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Potential of *Smallanthussonchifolius* (Yacon) Extract-Based Agar as SubstituteCulture Medium forMicro-organisms

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ABSTRACT

This study aimed to determine the viability of yacon extract-based agar (YEBA) as a substitute medium for micro-organisms. Three (3) species of bacteria were tested in this study: *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa*. The study involved the inoculation of these bacteria on Yacon extract-based agar at controlled environment conditions.

The yacon extract was prepared by treating the yacon juice with 95% ethanol for 72 hours then evaporating the alcohol using the rotavap. The resulting mixture was diluted into four concentrations: 25%, 50%, 75%, and 100% and were mixed with an appropriate amount of pharmaceutical agar to form the yacon extract-based agar. The pH of the YEBA was adjusted to 7.0 before inoculation with the test bacteria. Bacterial growth was determined through the number of colonies grown after overnight incubation at 37^0C.

The results of the experiment revealed that all the YEBA preparations showed signs of bacterial growth. It further revealed that the 75% and 100% extract concentrations exhibited the most significant number of colonies. Likewise, the colonies manifested characteristic pigments. Finally, it can be concluded that the Yacon extract-based agar, using ethanol as the extracting solution, can be used as a cheap and cost-effective substitute culture medium for micro-organisms.

Keywords: *Smallanthussonchifolius*, yacon extract, yacon extract-based agar, culture medium

Introduction

Robert Koch developed a method for isolating pure cultures of bacteria on solid media in 1883. He mixed meat broth with agar. The meat broth supplies the nutrients needed for the growth of micro-organisms. At the same time, the agar serves as a solidifying agent where the micro-organisms grow in the form of colonies representing different species of bacteria.

The isolation of specific species of bacteria from various sources is essential in all fields of microbiology because bacteria live in communities of mixed populations. These mixed populations of bacteria interact and cooperate to obtain nutrients from their environment with the waste products of one species of bacteria serving as food for another species.

Micro-organisms require specific conditions to grow and multiply. These are not limited to food or nutrients, and specific environmental conditions. Synthetic culture media, provide the food needed for bacterial
growth and maintenance. Nutrient agar is used to grow non-fastidious bacteria in laboratories. It is a primary medium composed of a simple peptone and a beef extract. Since commercially prepared culture media are costly and are not readily available, it is imperative to develop alternative or substitute culture media for laboratories with less facility.

The study of bacteria requires isolating them from their natural source and cultivating them in pure cultures using synthetic culture media or its equivalent. Therefore, any advancement in the field of Bacteriology necessitates expansion in the development of synthetic culture media and culture techniques. Bacteriologists employ culture media for different applications. They use culture media to cultivate these bacteria in pure cultures, reveal their morphological, metabolic, and biochemical properties, and allow long-term storage for future use.

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The culture media used in the laboratory setting may be classified as defined (simple) or undefined (complex). The medium is called simple if its specific ingredients are known. It is called complex if its components are not known. The commercially-available culture medium, Nutrient Agar, is an example of simple culture medium. It supports the growth of a wide range of non-fastidious organisms. It contains peptone (0.5%), which provides organic nitrogen; beef extract (0.3%), which is a common source of carbohydrates and salt; agar (1.5%), which makes the medium solid; and distilled water, which serves as a transport medium for the agar's various substances. Plant and animal extracts, egg yolk, blood, etc., on the otherhand, are typical examples of complex culture media.

Commercial culture media present variations in biological activity. Several of these have been developed to facilitate the study of micro-organisms. Bacterial culture media used in laboratories are generally synthetic, costly, and imported from different countries. Difco, a manufacturer of microbiological supplies and equipment which originated from the United States, but is distributing products in the country, offers hydrated culture media with prices ranging from US $200.00 to US $300.00 (Corpuz, 2017). Consequently, researchers embark on discovering and proving natural sources of bacterial culture, both agar and broth. One ofthe proven organic sources of bacterial cultureagar and broth is coconut water, from the fruit of coconut palm. However, it has not yet been developed as a commercial culture medium for bacteria. The sweet and refreshing coconut water contains sugars, sugar alcohols, lipids, and amino acids (Neerathilingam et al., 2013), making it an effective bacterial culture medium.

Smallanthussonchifolius(yacon), is also known as the "apple of the earth" because although it is grown underground like any other root crops, its fruit resembles an apple or pear. It is regarded as a "wonder-plant" in South America and Europe because of its numerous medicinal properties. In the Philippines, it is widely cultivated in the Cordilleras, Nueva Vizcaya, and other highlands (Valentova, 2001).

The yacon tuber contains 86-90% water and certain traces of phosphorus (22%), carbohydrates (11.1%), protein (0.8%), fiber (0.6%), lipids (0.6%), and cellulose (0.5%). One yacon tuber is also complete in other essential elements such as iron, calcium, sodium, potassium, carotene, magnesium, and vitamins A, B1, B2, and C (de la Cruz, 2005). The fructose content of yacon varies from 2-22 grams per 100 in fresh roots; alpha glucose is 2-7 grams; beta-glucose is 2-6 grams, and sucrose from 2-4 grams. The composition per 100 grams of edible portion: energy is 54 kcal; water is 86.6 g; calcium is 23 mg; phosphorus is 21 mg; iron is 0.3 mg; retinol is 12 mcg; thiamine is 0.02 mg; riboflavin is 0.11; niacin is 0.34 mg; ascorbic acid is 31.1 mg.

Yacon is recognized as a health food not only for humans but also for micro-organisms due to its nutritional value. There is an increasing demand foryacon due to the rising awareness among consumers of its beneficial effects.

Aside from its medicinal and nutritional values, yacon can be utilized in other ways. Thus, this study aimed to develop a substitute culture medium for micro-organisms from Yaconextract-based agar. Specifically, it (a) described the growth of the bacteria on the different concentrations of the YEBA in terms of colony count, pigmentation, and characteristics; (b) determined if a significant differenceexistsbetween the colony counts of each bacterium at different extract concentrations; (c) proposed a preparation of the YEBA that can be used as a substitute culture medium for micro-organisms, and (d) presented a cost-benefit analysis of the proposed product.

The findings of the study will be significant to the people who are in line with microbiological studies and experiments by providing a substitute culture medium for micro-organisms.

Materials and Methods

This study utilized the experimental study design. The experimental design was used because it involved the manipulation and control of the independentvariable concomitant to the

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dependent variables.

Preparation and Extraction of the Yacon

Fresh yacontubers were purchased at the local public market. They were transported to the laboratory and washed clean using tap water and finally with distilled water. Approximately 3.0 kilograms of the yacon tubers were peeled and cut into small pieces and processed using a sterile blender until a homogenous mixture is formed. The mixture was treated with 95% ethyl alcohol in 1:2 proportionand allowed to stand overnight, then filtered,and the filtrate was subjected to concentration using the rotavap. The resulting solution was further concentrated using a water bath maintained at 50^0C until a syrupy substance was produced. This is considered the 100% yaconextract.

Preparation of Culture Media

Nutrient agar was the control medium in the study. The preparation of the Nutrient agar was based on the manufacturer's instruction. Twenty-eight (28) grams of the powder was suspended in 1 liter of distilled water. The suspension was boiled with continuous stirring to dissolve the powder completely, then sterilized by autoclaving at 15 psi at 121^0C for 15 minutes.

The experimental medium was prepared using the yacon extract at different concentrations (25%, 50%, 75%, and 100%). To prepare the YEBA, 7 grams of Pharmaceutical agar was dissolved in 250 mL of the prepared concentrations of the extract. The pH of the prepared media was adjusted to 7.0 using an automated pH meter before autoclaving to ensure sterility of the medium. To adjust the pH, 0.1N NaOH (to increase the pH) or 0.1 N HCl (to decrease the pH) was added drop by drop to each prepared medium until the desired pH was obtained. The same sterilization technique, as in the preparation of the Nutrient agar was implemented.

Performing the Serial Dilution

Ten milliliters (10 mL) of the original cultures (OC) of each of the bacteria used were labeled OC. Six (6) dilution blanks were numbered 1-6, containing 9 mL of sterile distilled water each. Before performing the dilution, the microbial tube suspension was gently shaken to ensure that the cells were evenly distributed in the tube. One (1) mL was transferred from the OC tube to Tube 1 (10 mL total volume of liquid in Tube 1) and was shaken. The same sequence and procedure were done from Tube 1 until Tube 6 to complete the serial dilution. The same serial dilution technique was done on all the micro-organisms cultured.

Performing the Pour-Plate Technique, Colony Count and Gram Staining

The pour plate technique was used to determine the number of micro-organisms/mL. An automatic pipet with a sterile tip was used to transfer 100 µL of the microbial suspension (of each organism) from Tube 6 (with a dilution of 1:1,000,000) into a sterile empty petridish. Fifteen milliliters (15 mL) of each prepared medium (with different concentrations), cooled to 45^0C, was pouredout into the petri dish, and swirled to mix well. Each dish was allowed to solidify (without disturbing), after which it was inverted and incubated at 37^0C for 24 hours. The control medium was subjected to the same procedure.

After 24 hours of incubation, the colonies of the bacteria were counted and tabulated.Slide smear from each petri dish was likewise prepared to determine the Gram staining reaction of the bacteria. The fixed smear was stained with crystal violet for 1 minute and rinsed with running water. The smear was flooded with iodine for 30 seconds and rinsed with water. Few drops of

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decolorizer wereapplied to the streak for 30 seconds and once again rinsed with running water. The smear was air-dried and examined under the oil immersion objective of the microscope.

Biosafety Clearance

The experiment was conducted in a Biosafety Level 2 clinical laboratory of a private secondary hospital. This is required when conducting experiments involving pathogenic bacteria. It was ensured that no one was harmed during the entire process. Universal precaution was observed strictly and that all organisms and wastes generated from the experiment were treated appropriately based on standard procedures. Bacteria and culture media were autoclaved for 1 hour at 121^0C at 15psiwhile chemical wastes were disposed in appropriate containers.

Statistical Treatment of Data

Data gathered from the study were treated using the following statistical measures: mean was used to compute the average colony count of the bacteria; standard deviation (SD) determined how far the colony counts were from the mean; Analysis of variance (ANOVA) was used to determine if significant differences exist in the colony count of the different concentrations of the YEBA extract from one another. ANOVA computation was done personally by the researcher by referring to http://vassarstats.net/anova1u.html.

RESULTS AND DISCUSSION

Growth of the Bacteria

The growth of the bacteria on the different concentrations of the developed culture medium is presented in Table 1 (colony count) and Table 2 (characteristics).

In Table 1, one can observe the variation in the colony count of the three bacteria. As compared to the control medium (Nutrient Agar), there is no doubt that the control medium is still better than the YEBA in all concentrations. This is not surprising since Nutrient Agar contains all the essential nutrients required for the growth of bacteria.

Both the control and the test media were incubated at 37^0C. This is the optimum temperature for bacteria, meaning that bacteria grow best at this temperature. According to Ratkowsky,(1992), the logarithmic phase of bacterial growth is achieved when exposed temperatures where it is most favorable for their multiplication (optimal temperature). Below and above the optimal temperature, a significant decline occurred in the growth rate due to the inactivation or denaturation of proteins or inhibition of the synthesis of ribonucleic acid (RNA). It is a common fact that RNA translation is significant in the synthesis of proteins among bacteria.

Table 1. Growth of Bacteria on the YEBA at pH 7.0 and 37^0C

Culture Medium	Trial 1	Trial 2	Trial 3	Std. Dev.	Mean
Staphylococcus aureus					
Control Medium (Nutrient Agar)	53	49	52	2	51
100% YEBA	35	34	35	1	35
75% YEBA	33	33	33	0	33
50% YEBA	27	30	25	3	27
25% YEBA	22	25	29	4	25
					Escherichia coli

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Control Medium (Nutrient Agar)	60	59	53	4	57
100% YEBA	49	44	46	3	46
75% YEBA	51	49	49	1	50
50% YEBA	31	28	28	2	29
25% YEBA	23	25	26	2	25
Pseudomonas aeruginosa					
Control Medium (Nutrient Agar)	40	39	42	2	40
100% YEBA	28	28	25	2	27
75% YEBA	25	28	28	2	27
50% YEBA	19	18	17	1	18
25% YEBA	15	14	14	1	14

A closer look at the table, shows that bacteria grow best when the concentration of the extract is 75% or 100%. Likewise, in general, as the concentration of the extract increases, so is the colony count. Surprisingly for *Escherichia coli*, higher colony count was recorded at 75% extract than at 100%. This phenomenon can be explained by the fact that particular bacteria have specific nutrient requirements and, when the optimum nutrient requirement is already achieved, bacterial multiplication stops.

Figure 1 illustrates the growth of the bacteria on the different concentrations of the YEBA at pH 7.0 and 37^0C. Of the three bacteria tested, *Escherichia coli* manifested themost significant number of colonies in all concentrations of the extract. This should not come as a surprise since among the three bacteria, Escherichia coli, has the fastest doubling time. Doubling time is the time it takes for the bacterium to double its number. This is affected by the type of medium where the bacteria are inoculated, the temperature, the pH, and other environmental factors. The doubling time of *Escherichia coli* is 15-20 minutes, *Staphylococcus aureus* is 30 minutes, and *Pseudomonas aeruginosa* is 100-120
minutes.

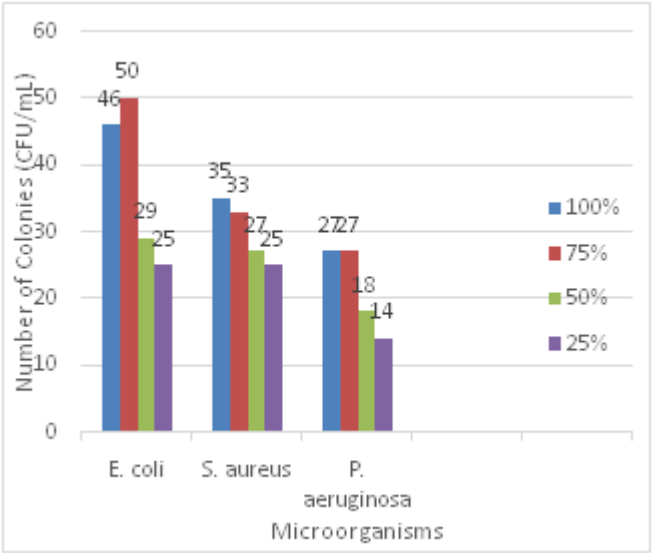

Figure 1. Growth (colony count) of micro-organisms on the yacon extract at ph 7.0 at 37^0C

Growth Characteristics of the Bacteria

Bacteria exist in nature under an enormous range of physical conditions such as oxygen concentration, hydrogen ion concentration (pH), and temperature. Like any other living system, bacteria also require a source of energy, carbon, nitrogen, oxygen, iron, other minerals, micronutrients, and water for growth and multiplication. According to Priyadarshini (2012), factors

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like variations in media components in a culture medium, pH, and temperature have a vital role in influencing microbial cell growth, and enzyme production. Hence the amount of a particular enzyme expressed by a specific bacteriumis directly dependent on the nutrients available and growth condition in a culture medium. In this study, the growth of the bacteria depends on the nutrients contained in the yacon extract-based agar and the growth conditions, e.g., pH and temperature.

Table 2 describes the different growth characteristics of the bacteria cultured on yacon extract-based agar. It shows that all three bacteria produced small-sized colonies, measuring about 2 mm in diameterthat is smooth in consistency, convex in elevation, and shiny in transparency. In terms of shape, both *Staphylococcus aureus* and *Pseudomonas aeruginosa*exhibited round-shaped colonieswhile *Escherichia coli*manifested irregular-shaped colonies. The majority of these growth characteristics of the bacteria agree well with most literature. However, *Escherichia coli* produced large colonies on Nutrient agar, while small colonies on the YEBA. This difference in the colony size can be attributed to the presence of a high concentration of proteins in the form of amino acids in the Nutrient agar, which is absent in the YEBA.

In summary, the growth of the three bacteria wasdue to the different nutritive elements found in the yacon, which served as the source of energy for the bacteria (Manrique et al. 2004).

Table 2. Growth Characteristics of Micro-organisms on the YEBA

Micro-organisms	Colony Growth Characteristics				
	Size	Shape	Elevation	Consistency	Surface features
Escherichia coli	Small	Irregular	Convex	Smooth	Shiny, mucoid, moist
Staphylococcus aureus	Small	Round	Convex	Smooth	Shiny, mucoid
Pseudomonas aeruginosa	Small	Round	Convex	Smooth	Shiny

Small = 1-2mm Large = 3-4 mm

Table 3 (below) presents the pigments produced by the three bacteria on the YEBA.

Table 3. Pigment Produced by the Bacteria on YEBA

Bacteria	Pigment Produced	
	On Nutrient Agar	On YEBA
Escherichia coli	No color produced	No color produced
Staphylococcus aureus	Yellow	Light yellow
Pseudomonas aeruginosa	Blue	Light blue

Among the micro-organisms, bacteria can develop and produce varied bio-products, and one such bio-products is pigment. These are colored inorganic substances that are insoluble in water.

In this experiment, both the *Staphylococcus aureus* and *Pseudomonas aeruginosa* developed pigmented colonies on YEBA similar to those produced when grown on Nutrient Agar. The former produced light-yellow pigments known as staphyloxanthin, while the latter had light blue pigments known as pyocyanin. On the other hand, *Escherichia coli*had non-pigmented colonies on both culture media.

Since pigment production is unique to certain species of bacteria, it can be used to identify the bacteria presumptively.

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Significant DifferenceBetween the Colony Countsof Each Bacterium at Different Concentrations of the Yacon Extract-Based Agar (YEBA)

Table 4 presents the ANOVA result on the significant difference between the colony countsof each bacterium tested at different concentrations of the YEBA.

Table 4. ANOVA Result on the Significant Difference Between the Bacteria's Colony Counts

Staphylococcus aureus					
Colony Count	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1262.66	4	315.667	67.643	.000
Within Groups	46.667	10	4.667		
Total	1309.333	14			
Escherichia coli					
Colony Count	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	2340.933	4	585.233	1078.055	.000
Within Groups	54.667	10	5.467		
Total	2395.600	14			
Pseudomonas aeruginosa					
Colony Count	Statistic	df1	df2	Sig.	
Welch	50.847	4	4.617	.000	

The ANOVA result shows that at least one pair of the colony counts significantly different from one another, F (4,10) = 67.643, p<0.01 for *S. aureus*, F (4,10) = 1078.055, p<0.01 for *E. coli*, and F (4,4.617) = 50.847, p<0.01 for *P. aeruginosa*. To determine which pairsare significantly different, Tukey HSD (for both *S. aureus* and *E. coli*) and Games-Howell Test (for *P. aeruginosa*) were used.

Multiple comparisons between the mean colony counts on the different concentrations of the YEBA for each bacterium tested revealed that bacterial growth on the Nutrient Agar (control medium) is significantly different from the mean colony count of all the bacteria grown on the different concentrations of the YEBA. This means that the control medium is still superior in facilitating the growth of non-fastidious bacteria as compared to the developed medium (YEBA). Inversely,the mean colony counts of the three bacteria are statistically the same on both the 75% and 100% YEBA, respectively. This means that either concentration can be used as substitute media to cultivate *Staphylococcus aureus, Escherichia coli*,and *Pseudomonas aeruginosa*.

Proposed Yacon-Extract Based Agar Formulation and Preparation

Based on the result of the study, the researcher proposes the following formulation of the YEBA, which can be used as a substitute culture medium for the growth of non-fastidious bacteria like *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

Table 5. YaconExtract-Based Agar Formulation

	Culture Medium for *Escherichiacoli, Staphylococcus aureus,*and *Pseudomonas aeruginosa*
100 % Yacon extract	50 ml

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Pharmaceutical agar	28 grams
Distilled water	1 liter

The extract is prepared by mixing the juice resulting from blending one kilogram of the yacon tuber and 95% ethyl alcohol in 1:2 solute to solvent ratio and allowing the mixture to stand FOR 72 hours. Filter the mixture and subject it to a rotary evaporator until all the alcohol is removed. The mixture that is left is considered the 100% yacon extract.

To prepare one liter of the YEBA, 50 mL of the 100% extract is added to 28 grams of the Pharmaceutical agar in a one-liter Erlenmeyer flask. Add sterile distilled water up to the one-liter mark, adjust the pH to 7.0 and sterilize. Fifteen to twenty milliliters of the prepared medium are dispensed in every petridish.

Comparative Cost Analysis Between the YEBA and Nutrient Agar

Table 6 presents a simple comparison of the cost of preparing one liter of the control culture medium (Nutrient Agar)as against the developed YEBA.

Table 6. Comparative Cost in the Preparation of 1 Liter Nutrient Agar as Against the YEBA

Components	Nutrient Agar (NA)	Formulated YEBA
Nutrient Agar	Php 840.00	-
Pharmaceutical Agar	-	Php 150.00
95% ethyl alcohol	-	Php 200.00
1 L distilled water	Php 30.00	Php 30.00
1-kilogram Yacon tubers	-	Php 75.00
Total Cost	Php 940.00	Php 455.00

The table reveals that the YEBA is almost 50% cheaper than the control medium. However, though cheaper, there are some advantages of the Nutrient Agar compared to YEBA. Since the former is in a powder form, it is more stable than YEBA. Nutrient Agar is stable for two years when stored at cool environment(20^0C and below) while the YEBA is stable for two weeks at refrigeration temperature. Furthermore, micro-organisms can survive up to two months on a Nutrient Agar plate/tubewhile three weeks on the YEBA plate/tube.

Conclusions and Recommendations

Based on the findings of the study, the researcher concludes thatthe YEBA can be used as a substitute culture medium for the growth of non-fastidious micro-organisms. For the cultivation of bacteria, specifically *Escherichia coli*, *Staphylococcus aureus*, *and Pseudomonas aeruginosa*, the 100% Yacon extract-based agar at pH 7.0 at 37^0C may be used.

The researcher recommends that this newly developed formulation of YEBA be tried in the laboratorywhen cultivating and isolating non-fastidious bacteria for instructional purposes. Other species of bacteria be tested to maximize its use, and the liquid extract be transformed into a powder form to improve its stability.

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