Batch Anaerobic Spontaneous Fermentation of Garlic: Time-Axis Analysis of Species Diversity

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***Abstract* — The batch anaerobic spontaneous fermentation of garlic was conducted over a 3 months period at room temperature 25°C in 31 identical 500ml mineral water bottles. Whole unpeeled garlic was grinded in a blender (Waring) together with 10% w/w sugar (sucrose) and distilled water. The product was made up with distilled water to produce 10% w/v solution of garlic and sugar in water. Samples were taken at days 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 15, 17, 22, 29, 37, 44, 51, 58, 65, 72 and 82 by harvesting a bottle and discarding it after use. Each sample was plated in different selective media at sample dilution of 10^0, 10^1, 10^2, 10^3, 10^4 and 10^5. The selective media comprise of Mc Conkey Agar, Urea Agar, and deMan Rogosa and Sharpe (MRS) agar. Potato Dextrose Agar (PDA) was used to detect fungi. Plate Count Agar (PCA) was used to determine the total viable cells per ml. Each growth was then subjected to gram staining. The results were then used in plotting species diversity versus time for the batch fermentation.**

Keywords— Anaerobic, Garlic, Fermentation, Selective media, PDA, MRS, Mc Conkey, Urea, PCA and Gram Staining.

I. INTRODUCTION

Spontaneous fermentation rely on the microbes available naturally on the substrates to carry out the fermentation and many Malaysian condiments [7] are produced via spontaneous fermentation [3]. Fermentation of medicinal fruits are thought to be beneficial in that it causes the compounds with the medicinal effects to be bioavailable for absorption into the bloodstream [4].

Garlic is defined as a fruit in Botany [7]. Garlic is known locally to have medicinal effects on various common human ailments [5]. So far there has been no further action to develop this potential into an emerging new product or medicine based on garlic. The main reason were because of the strong flavor and odor [6] but at the same time the procedure in making medicine is very complex [7]. The sample of garlic-based medicine need to undergo certain procedure such as toxicity test, and clinical test and to be acceptable there must be no negative effects to the consumer or else it will failed.

Fermentation of garlic is studied in this work with specific focus in the species diversity along the time axis of the batch fermentation. The data produced can be used to determine if a replication of the fermentation is successful. Ultimately it can be used for quality control and scale up of such fermentations.

In theory, on the outside of the skin of every fruit there are naturally occurring fungi and bacteria, both of the anaerobic and aerobic types. If the fruit is not consumed by humans or other living creatures but fall from the tree to the ground instead, the aerobic fungi will initiate the degeneration of the fruit to its basic elements by releasing enzymes to attack the skin. This is then followed some yeast or aerobic bacteria to digest the sugar to finally end with the basic elements. Most fruits have high water content and low pH and this will promote the spoilage by microorganisms such as fungi, bacteria and mould [1].

If the fruit is consumed by humans or other living creatures, the anaerobic bacteria which are transferred from the skin to the flesh of the fruit as the fruit is being peeled will be swallowed together with the fruit. These anaerobic bacteria will degenerate the fruit anaerobically as it passes through the digestive system, resulting in compounds that are suitable to be absorbed by the blood stream [7].

II. METHODOLOGY

A. Materials

Unpeeled garlic (Giant,Shah Alam), Sugar (CSR,Giant), Nutrient Agar (Merck KGaA, Germany), Potato Dextrose Agar (PDA) (Merck KGaA, Germany), Mc Conkey Agar (Merck KGaA, Germany), Urea Agar (Friendemann), deMan Rogosa (Merck KGaA, Germany), deMan Rogosa and Sharpe (MRS) (Merck KGaA, Germany), Plate Count Agar (PCA) (Merck KGaA, Germany), peptone water (Friendemann), distilled water, disposable mineral bottles (500 ml volume), alcohol 95% , Gram staining kit .

B. Apparatus

Disposable agar plate (Favorit), L smear, Bunsen burner, microcentrifuge tubes, pipette, pipette tips, Jar and tins, candle, autoclave.

C. Methods

1.5 kg of garlic was grinded together with 10%w/w sugar and some distilled water to facilitate the mixing. According to Breed [2], that the raw material must be 10% by weight. The garlic itself must not be peeled because it was expected that there are also microbes existing on the skin of the garlic. The other material need to grind with the garlic are sugar and distilled water. The grinded product is then made up with distilled water to produce the fermentation medium containing 3% garlic and 3% sugar.

31 mineral bottles each containing 350ml of fermentation medium were prepared. The preparation for each day of research project going on since this research is about anaerobic fermentation of garlic: time axis analysis. Based on calculation made, for the first week, 1 bottle per day for each medium. The number of water bottles depends on the test apply. As example only 1 bottle need for serial dilution, while for selecting media 6 bottles need for each

culture medium which are Mc Conkey agar, Potato Dextrose agar (PDA), Plate Count Agar (PCA), de Man, Rogosa and Sharpe Agar (MRS) and Urea agar respectively. To be alert that each bottles must be observe and open the bottle cap to release the gas produce form anaerobic fermentation process

1.0 Fermentation preparation

Dilutions were normally prepared in 9 mL of water. When 1 mL of garlic suspension is pipetted into 9 mL of water a 1:10 dilution is made. It was agreed with 5 time serial dilution until 1:100000. This also can be written as 10^{-1} and for the further dilution, 1 mL from 1:10 dilution is used in another 9 mL of water. This makes 10^{-2} dilution or 1:100. Next, the serial dilution were continue until reaching the final dilution which is 1:100000 using same procedure as before. Each dilution is culture on nutrient agar and last step was the cell was observed after 24 hour incubation using colony counter and the colony forming unit (CFU) was calculated, CFUs number for each dilution used on the nutrient agar.

2.0 Sampling and Analysis

2.1 Selecting Media Preparation

First and foremost, the nutrients must be calculated and weighted according to below calculation.

Calculation of agar samples:

40 g/L= 40 g dissolve in 1000 mL distilled water
2 petri dish of nutrient, amount of water and nutrient required are
= (2 x 25x 40g)/1000 mL
= 2 g

After all of the agar were distribute evenly, nutrient was dissolved into water and boiled. Not forget the important steps which was the nutrient must be steriled in autoclave at 121°C for 15 minutes. To suits with aseptic condition of this research methods, laminar flow was used. Pour nutrient agar into petri dish and allow nutrient to solidify under aseptic condition and normal environment condition. By using incubator, the samples were incubate at 37°C for 24 hours. This preparation steps is same as the other agar which are Mc Conkey agar, Potato Dextrose agar (PDA), Plate Count Agar (PCA), de Man, Rogosa and Sharpe Agar (MRS) and Urea agar. As all the selecting media agar are solidified, part of the requirement for all of the selecting media that been culture with the ferment medium must be stored in air tight jar to supporting the anaerobic condition [2].

2.2 Gram Staining

The determination of simple staining was to clarify the morphology and arrangement of bacteria cell. As prepared separate smear of the sample microbial from previous steps, a slide was placed on staining tray followed by flooding the smear. Carbol fuchsin 15-30 seconds; crystal violet, 20-60 seconds, methylene blue 1-2 minutes. Mildly the smear was washed with tap water to remove excess stain. The slide was hold parallel position to the stream of water in order to minimize the loss of organisms from the preparation. Further by next step was to bolt dry the sample using bibolous paper but not to wipe the slide. Therefore the stained slides were inspected using oil immersion microscope with scale of 100X.

III. RESULTS AND DISCUSSION

A. Number of colonies on agar plate spread with serially-diluted fermentation medium

Throughout 3 months fermentation which was to determine the microbial species diversity time-profiles of batch garlic anaerobic fermentation, samples were taken, serially-diluted and cultivated on various agar plates.

The results represent the first attempt at this experiment. There were few failed experiments, where the number of colonies was too numerous to count, and which was labelled as TNTC (too numerous to count).

Table 1 : Number of colonies of 10^1 serial dilution on PCA

Day 1	61	Day 9	17	Day 37	TNTC
Day 2	10	Day 10	TNTC	Day 44	TNTC
Day 3	TNTC	Day 11	TNTC	Day 51	34
Day 4	31	Day 15	90	Day 58	0
Day 5	80	Day 17	TNTC	Day 65	65
Day 6	2	Day 22	TNTC	Day 72	TNTC
Day 7	0	Day 29	TNTC	Day 82	TNTC

Table 2: Number of colonies of 10^1 serial dilution on MRS

Day 1	125	Day 9	5	Day 37	80
Day 2	0	Day 10	18	Day 44	33
Day 3	72	Day 11	TNTC	Day 51	0
Day 4	93	Day 15	TNTC	Day 58	1
Day 5	89	Day 17	TNTC	Day 65	2
Day 6	58	Day 22	65	Day 72	0
Day 7	262	Day 29	0	Day 82	0

Table 3: Number of colonies of 10^1 serial dilution on UREA

Day 1	136	Day 9	51	Day 37	53
Day 2	0	Day 10	0	Day 44	25
Day 3	86	Day 11	10	Day 51	1
Day 4	0	Day 15	20	Day 58	0
Day 5	0	Day 17	TNTC	Day 65	TNTC
Day 6	0	Day 22	0	Day 72	0
Day 7	0	Day 29	TNTC	Day 82	0

Table 4: Number of colonies of 10^1 serial dilution on MC CONKEY

Day 1	0	Day 9	17	Day 37	0
Day 2	0	Day 10	6	Day 44	0
Day 3	0	Day 11	121	Day 51	0
Day 4	0	Day 15	27	Day 58	0
Day 5	0	Day 17	125	Day 65	88
Day 6	0	Day 22	TNTC	Day 72	TNTC
Day 7	0	Day 29	0	Day 82	0

The second attempt of experiments were conducted as repetitions to replace the TNTC results. The purpose of repetition was to get the number of colonies in order to plot the species diversity and community dynamics graphs. Only failed results on Mc Conkey, MRS and Urea Agar were repeated. PCA agar results were not repeated because the number colonies at first attempt could be counted, and the main objective was only to observe the viable growth on the agar medium.

Table 5: Number of colonies of 10^1 serial dilution on MRS

Day 1	125	Day 9	5	Day 37	80
Day 2	0	Day 10	18	Day 44	33
Day 3	72	Day 11	31	Day 51	0
Day 4	93	Day 15	42	Day 58	1
Day 5	89	Day 17	57	Day 65	2
Day 6	58	Day 22	65	Day 72	0
Day 7	262	Day 29	0	Day 82	0

Table 6: Number of colonies of 10^1 serial dilution on UREA

Day 1	136	Day 9	51	Day 37	53
Day 2	0	Day 10	0	Day 44	25
Day 3	86	Day 11	10	Day 51	1
Day 4	0	Day 15	20	Day 58	0
Day 5	0	Day 17	15	Day 65	0
Day 6	0	Day 22	0	Day 72	0
Day 7	0	Day 29	0	Day 82	0

Table 7: Number of colonies of 10^1 serial dilution on MC CONKEY

Day 1	0	Day 9	17	Day 37	0
Day 2	0	Day 10	6	Day 44	0
Day 3	0	Day 11	121	Day 51	0
Day 4	0	Day 15	27	Day 58	0
Day 5	0	Day 17	125	Day 65	88
Day 6	0	Day 22	0	Day 72	22
Day 7	0	Day 29	0	Day 82	0

B. Time-Profile of number of colonies of selected microbes

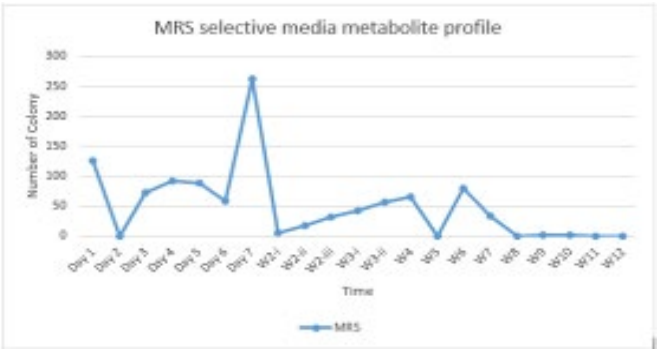

Figure 1: Time-Profiles of number of colonies of selected microbes on MRS agar

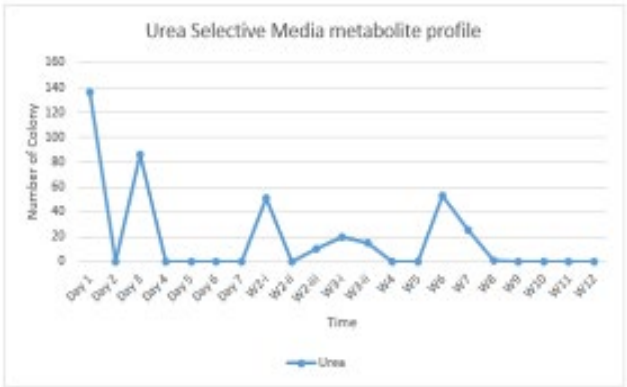

Figure 2: Time-Profiles of number of colonies of selected microbes on UREA agar

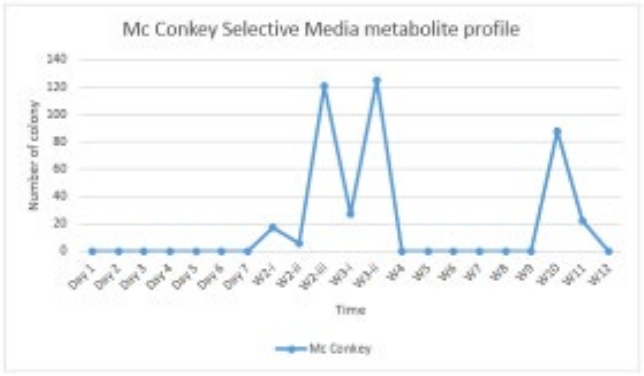

Figure 3: Time-Profiles of number of colonies of selected microbes on Mc Conkey agar

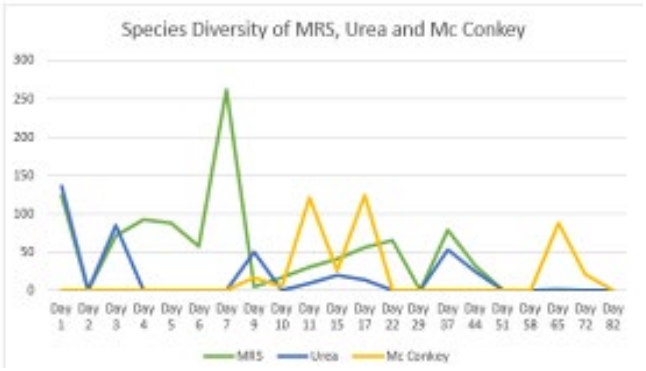

Figure 4: Time-profiles and Species Diversity of microbes selected by MRS, Urea and Mc Conkey Agar

By referring to the Fig.4 above, it was found that at the initial of the research, Day 1, both microbes in Urea Agar and MRS had better growth compared to microbes in Mc Conkey Agar. Even though the substrates comprising of the initial carbon source, was initially at high concentration. The assumption that can be made with this situation is the possibility that the microbes that exist at the beginning of fermentation as selected by Mc Conkey agar was not suited to the condition and therefore did not grow well.

However, the microbes selected by Mc Conkey agar grew well on days 10, 11, 17, 22, 65 and 72, indicating the presence of substrates suited for their growth at those times.

At the end of the fermentation days, the number colony that exist in each of those 3 agars were 0. This was due to concentration of all substrates having been fully depleted by the final day.

C. Gram Staining

The next step was to identify the microbes morphology by using the gram-staining method to identify the shape, cells arrangement and color. Most of the microbial shape were coccus, rod or spiral form. The tables below give the results of gram-staining samples along the 90 days fermentation.

DAY 1

MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Blue • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Rod • Color : Blue • Gram : Positive

DAY 2	
PCA	• Shape : Rod • Color : Red • Gram : Negative
PDA	• Shape : Coccus • Color : Blue • Gram : Positive

DAY 3	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Blue • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Rod Color : Blue/Purple Gram : Positive

DAY 4	
MRS	• Shape : Coccus Color : Red Gram : Negative
PDA	• Shape : Rod • Color : Blue • Gram : Positive
PCA	• Shape : Rod • Color : Blue/Purple • Gram : Positive

DAY 5	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Blue • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive

DAY 6	
MRS	• Shape : Rod • Color : Blue and Red • Gram : Positive/Negative
PDA	• Shape : Rod • Color : Blue • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive

DAY 7	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Coccus • Color : Red • Gram : Negative

DAY 9	
MRS	• Shape : Coccus • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Red • Gram : Negative
PCA	• Shape : Rod • Color : Red • Gram : Negative
UREA	• Shape : Coccus • Color : Red • Gram : Negative
MC CONKEY	• Shape : Rod • Color : Red • Gram : Negative

DAY 10	
MRS	• Shape : Coccus • Color : Blue • Gram : Positive
PDA	• Shape : Coccus • Color : Red • Gram : Negative
PCA	• Shape : Coccus • Color : Red • Gram : Negative
UREA	• Shape : Rod • Color : Red • Gram : Negative
MC CONKEY	• Shape: Rod • Color : Red • Gram : Negative

DAY 11	
MRS	• Shape : Rod • Color : Blue

	• Gram : Positive
PDA	• Shape : Rod • Color : Blue • Gram : Positive
PCA	• Shape : Coccus • Color : Red • Gram : Negative
UREA	• Shape :Rod • Color :Blue • Gram : Positive
MC CONKEY	• Shape :Coccus • Color :Red • Gram : Negative

DAY 15	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Coccus • Color : Red • Gram : Negative
PCA	• Shape : Rod • Color : Red • Gram : Negative
UREA	• Shape : Coccus • Color : Red • Gram : Negative
MC CONKEY	• Shape : Rod • Color :Red • Gram : Negative

DAY 17	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Blue/Purple • Gram : Positive
PCA	• Shape : Coccus • Color : Red • Gram : Negative
UREA	• Shape : Rod • Color : Red • Gram : Negative
MC CONKEY	• Shape : Rod • Color :Red • Gram : Negative

DAY 22	
PDA	• Shape : Rod • Color : Red • Gram : Negative
PCA	• Shape : Rod • Color : Blue • Gram : Positive
MC CONKEY	• Shape : Rod • Color :Red • Gram : Negative

DAY 29	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Red • Gram : Negative
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Rod • Color : Red • Gram : Negative
MC CONKEY	• Shape : Rod • Color :Red • Gram : Negative

DAY 37	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Blue/Purple • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Rod • Color : Blue • Gram : Positive

DAY 44	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Rod • Color : Blue/Purple • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Rod • Color : Blue • Gram : Positive

DAY 51	
PDA	• Shape : Rod • Color : Blue/Purple • Gram : Positive
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Rod • Color : Red • Gram : Negative

DAY 58	
MRS	• Shape : Rod • Color : Blue • Gram : Positive

DAY 65	
MRS	• Shape : Rod • Color : Blue • Gram : Positive
PDA	• Shape : Coccus

	• Color : Red • Gram : Negative
PCA	• Shape : Rod • Color : Blue • Gram : Positive
UREA	• Shape : Coccus • Color : Red • Gram : Negative
MC CONKEY	• Shape : Rod • Color :Red • Gram : Negative

DAY 72	
PDA	• Shape : Rod • Color : Red • Gram : Negative
PCA	• Shape : Coccus • Color : Blue • Gram : Positive
UREA	• Shape : Rod • Color : Red • Gram : Negative
MC CONKEY	• Shape : Rod • Color :Red • Gram : Negative

DAY 82	
PCA	• Shape : Rod • Color : Blue • Gram : Positive

The morphology of the microbes in the samples taken at the specified days were found to be somewhat different for different day, although for some different days the morphologies were similar. This relates well with the species diversity that existed throughout the 90 days of the anaerobic fermentation.

There were several limitations in this research project that need to highlighted such as each result of number of colony that appear too numerous to count and repetition was the only solution for this problem. As a backup plan, immediately counting the colony after incubation for 24 hours to confirm that the colony can be calculated. During the making of agar, serial dilution, contamination might happened. This problem also rise during preparation of the fermentation medium. As for the recommendation, the best way was to conduct the fermentation in the bioreactor. Bioreactor can secure there was absence of oxygen in the ferment medium. Next issue was when there was plentiful of colony, it was hard to get the single colony since it was clump together. So as recommendation, plate the higher dilution than 10^1 to get the single colony.

For future research, instead of using gram staining method, try to find out the best way for deeper morphology finding since gram staining method was not enough to identify the specific microbes that can survive in the anaerobic fermentation with garlic medium. Advanced technology or equipment such as Polymerase Chain Reaction (PCR) can be used instead of gram staining as an option.

IV. Conclusion

By looking thorough 90 days of this research, it was found that the anaerobic fermentation of garlic experienced both species diversity and community dynamics. The time-profiles of each selected microbes can be used as a standard in scale-up and quality control of this type of spontaneous fermentation.

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