our body contains both good and bad bacteria, probiotics are generally known as the "good" bacteria because they keep our gut in healthy condition (1).

Probiotics contain a large variety of microorganisms, the most common bacteria falls into groups called Lactobacillus and Bifidobacterium. Lactobacillus and Bifidobacterium are considered beneficial for certain nervous system disorders (*B. longum, B. breve, B. infantis, L. helveticus, L. rhamnosus, L. plantarum* and *L. casei*) (2) .

Nobel laureate Elie Metchnikoff known as the "father of probiotics" was the first person to suggest the possibility of expanding the gut with this "useful" or "good" microflora in the early 20th century. It was during that time in early 20th century, when milk was fermented with lactic-acid bacteria prohibited the growth of proteolytic bacteria produced by the lactose fermentation because of low pH. Metchnikoff suggested that ingestion of fermented milk would help increase the intestine with the beneficial lactic acid bacteria and decrease the pH of intestine (3).

Lactic acid bacteria were first discovered by Pasteur in 1857. Their isolation from rancid milk was reported in 1878 by Lister.

Bifidobacteria was first discovered by Henry Tissier in 1889 which was obtained and isolated from a breast-fed infant. The bacteria isolated was initially named as *Bacillus bifidus communis* and it

was renamed later on to the genus *Bifidobacterium*. Tissier also observed that bifidobacteria are found to be dominant in the gut-flora of breast feed babies and it helps in treating diarrhea in infants (4).

The first stable cultures of *Lactobacillus casei* strain Shirota were made in 1930 by Dr. Minoru Shirota.

Probiotics are effective for the treatment of a number of complications such as hypertension, cholesterolemia, diarrhea, eczema, inflammation, inflammatory bowel disease, irritable bowel syndrome, lactose intolerance, urinary tract, helicobacter pylori, necrotizing enterocolitis etc (5).

Eubacterium hallii is considered as the common gut microbe that contributes to intestinal propionate formation.

The bacterium belongs to the genus of the groups of Gram-positive in the family of Eubacteriaceae. These bacteria are represented by a rigid cell wall. They are non - motile.

Eubacterium hallii has been used in pharmaceutical, food, and feed industry. For instance, it has been used as a remedy in treating complications related to insulin resistance like dyslipidemia, type 1 diabetes mellitus, Cushing's syndrome, endocrine diseases, etc (6).

Eubacterium hallii is a human gut microbe with an adaptable usage of carbon sources. It can produce butyrate by consuming either glucose, acetate or lactate. When grown in presence of glucose, it produces equimolar amounts of butyrate. As butyrate is important for the gut microbiota, therefore they have effects on immune system. It has the potential to impact the metabolic balance between host health and host homeostasis. This saying is with respect to the intestinal metabolic balance because of its ability to utilize and produce lactate. It is normally present in human feces in concentrations normally upto < 3 mmol liter^{-1}. The concentrations can

be up to 100 mmol liter^{-1} if there is gut disorder like Ulcerative Colitis. *E. hallii* is capable of utilizing glycerol and leads to the formation of propanol, propanal, and propionate. It also forms butyrate from glucose but does not grow on or utilize more complex oligo and polysachharides. It has the capability of metabolizing glycerol to 3-hydroxypropionaldehyde forming reuterin. Reuterin has antimicrobial action against Gram Positive and Gram Negative bacteria, fungi, and yeast. This bacteria also has the ability to impact the ratios of acetate and lactate and of butyrate and propionate because they consume acetate and lactate, producing butyrate and propionate. Propionate is considered as the precursor for gluconeogenesis which helps in health promotion and influences various cell differentiation. Propionate and butyrate are considered important in maintaining homeostasis as it impacts the immune system thus rendering *Eubacterium hallii* beneficial for human health (7).

2. Factors affecting the growth of probiotics:

2a. Prebiotics

Prebiotics are non-digestible carbohydrates that can selectively promote the growth of gut probiotics. The most commonly known prebiotics are Fructans, Inulin, Fructo-oligosachharides (FOS), Galacto-oligosachharides (GOS). It moves through the digestive tract until it reaches the (probiotic) in the colon. Thus, prebiotics are commercially extracted out and produced from fruit and vegetables. To be precise, they are formed by the hydrolysis of polysaccharides from starch or dietary fibers. Prebiotics gives a feeling of fullness and helps to reduce blood sugar because of the non – digestible sugars; it also gives a calming effect to diseases like ulcerative colitis, colon cancer, inflammatory bowel disease, and acute infections (8).

2b. pH

Probiotics and pH are said to balance the gut. Probiotics are mainly related to a healthy digestive system and they help to conserve a beneficial pH balance in the gut. The lactic acid made by the lactic acid bacteria along with its other products like bacteriocins and hydrogen peroxide helps to maintain a healthy pH and inhibits the growth of harmful bacteria. The fermented food and the Lactobacillus Bulgaricus (LABs) in probiotic exist better due to the pH buffering effect, particularly in fat. Humans produce 2.5 liters of gastric juice on a day in a regular basis and fasting gastric pH is 1.5 which extends to about pH 3 and pH 5 after feeding (9).

2c. Temperature

Temperature plays a very important role in the growth and stability of probiotics. High heat can destroy the growth of these organisms. Cold air is not suitable for growth and survival of the bacteria, as well as having less moisture. The temperature range for the growth of probiotics ranges from approximately 37°C to 40°C, but for most probiotics the optimum temperature for growth is 37°C (10).

2d. Atmosphere

Probiotics are mostly anaerobic microorganism and most probiotics grow best under anaerobic gas moisture containing Nitrogen, CO2, and H2. Without the gut microflora, the human body wouldn't be able to use up the undigested carbohydrates because some of the gut flora contain enzymes that human cells do not have in order to break down certain polysaccharides. Probiotics ferment carbohydrates into short chain fatty acids (SCFAs) by saccharolytic fermentation (11).

Gases and organic acids, like lactic acid, are also produced by saccharolytic fermentation. They produce acetic acid which is used by muscle, propionic acid aids the liver in producing ATP, and lastly they produce butyric acid which provide energy to gut cells which might prevent cancer. The gut flora also synthesizes certain vitamins like biotin and folate which help in absorbing dietary elements.

2e. Short Chain Fatty Acid

Short chain fatty acids (SCFA) are also known as volatile fatty acids which has aliphatic tail of less than six carbon atoms. SCFA mainly include acetate, propionate and butyrate. SCFAs are obtained when dietary fiber is fermented in the colon. It promotes weight loss and various other health benefits. SCFAs promote better growth of probiotics. SCFA influences the blood flow of colon and uptake of electrolytes. It has been seen that large bowel SCFA are enlarged by carbohydrates (12) (13) (14).

2f. Bile Salt

The normal human GI tract is complex and has a large variety of microbial population. As a result, the microbes present in the gut gives a large range of metabolites from big molecules that host enzymes cannot convert. The two main types of molecules are cholesterol and bile acid. Bile is an aqueous solution of yellow-green color which is mainly composed of bile acid, cholesterol, phospholipids, and pigment biliverdin. Bile acids are obtained from cholesterol in the liver. Cholesterol is reduced to coprostanol and little amounts of coprostanone. The bile salt conversion occurs mainly via oxidation, conjugation, epimerization of hydroxyl groups at C3, C7 and C12, 7-dehydroxylation, desulfation and esterification. The steroids which include cholesterol and bile salts are commonly exposed to the gut microbiome. They undergo microbial metabolism.

Cholesterol is generally obtained from the diet in liver and other tissues. Bile works as a biological detergent which emulsifies lipids, thus helping in digestion of fat. This detergent property exhibit antimicrobial activity. On a regular day, approximately 5% of the total pool of bile acid escapes epithelial absorption (15) (16) (17).

2g. Freeze Drying or Lyophilization of Probiotics

Freeze drying is widely used nowadays in the food industry to assimilate probiotics and make probiotic formulations in food products although the viability of the freeze-dried product containing probiotic is largely affected during storage and processing. But, these freeze-dried probiotics are conserved by adding cryoprotectants, which increases the survival of the cells during storage as well as processing of the food (18).

CHAPTER 2: OBJECTIVE

The main objective of this study is to determine the optimum growing conditions of *Eubacterium hallii* for future development as a potential probiotics.

CHAPTER 3: MATERIALS AND METHODS

Sample Collection

The sample *Eubacterium Hallii* was obtained from *Eubacterium hallii* Holdeman and Moore (atcc® 27751™) ATCC. The strain which was used is VPI B4-27 (DSM3353). It was kept frozen at -80°C or colder and freeze dried at 2°C to 8°C.

Culturing of the Sample

In order to optimize the optimum growth conditions for this strain, culturing of the bacteria was performed. The sample was cultured by preparing 1869 PRAS-PYG media without tween 80 under anaerobic gas mixture 80% N2, 10%CO2 and 10%H2. After adding the gas mixture tween 80 was added before autoclaving. The media contained peptone (5.0gm), tryptone (5.0gm), yeast extract (10.0gm), 0.025% resazurin (4.0ml), salt solution(40.0ml), hemin solution (10.0 ml), Vitamin K1 solution (0.2ml), glucose (10.0g), tween 80 (0.25ml), L-cysteine (0.5g) in 945.55ml of distilled water. From the autoclaved media, 9 ml of media was transferred into hungate tubes by 10 ml pipettes under the same gas mixture. 0.2 ml of bacteria cultures was inoculated in the tubes. Five tubes were prepared to check the stability and growth of the bacteria. Duplicates were prepared to confirm the growth. Then the tubes were kept in incubators, one at 37°C (New Brunswick Scientific, Excella E24 Incubator Shaker Series) and one at 40°C (Quincy Lab, inc. Chicago IL 60639, Model 10-180) and rest like 43°C and 46°C were maintained at water bath. 25°C was the one at room temperature. The tubes were kept for 11 hours for growth. The turbidity was checked at 0 hour, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours and 11 hours under turbidity meter.

Temperature

The optimum temperature for growth of *Eubacterium hallii* was observed. After culturing the tubes with the bacterial strain, two of the hungate tubes were kept in the incubator (New Brunswick Scientific, Excella E24 Incubator Shaker Series) at 37°C and 40°C incubator (Quincy Lab, inc. Chicago IL 60639, Model 10-180) respectively. The rest of them were kept in water bath to maintain temperatures at 43°C and 46°C respectively. Room temperature was kept at 25°C. Although, the optimum growth was obtained at 43°C, it also showed growth at 40°C.

Determination of Optimum pH

Tubes with bacterial strains were prepared and different pH values ranging from pH 7 to pH 2 were tested for this bacteria. 48 sterile micro well plate (Perkin Elmer) was used. pH ranges were adjusted by HCL in hungate tubes initially. Thereafter, inoculation was followed. Under anaerobic gas mixture, 0.9 ml of adjusted pH media with bacteria samples (24 h cultures) were added into the wells. Duplicates were prepared to confirm the reading. Control samples were prepared to confirm the growth. This whole process was done inside the anaerobic chamber (By PLAS LABS, Lansing, Michigan). It was then allowed to grow for 1 hour at 37°C (New Brunswick Scientific, Excella E24 Incubator Shaker Series). The plate was then put into the spectrophotometer (Perkin Elmer HTS 7000 Bio Assay Reader) at 595 nm for the absorbance reading and the spectrometric reading was observed for the optimum pH of the bacteria.

Bile Salt Treatment

Bile salt treatment was performed on the strain. Bile salt of concentrations 0.01% (w/v), 0.05% (w/v), 0.1% (w/v), 0.2% (w/v) and 0.3% (w/v) was used. 48 sterile micro-well plate (Perkin Elmer) was used. Under anaerobic gas mixture, 0.9 ml of bile salt along with bacteria (24 h cultures) was

put inside the wells. Similarly, a control was prepared in order to confirm the growth. A positive control was prepared to confirm growth in presence of bile salt. The plate was then allowed to grow for 1 hour at 37°C (New Brunswick Scientific, Excella E24 Incubator Shaker Series). The plate was then put into the spectrophotometer (Perkin Elmer HTS 7000 Bio Assay Reader) at 595 nm for the absorbance reading for 19 – 26 cycles which shows the growth of the bacteria. Spectrometric reading was observed for the optimum growth of the bacteria.

Prebiotics on the growth of *Eubacterium hallii*

There were ten types of prebiotics which were used for this study. The prebiotics are Isomaltooligosachharide (IMO), Guar Gum, Oligochitosan, Acacia Gum, Fructo-oligosachharide (FOS), Wheat Bran, Xylooligosaccharide (XOS), Citrucel, Benefiber, Gluco-oligosaccharide (GOS) and Psyllium. Each of the prebiotics was weighed and dissolved in water in respective centrifuge tubes. They were kept in a warm water bath until the prebiotics were dissolved completely. Then, these respective ten prebiotics were mixed with PYG media without glucose in hungate tubes. PYG media with bacteria without glucose and PYG media, bacteria along with glucose were used as a reference to compare the growth of ten different prebiotics. 48 sterile micro well plate (Perkin Elmer) was used. 0.9 ml of individual prebiotics were added into the wells from the tubes along with bacteria (24 h cultures) in an anaerobic chamber (By PLAS LABS, Lansing, Michigan). Duplicates were prepared to confirm the growth. It was then allowed to grow for 1 hour at 37°C (New Brunswick Scientific, Excella E24 Incubator Shaker Series). The plate was then put into a spectrophotometer (Perkin Elmer HTS 7000 Bio Assay Reader) at 595 nm for the absorbance reading for about 19-26 cycles, which shows the growth of the bacteria and the spectrometric reading of the prebiotic that influences the optimum growth of the strain.

Acetate on the Growth of *Eubacterium hallii*

The strain which was used for this study consumes acetate and produces butyrate; therefore, different concentrations of acetate were used for this study. 33mM, 66mM, 99mM and 132mM acetate concentrations were used to determine how the concentration of acetate benefits this bacterial strain. These different acetate concentrations were prepared under anaerobic condition in an anaerobic chamber (By PLAS LABS, Lansing, Michigan). Under the same anaerobic conditions 0.9 ml of different acetate concentrations were added in to the 48 sterile micro well plate along with bacterial strain (24 h cultures). Duplicates were prepared to confirm the growth. The plate was then allowed to grow for 1 hour at 37°C (New Brunswick Scientific, Excella E24 Incubator Shaker Series). The plate was then put into spectrophotometer (Perkin Elmer HTS 7000 Bio Assay Reader) at 595 nm for the absorbance reading for 19-26 cycles, which shows the growth of the bacteria. The spectrometric reading was obtained, which showed the optimum acetate concentration on the growth of *Eubacterium hallii*.

The Effect of Freeze – drying on Viability of *Eubacterium hallii*

For this procedure, the strain of *Eubacterium hallii* was freeze dried by the protocol mentioned here. After obtaining the cell mass, it was freeze dried at 2°C to 8°C. The viability of the freeze dried sample was studied. Two types of samples were prepared to check the viability. They were treated sample and control sample. Two hungate tubes were assigned for each of the group of samples. 0.05gm of freeze dried sample was measured aseptically in a centrifuge tube with a spatula. Thereafter, the samples were inoculated with 10ml of PYG media. The treated sample was exposed to normal air for 15 mins and then inoculated, whereas the control samples were inoculated in the anaerobic chamber (By PLAS LABS, Lansing, Michigan). After this, the tubes

were incubated at 37°C incubator (New Brunswick Scientific, Excella E24 Incubator Shaker Series). The turbidity was checked at regular intervals after 24 hours to confirm the viability of the cells and also to check whether the cells exposed to air were damaged by oxygen, as *Eubacterium hallii* is an anaerobic microorganism.

CHAPTER 4: RESULTS AND DISCUSSION

Culturing of the Sample

A single strain of *Eubacterium hallii* was studied for the growth activities in the PYG media. Five tubes were prepared for this study. Duplicates were prepared for this method. Turbidity was checked using a turbidity meter. At 0 hour, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours and 11 hours; the tubes were checked for determination of turbidity. Five temperatures were used to see the growth after culturing, which were 25°C, 37°C, 40°C, 43°C and 46°C.

However, a previous study showed that there are other medias used for culturing this strain of probiotic such as Man Rogosa Sharpe culture medium (MRS media), YCFA, BHI, Brucella blood agar, TPY medium, Reinforced clostridium medium (RCM), RCB etc. This strain of bacteria has been found to produce butyric acid during bacteria growth into stationary phase per 24 hours at 37°C. This strain is generally isolated from human faecal sample and *Eubacterium hallii* is generally considered as a representative of intestine which is capable of producing butyrate (19).

Generally, to investigate the gut microbiota, culturing of strains are done. There is a new method which has been used in many studies to culture medium and that is the three – stage continuous culture medium, which has the advantage of accessibility and ability to produce a large range of nutritional and environmental parameters characteristic of the colons because the rate of supply of nutrients in this type of growth medium can be easily controlled. A variety of molecular techniques have been used in many studies for culturing this type of anaerobe. Culturing is done in absolute anaerobic medium and the visibility of pink color indicates that oxygen is present in the media and which should be discarded (20).

Effect of Incubation at Different Temperatures

In this study, the growth behavior of *Eubacterium hallii* has been carefully studied. The bacterial strains with the PYG media in hungate tubes were kept in 25°C, 37°C, 40°C, 43°C and 46°C in the incubators respectively. A graph has been plotted between absorbance and time (hours) which can be seen from (Figure 1). The turbidity was checked at 0 hour, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours and 11 hours. The optimum temperature for the growth of this strain was found to be at 43°C. Many studies showed optimum growth for *Eubacterium hallii* at 40°C (21). In this study, the growth at 43°C can be compared to the growth at 40°C because the absorbance value was almost found to be similar. As time increased to 11 hour, optimum growth was obtained at 43°C.

Other studies cultured *Eubacterium hallii* with an incubation temperature range of 15-45°C, it was found out that the optimum temperature for growth of this strain was at 43°C.

Effect of pH

In this study, the pH tolerance test was performed on the strain of *Eubacterium hallii.* A graph has been plotted between absorbance and time (min). The graph for the pH tolerance test can be observed from (Figure 2). From the graph, it can be seen that this strain has the optimum growth at pH 6 with the absorbance value of almost 1.86. Then at pH 7, growth is almost similar to pH 6 with the absorbance value of more than 1.65. The least growth was obtained at pH 2, which means this strain is not tolerant to low pH. At pH 3 and pH 4 the growth is almost the same, and at pH 5 the absorbance value is 1.45, which means that this strain is capable of growing at pH 5. Viability of these strains depends on pH and exposure to acid (22).

Several experiments have been done on the pH tolerance of these butyrate producing bacteria, such as *Eubacterium hallii*. The studies show that they have the greatest pH tolerance at 5.5 and optimum growth at pH 6.5-7.0. The pH tolerance for this strain ranges from 5.5-9.0, but the optimum pH was 6.5-7.0. In several studies, a pH of 6.5 has been used. However, this bacterial strain showed the best pH tolerance at 5.5, 6.2 and 6.7. Many studies have been conducted to show that the pH of the human proximal colon is lower than the pH of faeces and the distal colon, which is closer to neutral. The low pH of the human proximal colon is mainly because of the active fermentation of dietary components leading to the formation of short - chain fatty acids. There even exists evidence that a low pH is due to large consumption of fermentable carbohydrates like lactulose and oligo fructose, which lowers the pH and therefore the colonic pH is low. Colonic pH largely depends on the dietary intake (23).

Effect of Bile Salt

Tolerance to bile salt is considered to be an important factor for the probiotic in the intestine, and after experiments were performed, a graph was plotted between absorbance and time (min). The graph can be seen from (Figure 3). The tolerance to bile salt at 0.01% (w/v) deferred to that of the concentration at 0.2% (w/v). This strain was found to be sensitive to bile salt concentration. The bile salt concentration of 0.01% (w/v) and 0.05% (w/v) showed good absorbance and tolerance, and they are almost at the same range and showed inhibition of growth at a concentration of 0.1% (w/v), 0.2 % (w/v) and 0.3% (w/v).

The human liver synthesizes bile acid into the small intestine, and it is estimated that 90 to 95 % of secreted bile acid is absorbed by the small intestine. The concentration of bile acid approximately ranges up to 0.05 to 0.3 % and this might be the reason for restricted growth of this strain of *Eubacterium hallii* in the small intestine.

Sensitivity to bile salt can be different for different strains and this strain showed resistance to growth in high concentrations of bile salt. Nevertheless, there remain many strains of probiotics which are unaffected with the stringency of passage through upper GI tract and turn into a viable state before entering the colon in reasonable amounts and finally affect its metabolism (24).

Effect of Prebiotics

Prebiotics stimulate the growth of this strain of *Eubacterium hallii*. Analysis of different types of prebiotics like Isomaltooligosachharide (IMO), Guar Gum, Oligochitosan, Acacia Gum, Fructo-oligosachharide (FOS), Wheat Bran, Xylooligosaccharide (XOS), Citrucel, Benefiber, Gluco-oligosaccharide (GOS) and Psyllium were studied with the growth of this strain. A line graph was plotted between absorbance and time (hours) which can be seen from (Figure 4). A bar graph (Figure 5) was also plotted with the absorbance and different prebiotics to show the growth of *Eubacterium hallii*. This bar graph was obtained by checking the hungate tubes for turbidity under turbidity meter over 12 hours of time period.

Gluco-oligosaccharide (GOS) influences optimum growth of the strain. It is comparable with the PYG media with glucose, and it can be assumed that the prebiotic GOS influences same amount of growth like glucose, so glucose can be substituted with GOS in the media. It is also seen that PYG media without glucose has a relatively low growth and thus it can be said that the addition of glucose stimulates the growth of the bacterial strain. Benefiber, Isomaltooligosachharide (IMO), Guar Gum, Fructo-oligosachharide (FOS), Pectin grapefruit and Acacia gum influences almost similar growth on the strain. Wheat bran doesn't influence the growth of strain. Citrucel being the next lowest following wheat bran, and finally it can be seen that oligo chitosan influences the least amount of growth on the probiotic strain *Eubacterium hallii*.

Prebiotics have an additional benefit because they are usually fermented in the large intestine and there has been a lot of in vitro and in vivo studies that human enzymes cannot digest prebiotics, and are fermented in the large intestine by the anaerobic bacteria. This fermentation leads to the formation of short chain fatty acids which influences the growth of probiotics of the large intestine. Prebiotics have the most important effect in the body that is to fight against harmful diseases in the body, including reduction in the risk of rotavirus causing colon cancer or diarrhea. Prebiotics are considered beneficial in treating osteoporosis, obesity, type 2 diabetes etc. (25).

Effect of Acetate

The effect of acetate on the growth of this strain of *Eubacterium hallii* was carefully observed and a graph was plotted between absorbance and time (min) which can be observed from (Figure 4). This strain of probiotic has been observed to consume acetate (33, 66, 99, 132 mM) and produce butyrate. The optimum growth was obtained in the 66mM concentration of acetate. The least growth was obtained in the concentration of acetate, 33mM. There was a doubling of concentration of acetate during the study to check how the concentration of acetate stimulate the growth of the strain. In high concentration of acetate that is 132mM, acidity of the media was increased resulting in less growth of the bacterial strain. For cost effectiveness, 66mM can be used.

This bacterium has been seen to produce at least 10mM of butyric acid during growth to the stationary phase per 24 hours at 37°C and consumes at least 10mM of lactic acid per 24 hours at 37°C during growth to stationary phase. The bacteria which utilizes lactic acid is capable of converting lactate produced by another gut bacteria from dietary sources like resistant starch (26).

Another aspect of invention of these lactic acid utilizing bacteria is that they are beneficial in treating diseases associated with high doses of lactic acid such as short bowel syndrome, Crohn's disease etc. (27).

Under normal conditions, in the gut the concentration of the lactic acid is low in spite of the fact that many bacterial species like bifidobacteria, lactobacillus, and streptococcus, present in the intestine produce lactic acid as a fermented end product. It has been hypothesized that there are certain bacteria which prevents the accumulation of lactic acid in the intestinal gut and they consume it as an energy source. The identification of these organisms has not been elucidated earlier and *Eubacterium hallii* falls into this category of organisms which consume lactic acid as an energy source and produce large quantities of butyric acid in the gut. These type of probiotic organism removes the harmful accumulation of lactic acid in the intestinal gut (28).

High concentrations of lactic acid are said to cause toxicity in the human blood stream and cause diseases like ulcerative colitis and short bowel syndrome whereas production of butyric acid is considered to be beneficial for humans which is a preferred energy source for cells and provides precaution against colorectal cancer and thus *Eubacterium hallii* is considered to be a potentially beneficial probiotic for humans (29).

Viability of Freeze-Dried sample

Viability of the freeze dried sample of *Eubacterium hallii* was studied. The turbidity values of treated and control samples were studied. The values are listed in (Table 1). The values were obtained after 0, 7, 14 and 24 hours. At 0 hour, treated and control samples both had a turbidity of 0. At 7 hours, the control sample had a turbidity of 0.22 while the treated sample had a turbidity of 0.07. At 14 hours, the control sample had a turbidity value of 0.30 while the treated sample had

a turbidity value of 0.20. At 24 hours, the control sample had a turbidity of 0.65 while the treated sample had a turbidity of 0.55. The reason why treated sample had lesser turbidity value than control samples is that they were exposed to air while the control samples were inoculated in the anaerobic chamber (By PLAS LABS, Lansing, Michigan) without any exposure to air. It can be inferred that this strain of *Eubacterium hallii* is sensitive to oxygen which resulted in lesser turbidity in treated samples.

Several studies have shown that viability of probiotics decreases in fermented food products due to storage temperature, acidity, storage time and as a result they have limited shelf life. This is why probiotics are encapsulated in foils. There is not much information about the viability of *Eubacterium hallii* in probiotic products at various temperature so more research needs to be performed in freeze drying at different temperatures (30).

CHAPTER 5: CONCLUSION

Eubacterium hallii is considered to be a potential probiotic because it has the ability to maintain metabolic balance as well as gut homeostasis in the intestine by the formation of various types of short chain fatty acids. This is the reason behind choosing this bacteria in this study. Optimization of growth conditions were performed on the bacterial strain of *Eubacterium hallii*. The media composition was important during this study. This bacteria is sensitive to oxygen therefore, precautions were taken while culturing and also during other techniques like prebiotic treatment, pH treatment etc. This bacteria was seen to consume acetate and thus in the PYG media addition of acetate can enhance the growth of this bacteria.

There were four different concentrations of acetate used in the study. Doubling of concentration was performed to check how acetate benefits the bacterial growth. 66mM of acetate had the optimum growth therefore for cost effectiveness 66mM can be used. As the concentration was raised to 132 mM there was less growth of the strain because the acidity of the media was increased due to high concentration of acetate. The least growth was obtained at 33mM concentration of acetate.

The optimum growth was obtained at incubation temperature of 43°C. Maintaining pH of the medium is an important factor while culturing and optimum pH for this strain was found to be 6.5. Addition of glucose promotes the growth of the bacteria and including acetate would enhance more growth (31).

Prebiotics stimulated the growth of *Eubacterium hallii*. Gluco-oligosaccharide (GOS) was found to be the best in the growth of *Eubacterium hallii* compared to other selected prebiotics. Gluco-

oligosaccharide (GOS) is comparable to glucose in the media which acts as an enhancer in the growth of *Eubacterium hallii* (32).

Tolerance of the probiotic strain to bile salt was observed too, because tolerance to bile salt is considered to be important while determining a probiotic to be beneficial. This strain was observed to be tolerant to low bile salt concentration (0.01%) w/v and it was not tolerant to high bile salt concentration (0.3%) w/v. The concentration of bile acid in the large intestine approximately ranges up to (0.05 to 0.3) % and this might be the reason for restricted growth of this strain of *Eubacterium hallii* in the high bile salt concentration. Additional studies should be carried out to determine the reasons for tolerance to high bile salt concentration (33) (34).

For future studies, identifying the generation time using a proper method of counting cells under fluorescence microscope should be carried out for determining colony forming units. Quantification of acetate consumption should be studied more extensively along with identifying other metabolically important products. More animal studies should be performed to identify the health benefits of *Eubacterium hallii*.

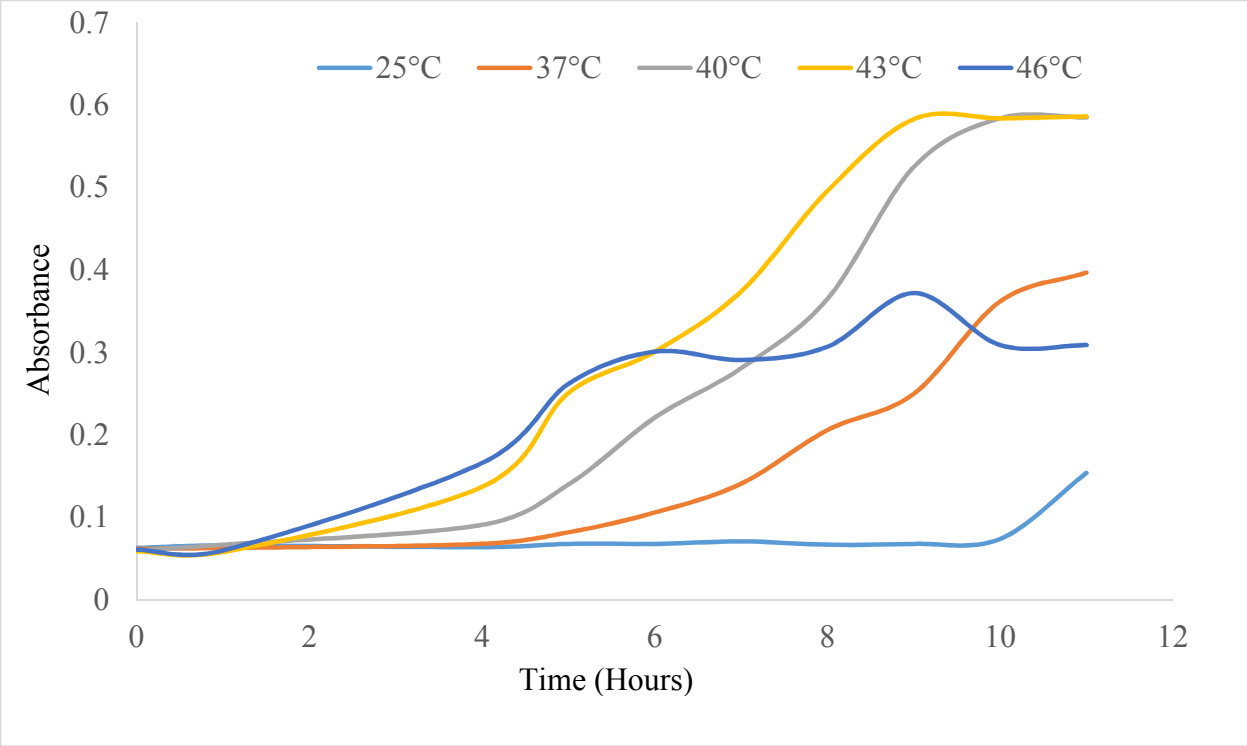

Figure 1. Graphical representation of turbidity exhibited by *Eubacterium hallii* at 25°C, 37°C, 40°C, 43°C & 46°C.

Five tubes were prepared with the same concentration of media 9ml and 0.2ml of bacterial cultures. Aseptically inoculation was performed. The turbidity was checked from 0 to 11 hours after every 1 hour interval to determine the optimum growth of *Eubacterium hallii*. Different colors indicate the growth of the strain at different temperatures.

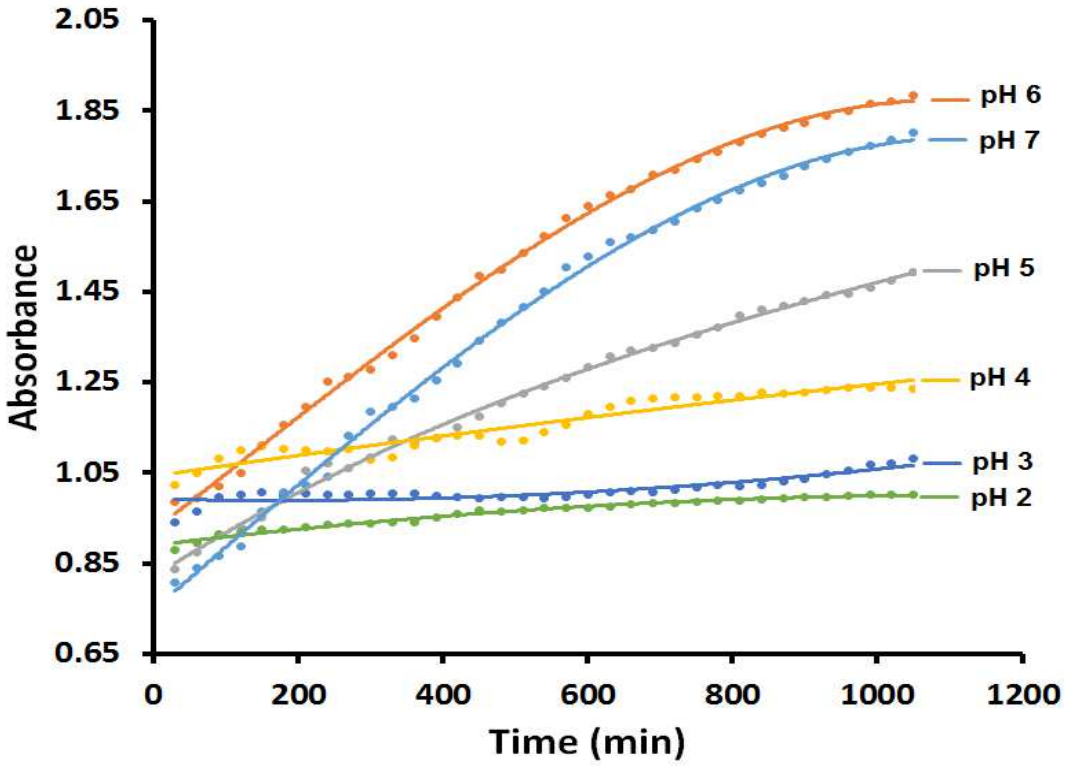

Figure 2. Effect of different pH on *Eubacterium hallii* **over 1200 min of time. The absorbance being plotted on the x-axis which shows the growth of this strain of** *Eubacterium hallii* **at different pH.**

pH 7 to pH 2 were used for this figure. Six different pH were used to determine the optimum growth of *Eubacterium hallii*. Different colors show the growth of this strain at different pH.

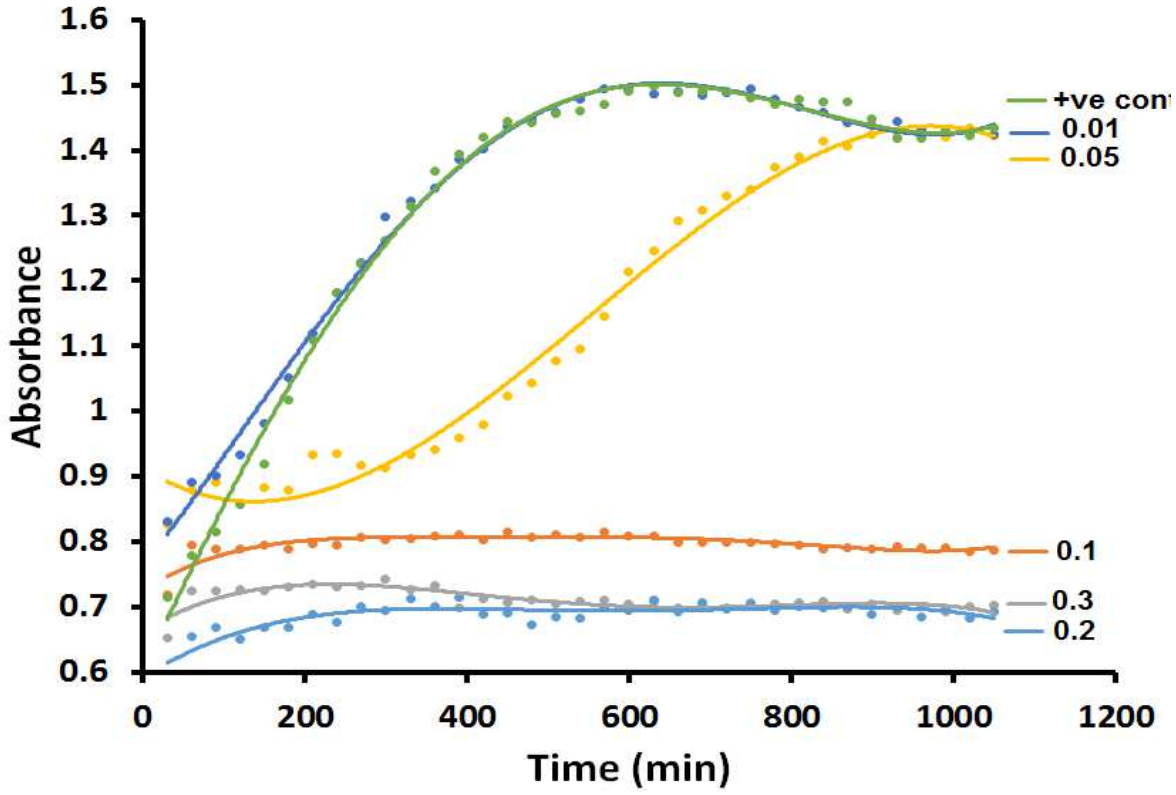

Figure 3. Effect of different bile salt concentration on *Eubacterium hallii*. **The optimum growth was observed in the spectrophotometer at 595 nm over 1200 minutes of time with absorbance on the x-axis showing the growth of** *Eubacterium hallii* **under five different bile salt concentrations.**

Eubacterium hallii was used as the strain and the strain was treated with five different bile salt concentration 0.01% (w/v), 0.05% (w/v), 0.1% (w/v), 0.2% (w/v), and 0.3 % (w/v). A positive concentration was used. Different colors show the growth of this strain at different bile salt concentration.

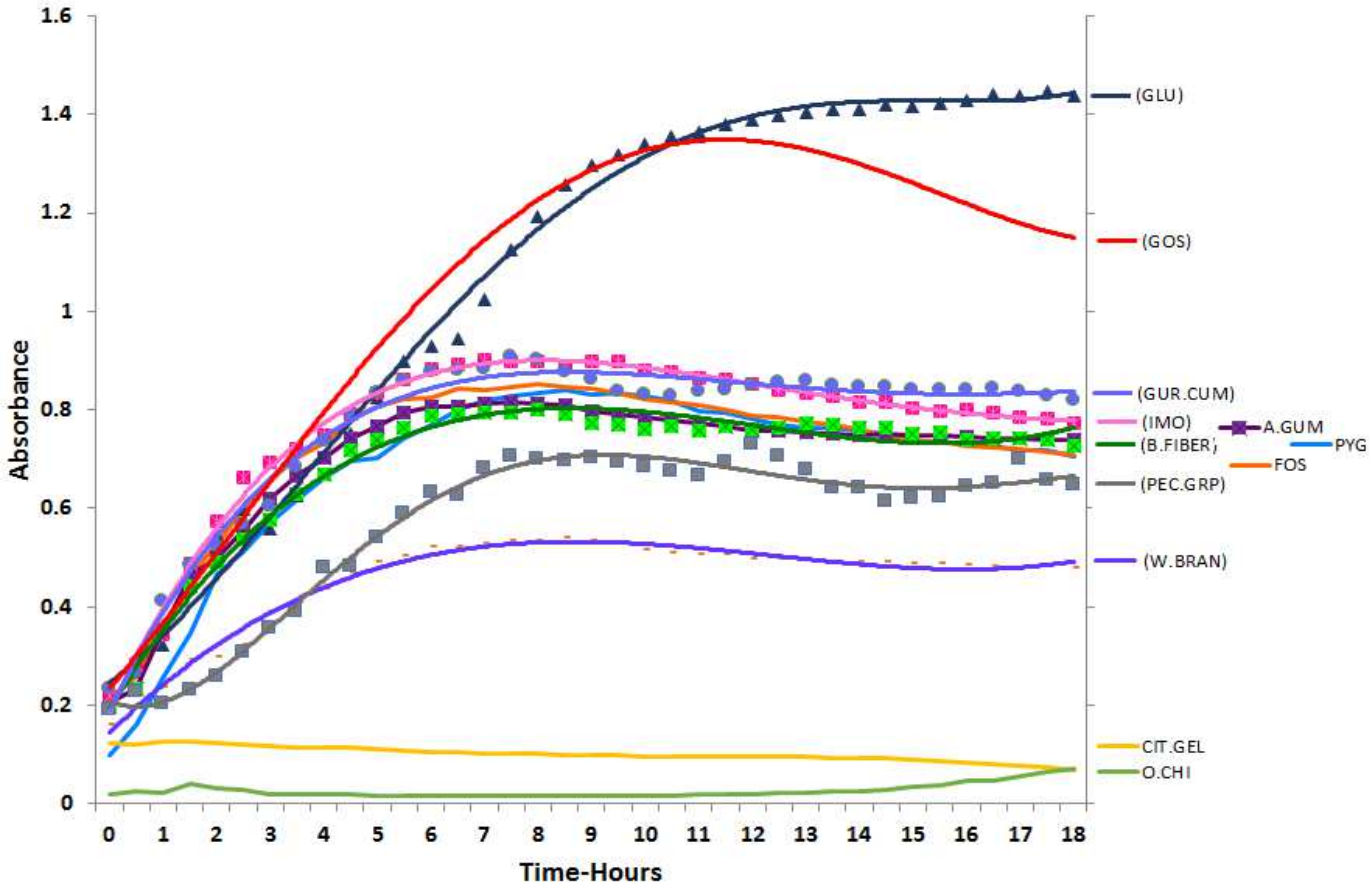

Figure 4. Line graph showing the effect of different prebiotics on *Eubacterium hallii* over 18 hours of time with x axis showing the absorbance of this strain.

Different colors show the growth of the strain under the treatment of ten different prebiotics over the time period of 18 hours in spectrophotometer under 595 nm.

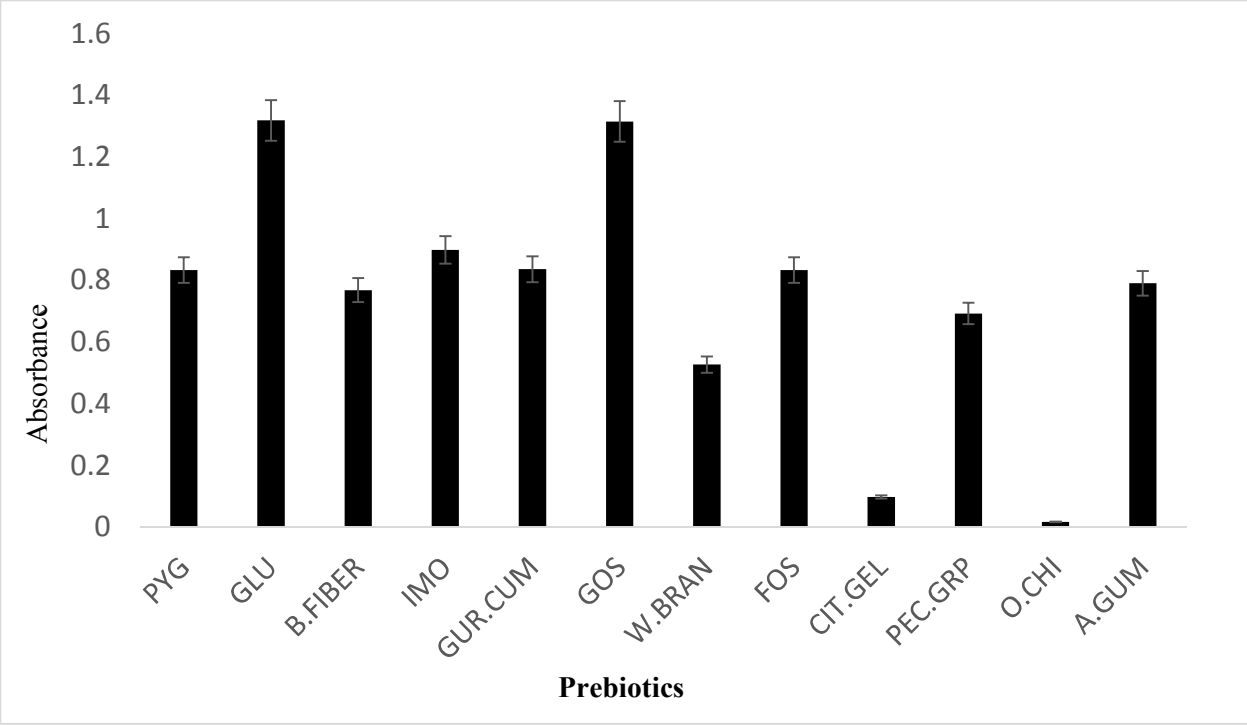

Figure 5. Bar graph showing the effect of different prebiotics on *Eubacterium hallii* **with the absorbance being recorded by turbidity meter over 12 hour period of time.**

The different bars showing growth of *Eubacterium hallii* under the treatment of ten different prebiotics over 12 hour period of time. This growth was observed by measuring turbidity under turbidity meter to confirm the spectrophotometric reading.

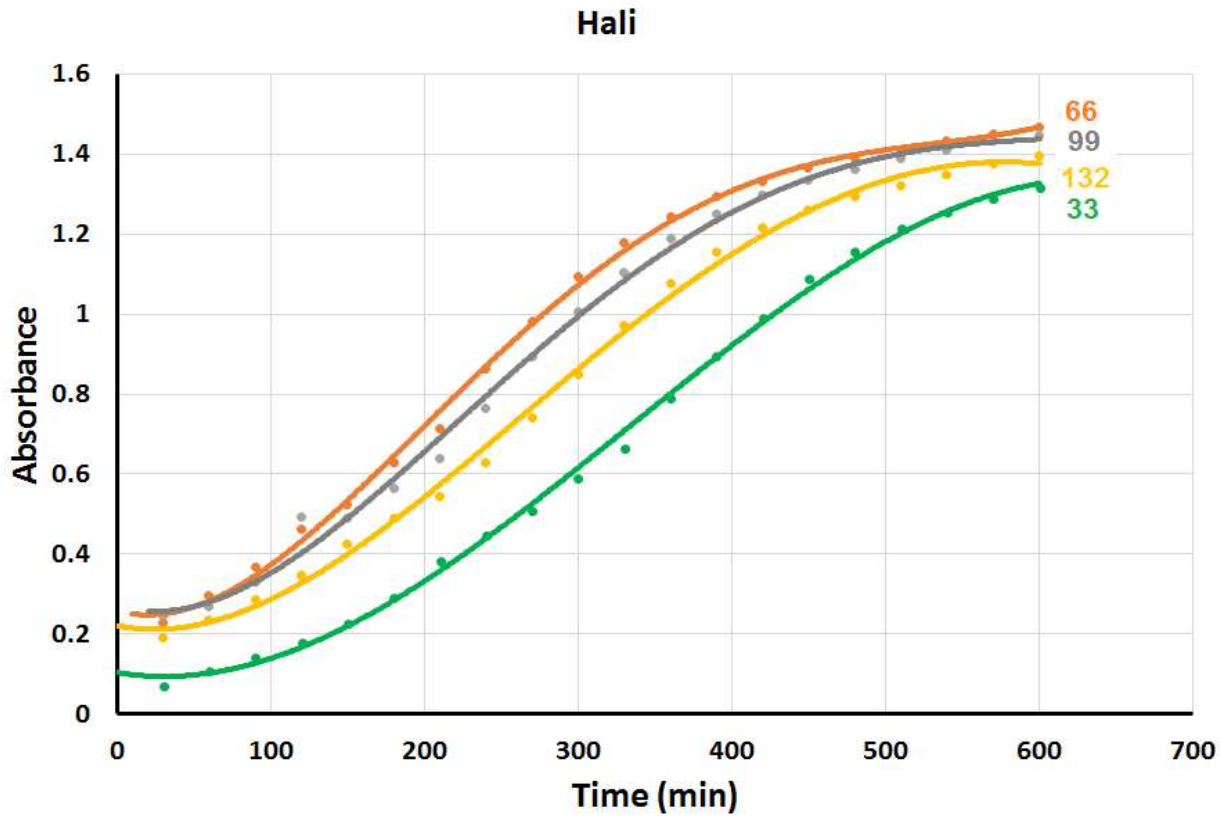

Figure 6. Effect of different acetate concentrations on *Eubacterium hallii* **over a 700 minutes of time period. The x-axis represent the absorbance which indicates the growth of the strain.**

Four different concentrations of acetate were used like 33mM, 66 mM, 99mM, and 132mM. Different colors represent the growth of *Eubacterium hallii* under different concentrations of acetate.

Table 1. Viability of the freeze-dried sample

Time (hours)	Turbidity of Treated Sample	Turbidity of control sample
0	0	0
7	0.07	0.22
14	0.20	0.30
24	0.55	0.65

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ABSTRACT

OPTIMIZATION OF GROWTH CONDITIONS OF "*Eubacterium hallii*" AS A POTENTIAL PROBIOTICS

by

SAMPURNA GUHATHAKURTA

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Advisor: Dr. Kequan Zhou

Major: Nutrition and Food Science

Degree: Master of Science

Probiotics are becoming popular to a greater extent over the past two decades for their potential benefits on human health. Therefore, they are widely used in the food industry by incorporation into food products. Nowadays, there are a lot of probiotics which are famous in the market. In this study, the strain of *Eubacterium hallii* was used because it has a significant importance in the pharmaceutical or food industry especially for treating irritable bowel syndrome and ulcerative colitis. This strain has the property of utilizing lactate and acetate, and produce butyrate. Utilization of lactate helps to keep pH balance of gut epithelium and benefit for other healthy bacteria, while the utilization of acetate reduces metabolic syndrome, obesity, diabetes. The production of butyrate has an anti – inflammatory action. The present objective of the study was to determine the optimum growth conditions. PYG media was used for culturing this strain and it was incubated at 25°C, 37°C, 40°C, 43°C, and 46°C. Optimum growth was obtained at 43°C. Effect of different pH treatment was observed on this strain ranging from pH 7 to pH 2.

The growth was significantly different from high pH to low pH. Other growth parameters like bile salt tolerance test was performed to determine the potential of its sustainability in the human gut. The strain was treated with four different concentrations of acetate and it showed to consume a concentration of 66mM of acetate for optimum growth. It was observed to grow at a concentration of 66mM of acetate, therefore for cost effectiveness 66mM can be used. As the concentration was raised to 132 mM, there was a decline in growth because the media was acidic. The least growth was obtained at 33mM concentration of acetate. Ten different prebiotics were used in this study which influenced the growth of the strain, and Gluco-oligosaccharide (GOS) was found to be the best among the prebiotics providing optimum growth. Freeze drying of the strain was performed at 2°C to 8°C and viability of the freeze dried sample was observed. Further investigation should be conducted to determine its potentiality through animal studies.

AUTOBIOGRAPHICAL STATEMENT

I completed my undergraduate degree in Food Technology from West Bengal University of Technology, India, in 2014. During my undergraduate degree, I was exposed to five internships in reputed food companies of India in the quality control and research and development department, which helped me to gain a lot of knowledge as to how food is being processed, manufactured and maintained safe at an industrial level. Thus to progress further and refine my knowledge in the world of food science, I was accepted in 2015 to pursue a Master of Science degree in Nutrition and Food Science at Wayne State University, Detroit MI and will be completing the degree in May 2017.