Batch Anaerobic Fermentation of Pomegranate Fruit: Time-Axis Analysis of Species Diversity

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Abstract— **Batch anaerobic spontaneous fermentation based on a medium of 10% grinded pomegranate fruit and 10% sucrose in distilled water was carried out over a period of 12 weeks to study the species diversity along the time axis. In this research, streaking on nutrient agar, potato dextrose agar and a few selective media as well as Gram staining were applied to obtain the results. Before the fermentation samples are streaked on the various media, they were diluted in a series. The selective media used were MacConkey agar, Urea agar and MRS (de Man, Rogosa and Sharpe) agar. Potato Dextrose Agar (PDA) was used for the growth of fungus whereas nutrient agar was used for the total growth. Gram staining was carried out for the growth on the selective media to determine if the bacteria was Gram positive or Gram negative.**

Keywords— Fermentation, Anaerobic, Pomegranate, Species Diversity

INTRODUCTION

Theoretically, on the outer skin layer of every fruit there would exist a natural fauna of aerobic and anaerobic microorganisms including bacteria and fungi which could come from the soil, water or air. When a fruit is left uneaten beyond its time of being ripe, it would fall to the ground. Then, the fungi will initiate the disintegration of the fruit by releasing enzymes which will attack the skin of the fruit. The reason being, most fruits have quite a hard layer of skin for proctection. Once the skin is broken and the juicy inside of the fruit is exposed, the aerobic bacteria from the skin would proceed to slowly disintegrate the whole fruit to its basic elements.

When a ripe fruit is taken and eaten by humans or other creatures, the aerobic and anaerobic bacteria that are transferred from the skin of the fruit to the juicy inside of the fruit by the eater's hand upon breaking the skin are swallowed together with the fruit into the stomach. Since there is no oxygen in the alimentary canal, only the anaerobic bacteria will play a role in breaking down the fruit through an anaerobic fermentation. The end point of this anaerobic fermentation coincides with the production of metabolites which are useful to the eater, and which are bioavailable and readily absorbed through the walls of the alimentary canal and into the blood stream.

The term 'spontaneous fermentation' is used to describe this fermentation process because there was no inoculation done, and the inoculum comes from the naturally occurring microorganisms residing on the outer skin of the fruit.

The medicinal effects of the pomegranate fruit have been well-documented. When a person eats a pomegranate fruit, the benefits of the fruits will be experienced by the body through absorption of the bioavailable metabolites of the anaerobic fermentation occurring in the alimentary canal. A good idea to be tested is to carry out this spontaneous anaerobic fermentation of pomegranate fruit outside the body to produce the same bioavailable metabolites as commercial products.

The types of bioavailable metabolites produced will be a function of the anaerobic microbes present in the batch spontaneous anaerobic fermentation of pomegranate fruit along the time axis.

This research focuses on the batch spontaneous anaerobic fermentation of grinded whole pomegranate fruit to study the species diversity profiles along the time axis over a period of 12 weeks. Throughout the period of 12 weeks, there will be different types of fungi and bacteria present in the fermentation sample, therefore a time profile for each of the different types of fungi and bacteria will be needed to characterize the fermentation. This in turn can be used as a future reference in the fermentation industry for scaling up this fermentation process as well as for quality control.

I. METHODOLOGY

A. Materials

The pomegranate fruit used were ripe fruits imported from Turkey. The pomegranates were cut into smaller pieces before they were grinded with their skin unpeeled. Sucrose (MSM) was purchased Giant Supermarket. Gram staining kit (Merck, Darmstadt, Germany) and Microscopy oil (Merck, Darmstadt, Germany) were used during the Gram staining sample analysis. 98% ethanol (J. Kollin Chemicals) was used for sterilization purposes. The Nutrient agar, Potato Dextrose agar (PDA), MRS (de Man, Rogosa and Sharpe) agar, Plate count agar (PCA), Urea agar, MacConkey agar used were from Merck, Darmstadt, Germany. The peptone water which was used for dilution was also from Merck.

B. Apparatus

Disposable plastic bottles with volume of 500 ml were purchased from Tesco Supermarket. The agars were incubated in an incubator (Thermo Scientific^{TM}) for 24 hours. An all-purpose laboratory Parafilm M tape from Bemis Flexible Packaging was used around the petri dishes containing agar to prevent contamination. A colony counter was used for the counting of colony growth on the agar.

C. Methods

Fermentation Process

In this research, the process of batch spontaneous anaerobic fermentation was conducted. 500 ml batch spontaneous anaerobic fermentations were carried out in 31 separate tightly capped disposable plastic bottles, each containing 10% (w/v) pomegranate fruit and 10% (w/v) sucrose in distilled water. A total of 1.55 kg of fruit were used and grinded together with 1.55kg sucrose and some distilled water. The resulting mixture was then made up to 10.5L with distilled water, mixed thoroughly, and divided into 500ml portions which are filled into the 31 plastic bottles.

Each bottles were then labelled according to the days when the sample will be analyzed, which are, Day 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 18, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84. The bottles from

day 28 onwards were done in replicates just in case of contamination. During fermentation, the bottles were left at a room temperature of 25˚C and away from direct sunlight for a period of 12 weeks. Periodically, the cap of each of the plastic bottles were opened to release the pressure of accumulated gases inside the bottle in order to prevent it from exploding.

Sample Analysis

In the first week of fermentation, a sample was taken every day and analysed. For the second week of the fermentation, a sample was taken every two days and analysed. For the third week of the fermentation, a sample was taken every 3 days and analysed. From week 4 to week 12, the fermentation samples were analysed once a week. In the first week of sample analysis, data was taken every day because the early stages of fermentation are usually when the growth of microorganisms is at its peak.

The sample analysis includes a serial dilution of 10^0, 10^1, 10^2, 10^3, 10^4 and 10^5, which were diluted with peptone water. After dilution, each diluted samples were streaked on nutrient agar, PDA, PCA as well as streaked on selective media agars using a smearing tool. After each agar has been streaked with samples, the petri dishes were sealed with parafilm tape and then incubated for 24 hours. During incubation, the petri dishes were placed inside a jar or tin container that was deprived of oxygen. When the incubation process ended, the petri dishes were taken out and then the morphology of the growth of any bacteria or fungi on each petri dish was observed. Plate counting was then initiated by using a colony counter to obtain the number of colony forming units (CFU) on each plate.

Gram staining is the final laboratory procedure for the analysis of sample in this experiment. The purpose is to differentiate whether a Gram positive or a Gram negative bacteria exists in a sample. To prepare for Gram staining, a smear on a glass microscope slide will be prepared first. In order to obtain a perfect smear, only a thin layer from the culture is required because if the smear is too thick, the amount of light that can pass through the glass slide will be reduced which makes it difficult to visualise the morphology of a cell. To ensure the smear does not wash away during the staining procedure, heat fixation is applied so that the smear will stick on the glass slide. This is done by rapidly passing the glass slide with the smear over the flame of a Bunsen burner for 3 times while taking caution as to not burn and kill the cells in the smear. Next, the Gram staining begins with flooding the glass slide with crystal violet for one minute and then pouring off the excess dye and washing it in tap water gently before drying the glass slide using a paper towel. Then, the glass slide will be flooded with Gram's iodine continuously for one minute. The glass slide is then washed with tap water and drained carefully and then washing it with 95% alcohol for 30 seconds. Then, to remove excess alcohol, the glass slide will be washed with tap water again for 30 seconds to stop the decolourization. Next, the glass slide will be counterstain with 0.25% of safranin for 30 seconds. Then it will be washed, drain and blot with paper towel before examining it under a microscope.

II. RESULTS AND DISCUSSION

A. The number of colony count on the MRS agar at a specific day of the spontaneous fermentation.

The MRS agar was used to see if there were any growth of *Lactobacilli*. Sodium acetate is a part of the ingredients for this agar which helps in inhibiting the growth of competing bacteria. Throughout the 12-week period, the growth of microorganisms on the MRS agar was very rapid in the beginning. It started with zero growth on the first day which then rapidly increased to 179 colonies by the 6th day. There was a growth decrease on day 7 till day 9 but by the 11th day, there was an overgrowth of microorganisms on the agar plate till the 42nd day. During this period, the colony count was too numerous to count and it was denoted as TNTC. As shown on the graph in Figure 1, the period when the colony count was TNTC was considered as zero colony count because it does not show any accuracy for the microorganism growth. It is possible to consider that the reason for this TNTC (too numerous to count) result is due to contamination on the agar that was used. To prevent this type of result from happening in the future, duplications should be performed to confirm the possibility of the agar contamination or to decline this possibility. At the 49th day, there was zero growth on the MRS agar, however, after this day, there was growth again and it continues until the last day.

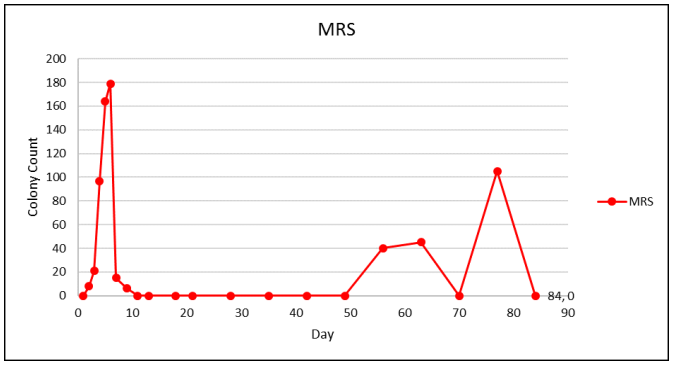

Fig 1: The graph shows the of colony count on the MRS agar at a specific day of fermentation

B. The number of colony count on the MacConkey agar at a specific day of the spontaneous fermentation.

MacConkey agar favors the growth of bacteria that are able to ferment lactose. Based on the results obtained from this fermentation, the growth of microorganisms on this agar is quite rare, only 2 different days has a TNTC result while most of the days throughout the 12-week period there were zero growth. This makes perfect sense because pomegranate is a fruit and lactose is a type of sugar that is found in milk and there would be no reason for a lactose-metabolizing microorganisms to exist in pomegranate fruits. The days with a TNTC result, which were the 21st and 77th day could have resulted from contamination on the agar.

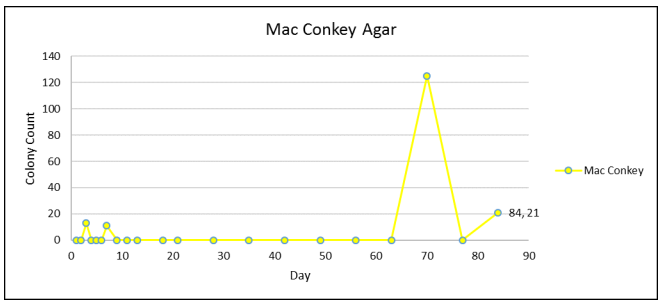

Fig 2: The graph shows the of colony count on the MacConkey agar at a specific day of fermentation

C. The number of colony count on the Urea agar at a specific day of the spontaneous fermentation.

The Urea agar was used to detect the growth of urea-metabolizing microorganisms from this spontaneous fermentation.

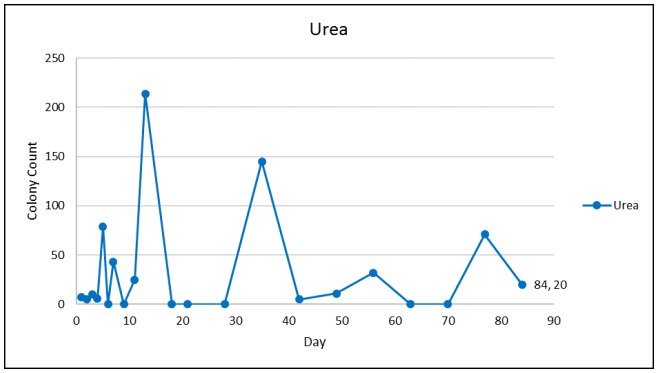

Fig 3: The graph shows the of colony count on the Urea agar at a specific day of fermentation

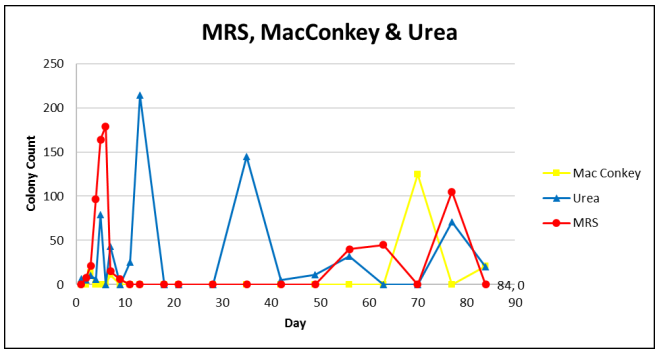

Fig 4: The graph shows superimposed data for MRS, MacConkey and Urea agar.

D. Gram Staining

This analysis was carried out to identify whether the microorganism present on the selective media agars were either a Gram-positive or a Gram-negative. This can be identified by looking at the colour change of the sample under the microscope, which would be from colourless to either pink or violet. If the sample on the microscope slide turns violet, it is a Gram-positive bacteria. Whereas, if the sample turns pink, it is a Gram-negative bacteria. In order to further identify the species that had grown on the selective media, its shape, either a rod shape or a coccus shape was also taken into attention. The following tables below will show the results obtain from each Gram staining test for each selective media and for each day.

Day 1	
Urea Agar	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 2	
Urea Agar	
Colour	Pink (Gram negative)
Shape	Vibrio
Possible Bacteria	• Bacteroides • Fusobacterium
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci

Day 3	
Urea	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MacConkey Agar	
Colour	Purple and Pink (Contaminated)
Shape	Rods
Possible Bacteria	Null

Day 4	
Urea	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MacConkey Agar	
Colour	Pink (Gram negative)
Shape	Coccus
Possible Bacteria	• Veillonella

Day 5	
Urea	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MacConkey Agar	
Colour	Pink (Gram negative)
Shape	Coccus
Possible Bacteria	• Veillonella

Day 6	
Urea	
Colour	Pink (Gram negative)
Shape	Coccus
Possible Bacteria	• Veillonella
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci

Day 7	
Urea	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces

	• Clostridium
MacConkey Agar	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium

Day 9	
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 11	
Urea	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 13	
Urea	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci

Day 18	
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 21	
Urea	
Colour	Pink (Gram negative)
Shape	Coccus
Possible Bacteria	• Veillonella
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MacConkey Agar	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium

Day 28	
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 35	
Urea	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci

Day 42	
Urea	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci

Day 49	
Urea	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 56	
Urea	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 63	
Urea	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium

Day 70	
Urea	
Colour	Pink and Purple (Contaminated)
Shape	Coccus for Pink, Rods for Purple

Possible Bacteria	• Null
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium
MacConkey Agar	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium

Day 77	
Urea	
Colour	Pink and Purple (Contaminated)
Shape	Coccus for Pink, Rods for Purple
Possible Bacteria	• Null
MRS	
Colour	Purple (Gram positive)
Shape	Rods
Possible Bacteria	• Actinomyces • Clostridium
MacConkey Agar	
Colour	Pink (Gram negative)
Shape	Rods
Possible Bacteria	• Bacteroides • Fusobacterium

Day 84	
Urea	
Colour	Pink and Purple (Contaminated)
Shape	Coccus for Purple, Rods for Pink
Possible Bacteria	• Null
MRS	
Colour	Purple (Gram positive)
Shape	Coccus
Possible Bacteria	• Peptostreptococci

III. Conclusion

By the end of the fermentation period, which is at Day 84, there were still some growth occurring in the fermentation. This could mean that the microorganisms are still growing due to the existence of sufficient amount of substrate in the fermentation medium. The substrate could also be the metabolites produced earlier. If it is due to the existence of sufficient amount of substrate remaining in the medium, the fermentation should either be extended until all growth have ceased, or the amount of sucrose should be reduced in subsequent fermentations.

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References

[1] Downes and Ito. 2001. *Compendium of methods for the microbiological examination of foods, 4th ed*. American Public Health Association, Washington, D. C.

[2] Gumienna, X, Malgorzata, X, Artur Szwengiel, and Barbara Gorna. "*Bioactive Components of Pomegranate Fruit and Their Transformation by Fermentation Processes."* European Food Research and Technology 242.5 (2016): 631-40. Web.

[3] Curiel, Jose Antonio, Daniela Pinto, Barbara Marzani, Pasquale Filannino, Giovanni Antonio Farris, Marco Gobbetti, and Carlo Giuseppe Rizzello. *"Lactic Acid Fermentation as a Tool to Enhance the Antioxidant Properties of Myrtus Communis Berries."* Microbial Cell Factories 14.1 (2015). Web.

[4] Mousavi, Zeinab E., Seyed Mohammad Mousavi, Seyed Hadi Razavi, Mehri Hadinejad, Zahra Emam-Djomeh, and Mojgan Mirzapour. *"Effect of Fermentation of Pomegranate Juice ByLactobacillus PlantarumandLactobacillus Acidophiluson the Antioxidant Activity and Metabolism of Sugars, Organic Acids and Phenolic Compounds."* Food Biotechnology 27.1 (2013): 1-13. Web.