Batch Spontaneous Anaerobic Fermentation of Ginger: Time Axis Analysis of Population Dynamics and Microbial Diversity

Nurul Faezah Tuseran and Jailani Salihon

Faculty of Chemical Engineering, Universiti Teknologi Mara

***Abstract*—Ginger (Zingeber officinale) has lot of promising benefits especially for medicinal purposes. Naturally, fungi and bacteria can live on the outer skin of ginger as it grow on the soil and these microbe can be both aerobic and anaerobic. If ginger let to rot naturally, it will break into its basic elements by enzyme that is secreted by fungi. The enzymes will break the skin, followed by aerobic bacteria degrading it to its basic elements. However if ginger consumed by humans or other living creatures, the anaerobic microbes will be digested in the alimentary canal into bioavailable products which are readily absorbed by the body. This anaerobic fermentation in the gut can be approximated by spontaneous anaerobic fermentation of ginger to produce bioavailable products to be consumed by humans for their health benefits. In this research, the batch spontaneous fermentation of ginger is characterized by tracking the microbial diversity and population dynamics along time axis. This will create the basis for precise identification of the species in further advanced work. Together with the metabolite profile, microbial diversity and population dynamics profiles will form the basis for quality control and scale up for this fermentation. To achieve these goal, the steps that need to be carried out on each sample are serial dilutions, streaking on each agar plate with the selective media MRS, Urea, and Mac Conkey agar, incubation, colony counting, gram staining, gram staining and finally identification of cell morphology and plotting of populations dynamics and microbial diversity. 31 batch spontaneous anaerobic fermentations of 10%(w/v) sucrose and 10%(w/v) grinded ginger in distilled water of volume 500ml were carried out for 12 weeks. Samples were taken on days 2,3,4,5,7,8,10,14,15, and week 3,4,5, 6, 7,8,9,10,11,12. After carrying out the above steps, the results shows that selected microbe that grows on each agar especially on selective medium has its own population dynamics along the time axis. Superimposing the population dynamics time-profile of each selected microbe results in the microbial diversity profiles of the spontaneous fermentation. Gram staining results allow cell morphology to be observed. The cell morphology were too varied and different each day. This research has fulfilled its objectives which were carrying out batch spontaneous anaerobic ginger fermentation, obtaining population dynamics and microbial diversity and recording the cell morphology.**

***Keywords*: Spontaneous anaerobic ginger fermentation, population dynamics, microbial diversity,**

I. INTRODUCTION

Ginger (Zingeber officinale) has a lot of useful benefits, long before modern medicinal. Ginger used to treat nausea, motion sickness, relieving inflammation, lowering blood cholesterol, reducing blood glucose level, relieving hypertension and may take part in cancer preventions (Keith Singletary, 2010). All of these benefits has been studied and some of it were proved to be true.

Ginger rhizome grows beneath the soil and soil are the habitat for fungi and bacteria. The ginger rhizome skin may become their habitat. Those microbe may posses as aerobic or anaerobic. If ginger rotten naturally, it will break into its basic elements. Fungi secreted enzyme that will degrade ginger's skin because the skin of each plant was made by cell wall which is hard to degrade, cell wall composed of pectin, cellulose, and hemicellulose (Jaeyong Choi et al. , 2013). After that aerobic bacteria will follow on degrading the ginger.

If ginger were consumed by human or other living things like animals, anaerobic microbes will be able to survive as the conditions of our alimentary canal are anaerobic which less in oxygen as stated by the article of healthy gut bacteria. The anaerobic microbes will be digested in alimentary canal and turn into bioavailable products which will be absorbed by the body. The article of healthy gut bacteria also specified that the anaerobic bacteria does not need oxygen in energy–releasing reaction instead they release energy that locked up inside nutrient by fermentation process. Therefore, the anaerobic fermentation inside gut can be approximated by spontaneous anaerobic fermentation of ginger and producing bioavailable products that can be consumed orally by humans for health benefits.

There are other fermented products that successfully being produced in industrial scale like sauerkraut, pickles, olives and kimchi. Actually there are many vegetables that involve in fermentation process but they are not being produced in large scale due to certain restriction. Certain production that involving fermentation process in Malaysia like the making of tempeh (fermented soy bean) and budu (fermented fish & shrimps) usually are being produce in batch production. This process usually are not a continuous process as it difficult to achieve the best standard of the products and the quality control still at its foundation.

The batch spontaneous anaerobic fermentation is carried out to know the populations dynamics and microbial diversity. This will become the pillars of the quality control for the scaling up ginger fermentation process. The basis of quality control is important in order to avoid any loss, waste or failure during operations. By knowing the population dynamics, the growth pattern of microbe were able to be identified. Superimposing the

population dynamics create the microbial diversity profile of ginger fermentation.

II. METHODOLOGY

A. Materials

Material used for this research are ginger rhizome, sugar and distilled water. Gingers were purchased from the local store.

B. Batch Spontaneous Anaerobic Fermentation

Gingers that has been purchased was cut into a small pieces to ease the blending process. The ginger does not need to be washed. The gingers were put inside the blender along with water, enough to make all the ginger blended nicely. The blended ginger and sucrose or sugar then weighed for 50g. Both ginger and sugar that has been weighed was poured into a 500ml plastic bottle. Then filled up the bottle with distilled water. Put on the cap. The procedure was repeated for the next 30 bottle. The 31 bottle including the backup sample. The bottle was left until it reached the assigned time and ready for sampling. Fermentation process were carried out for 12 week.

C. Preparation of sample

After the fermentation has reached certain time, the sample was taken. Samples were taken on days 2,3,4,5,7,8,10,14,15, and week 3,4,5, 6, 7,8,9,10,11,12. Serial dilution were carried out. After all the material has been assembled, the tubes were labelled from dilution 10^1 to dilution of 10^6. For dilution 10^1 10ml of solution from the fermented ginger was taken and add 90ml of peptone water into the test tube. The solution was mixed very well. For dilution 10^2, take 10ml of sample from dilution 10^1 and tube was filled with 90ml of peptone water. Procedure was repeated for the dilution 10^3, 10^4,10^5 and 10^6 and took each sample from previous serial dilution tube. After serial dilution step has finished, sample can be streaked on agar.

D. Isolation of fungus and bacteria

Two different media was used to distinguish between fungus and bacteria, which are nutrient agar and potato dextrose agar. Nutrient agar was used to detect any bacteria, while potato dextrose agar was used to detect fungus. The agar were prepared according to the instructions on the bottle of agar. Beaker was placed on hot plate for 15 minutes and ready for autoclave. After autoclave process, agar was poured into petri dish and stored in refrigerator. The streaking process was carried out for all of the dilution factor. Only 100ml of sample was dropped on the agar and streaked using L-shaped spreader. Each time before streaking on agar, the L-shaped spreader need to be sterilized. The plate was sealed with parafilm and put into a jar. The jar need to free from air especially oxygen. A candle was light before closing the jar. After that, each streaked plates were placed inside the incubator for 24 hours at 37°C.

E. Selective medium

Selective medium used for this experiment were Mc Conkey agar, plate count agar, urea agar, and de Man Rogosa agar. The preparation of agar were almost the same as preparation of nutrient agar or PDA agar. 100ml of sample was dropped on the agar and streaked using L-shaped spreader. L-shaped spreader need to be sterilized before and after used. The plate was sealed with parafilm and put into a jar. The jar need to free from air especially oxygen. A candle was light before closing the jar. After that, each streaked plates were placed inside the incubator for 24 hours at 37°C. Mc Conkey agar is specialized in isolation & differentiation of gram negative bacteria specifically family of Enterobacteriaceae and genus of Pseudomonas. De Man Rogosa and Sharpe agar used to isolation, enumeration and cultivation of Lactobacillus species. Plate count agar is used to determine total growth of microorganism. Lastly, urea agar were used for the detection of any urease production and precisely for the members of genus Proteus.

F. Colony Counting

To create a populations dynamics profile and microbial, after each incubation process, all the bacteria inside each plate will be counted in order to know the colony forming unit (log(cfu/ml)) and the growth pattern of the microbe inside the ginger fermentation. The graph of selective medium agar were plotted against time. The population dynamics time-profile of each selected microbe were superimposed results in the microbial diversity profiles of the spontaneous fermentation

G. Gram staining

Any microbe alive after the incubation process were proceeded to gram staining procedure. A sterile water was dropped on the slide and a loopful of microbe from the plate culture was smeared evenly. Smeared properly to avoid any thick smears. The slide was heat fixed to kill bacteria so that the smear later on easy to take up the stains. Solution of crystal violet, grams iodine, ethyl alcohol and safranin are smeared on the slides respectively. Slide was placed on the microscope. During observation, the colour of microbes, shape and arrangement were recorded to get the cell morphology result

III. RESULTS AND DISCUSSION

A. Population Dynamics

Generally, microbes reached the highest point or grow exponentially when the substrate or food source is abundant. Once the substrate has finished consumed by the microbes, the growth will be decreased. However, there is a possibility where other microbe may consumed others microbe's waste product. This condition allowing certain microbes to keep on living.

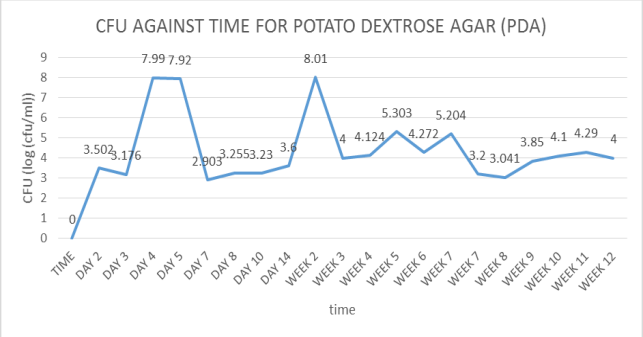

Fig 1: Graph of CFU against time for potato dextrose agar (PDA)

The graph shows increasing pattern until day 4 and 5 but experienced plunged reading in day 5. Reached its peak in week 2 that gave a reading of 8.01 log (cfu/ml). After week 3, the graph continue fluctuate sharply between week 5 and week 7 but fluctuate gently from week 7 until week 12. The fluctuation from week 9 to 12 may indicate the stationary phase of microbial growth and the differences between the readings were not that big.

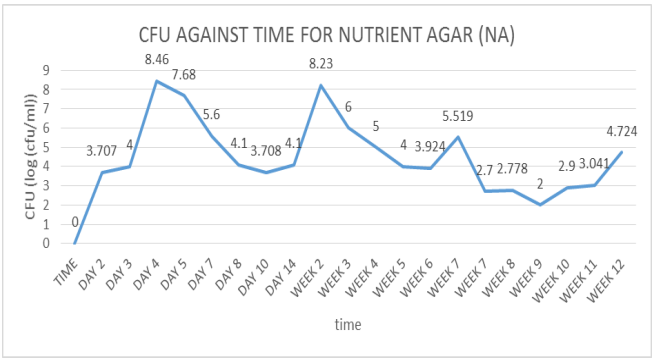

Fig 2: Graph of CFU against time for nutrient agar

The graph fluctuated over time. The reading is increasing until day 4 and then fluctuates for the consecutive weeks. Its peak is at day 4 and week 2 which gives reading of 8 log (cfu /ml). The final reading is 4.7 log (cfu /ml) which means that there are still microbes able to survive up until that point. Nutrient agar is an agar that support growth of non-fastidious microbes, literally it can support any kind growth of bacteria. That may become the reason of fluctuation because as one specific nutrient is finished, the specific microbe will undergo death phase causing the graph to drop gradually. The next increment may show the exponential growth of other bacteria and that bacteria consumed other available nutrient. This also show the interaction between certain bacteria

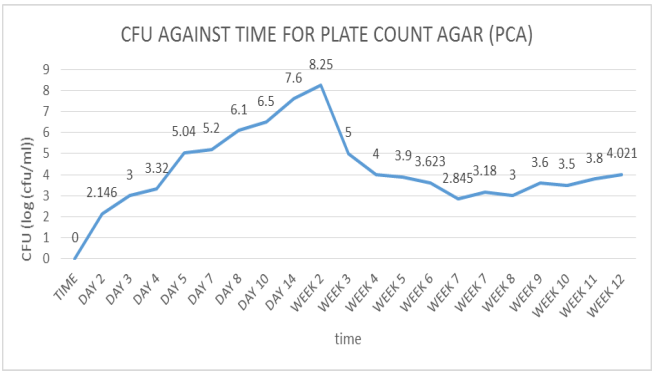

Fig 3: Graph of CFU against time for plate count agar (PCA)

From this graph, it can be seen that the reading keep on surging until week 2 where it reaches its peak, 8.25 log (cfu/ml) and start to declining and fluttered slightly until week 12th. The true purpose for this agar is to monitor total bacterial growth therefore it can be explained that this whole fermentation was involved in exponential growth from day 2 until week 2, this indicate that most of the bacteria are at their efficient state and probably same goes to the fermentation process

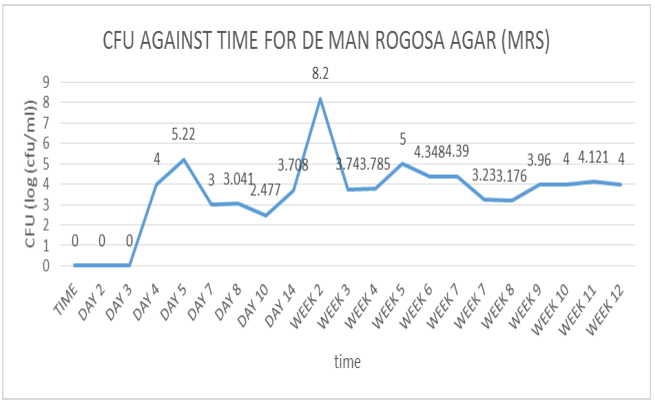

Fig 4: Graph of CFU against time for De Man Rogosa and Sharpe (MRS) agar

From this figure, the microbes only start to grow on this medium on day 4 and continue to increase until day 5. The lactic acid bacteria grow quite late compared to any other microbe probably this signify that the real fermentation start only after 4 days had passed. Lactic acid bacteria proves that the fermentation process has taken place.In week 2, it reaches its peak that gives reading of 8.2 log (cfu/ml). After that declining and fluctuate a little until week 9. In week 9 until week 12, the graph almost stabilize, which means viable microbes is equally with ceased microbes.

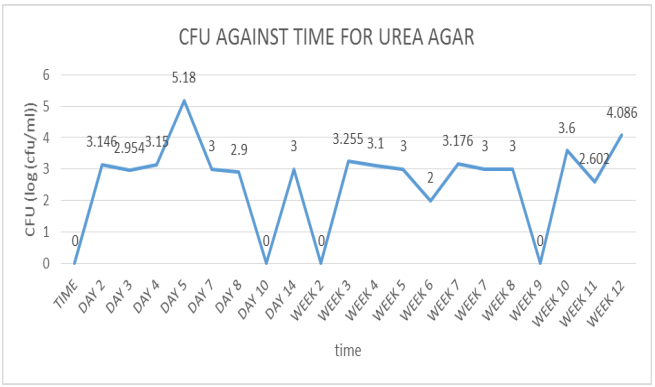

Fig 5: Graph of CFU against time for urea agar

This graph fluctuated sharply. Peak reading was at day 5 that gave reading of 5.18 log (cfu/ml). However there is time the graph gives reading of 0 in day 10th, week 2, and week 9, maybe this occur due to contamination problem when streaking procedure was carried out because if the population has reached 0 log (cfu/ml), supposedly in the next week there will be 0 log (cfu/ml) reading as well. At the end of fermentation, it still gives reading which probably indicate the fermentation is still not over in just 12th week.

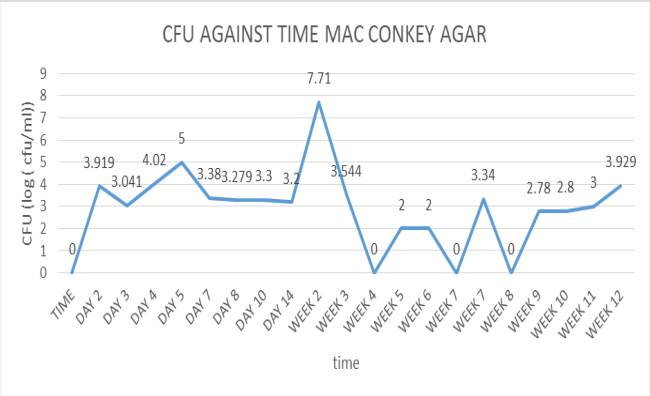

Fig 6: Graph of CFU against time for Mac Conkey agar.

This graph almost the same with graph of urea agar as it fluctuated sharply many times. This graph reaches its peak in week 2 that gives reading of 7.71 log (cfu/ml). Zero reading in week 4, week 7, and week 8 probably due to contamination or error in streaking procedure because if the microbe has reached its death phase therefore in the next week reading it will gives zero reading as well. At week 12th, CFU reading is 3.929 log (cfu/ml). Although the graph indicates reading of microbes but these microbes are not able to produce urease as the result were negative.

B. Microbial Diversity

The community dynamics of ginger fermentation are shown as below:

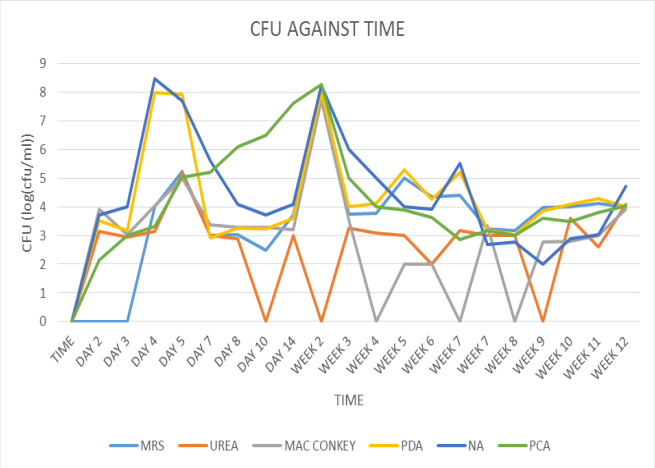

Fig 7: graph of colony forming unit (log(cfu/ml)) against time

The graphs shows abundance of microbes that lives inside ginger spontaneous fermentation. Each agar media shows a different pattern of graph and has their own fluctuation pattern. From here, the peak of fermentation is at week 2 or 3 where the graph line is at its highest reading which indicates the highest presence of microbes. This probably their peak of exponential growth, microbe has its own graph curve. Basically, the growth also peaked due to presence of useful substrate inside all the agar.

Although the fermentation has been carried out for 12 week, the CFU reading on week 12th still does not indicate 0 log (cfu/ml) which means that potentially there are microbes that can survived for a longer time than 12 week but whether it is helpful to the successfulness of fermentation, that's need to be investigated. However, there is a probability that the microbe may not be helpful as in week 12, the reaction of fermentation are dropped as less gas are being produced. However as for Mac Conkey and urea agar the graph are quite differ from potato dextrose agar, nutrient agar, plate count agar and MRS agar.

C. Cell morphology

The morphology of cell were different for each day. The cell morphology of microbes from ginger fermentation can be summarized as this:

No	Agar type	Species / usage	Physical observation	Gram staining
1	MRS agar	• To detect Lactobacillus species	• White colony and viscous • The colour of agar does not undergo any changes after incubation	Gram positive bacteria • Blue in colour • Rod shape or bacilli (coccobacillus, bacillus, diplobacilli, palisades, streptobacilli) • Cocci shape (coccus, diplococci, staphylococci) • Odd shape (hypha, enlarged rod fusobacterium) Gram negative bacteria • Red in colour • Rod shape or bacilli (bacillus, coccobacillus, diplobacilli, palisades, streptobacilli) • Odd shape (hypha)
	PDA agar	• To detect Fungi, yeast, mould	• White colony • Viscosity of colony varies in ranges of time • The colour of agar does not undergo any changes after incubation	Gram positive bacteria • Blue in colour • Rod shape or bacilli (coccobacillus, bacillus, diplobacilli, palisades, streptobacilli) • Cocci shape (coccus, diplococci, streptococci, staphylococci) • Odd shape (hypha, enlarged rod fusobacterium, filamentous, club rod corynebacteriaceae) Gram negative bacteria • Red in colour • Rod shape or bacilli (coccobacillus, bacillus, diplobacilli, palisades, streptobacilli) • Cocci shape (cocci, staphylococci) • odd shape (hypha, filamentous)
3	Mac Conkey agar	• Gram negative rods, family Enterbacteriaceae, genus pseudomonas • Differentiation of lactose fermenting gram negative-bacteria • To differentiate bacteria that can ferment sugar other than lactose	• Purple or pale purple colony • Colony less viscous • The colour of agar does not undergo any changes after incubation	Gram positive bacteria • Blue in colour • Rod shape or bacilli (coccobacillus, bacillus, palisades, streptobacilli) • Odd shape (hypha) Gram negative bacteria • Red in colour • Rod shape or bacilli (bacillus, palisades, diplobacilli, streptobacilli) • Cocci shape (cocci, diplococci, staphylococci) • Odd shape (filamentous)

No	Agar type	Species / usage	Physical observation	Gram staining
4	Nutrient agar	• Support growth of non-fastidious microbes	• White colony • The colour of agar does not undergo any changes after incubation • Viscosities of colony varies in range of time	Gram positive bacteria • Blue in colour • Rod shape or bacilli (coccobacilli, bacillus, palisades, diplobacilli, streptobacilli) • Odd shape (hypha, filamentous) Gram negative bacteria • Red in colour • Rod shape or bacilli (bacillus, palisades, diplobacilli, coccobacillus)
5	Plate Count Agar (PCA)	• To monitor total bacterial growth of sample	• White colonies • The colour of agar does not undergo any changes after • Incubation • Viscosities of colony varies	Gram positive bacteria • Blue in colour • Rod shape or bacilli (coccobacillus, bacillus, palisades, streptobacilli, diplobacilli) • Cocci shape (coccus, staphylococci) • Odd shape (hypha, filament) Gram negative bacteria • Red in colour • Rod shape or bacilli (bacillus, palisades, diplobacilli, streptobacilli, coccobacilli) • Cocci shape (cocci, diplococci, streptococci, staphylococci)
6	Urea agar	• Used for differentiation of microorganism based on urease activity	• Colourless colony • White colony • Colony less viscous • The colour of agar turn to yellow colour. (negative for urease production)	Gram positive bacteria • Blue in colour • Rod shape or bacilli (coccobacillus, bacillus, palisades, streptobacilli, diplobacilli) • Cocci shape (coccus, staphylococci, diplococci) • Odd shape (hypha) Gram negative bacteria • Red in colour • Rod shape or bacilli (bacillus, palisades, streptobacilli,) • Cocci shape (coccus, staphylococci) • Odd shape (filament)

IV. Conclusion

In this experiment, all objectives were able to be acquired. The population dynamics of microbe on each agar shows different pattern and does not really follow the general microbial growth curve. Superimposing population dynamics, the microbial diversity was assorted. As for the cell morphology, each day has different microbe as it too diverse. From the microbial growth on plate count agar (PCA) at highest dilution factor, the growth is still too numerous. From here, it can be concluded that lots of microbe able to live on spontaneous ginger fermentation. The peak of microbial growth is during week 2 and 3. When the graph is at its peak its shows that he microbe are growing rapidly as their food source is abundant. However, there is a case where other microbes may produce other substrate for another microbes. For future recommendation, the dilution factor need to be higher than 10^{6} so that the colony able to form nicely leading to a pure strain and the exact growth able to be recorded. Next, The microbe able to grow on each different agar, it is suggested that the use of different types of agar for growth of microbes need to be broadened to study the microbial diversity of batch spontaneous anaerobic ginger fermentation. The fermentation process time need to be extended for more than 12 week .Lastly, this kind of experiment need to be proceed and improve its method by knowing exactly the name of species through PCR process or any advanced technique

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