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RESEARCH PAPER

Formulation of Alternative Culture Media from Natural Plant Protein Sources for Cultivation of Different Bacteria and Fungi

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A B S T R A C T

This study was carried out to find out some semisynthetic new media for routine microbiological tests in microbiology laboratories, with cheap natural protein source. Agar culture media were formulated using natural plant sources such as cowpea, lentil, split pea, chickpea, soy protein, mung beans and rice and used for cultivation of *Staphylococcus aureus*, *Escherichia coli*, *B. cereus*, *Pseudomonas aeruginosa*, *Penicillium sp.* and *Aspergillus sp.* All tested microorganisms grew well on most plant sources except on rice. *B. cereus* showed the best growth and large colonies on formulated media. *E. coli* and *P. aeruginosa* also showed good growth, and Pigmentation of *P. aeruginosa* was not affected. *S. aureus*, *P. aeruginosa* and *B. cereus* produced beta hemolysis on all formulated media. Each bacteria exhibit the same diameter of inhibition of antimicrobials used zone on all formulated media. Results showed that most protein sources used were suitable for cultivation, hemolysis detection and antimicrobial susceptibility test.

KEY WORDS: Alternative media; formulated; protein source; bacteria cultivation.
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1. INTRODUCTION

Generally microorganisms grow naturally in almost all types of habitats. They need essential sources of nutrients to maintain growth and reproduction. Microorganisms generally, need sources of energy, carbon, nitrogen, phosphorus, sulfur and various minerals (Harvey *et al*., 2007). Culture media is a nutrient material prepared for the growth of microorganisms in a laboratory. Nutrient agar medium is commonly used as general purpose medium for the cultivation of broad range of bacteria.

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It is a basic medium composed of peptic digest of animal tissue, beef extract, yeast extract, sodium chloride and agar. Microbiological researches are carried out at high cost and scarcity of culture media (Adesemoye and Adedire, 2005). It is one of the serious problems for developing country. Microbial researches are hindered by high cost of culture media (Mekala *et al*., 2016).

The search for alternative, cheap media for use in laboratory agents for routine microbiological experiments is going on. Recent research has been focused on replacing the culture media with alternatives cheap materials (Mateen *et al*., 2012; Abbott and Chapman, 1981; Babbar and Jain, 1998; Babbar and Jain, 2004; Bromke and Furiga, 1991). Legume seeds serve as a good protein source and they are very cheap and widely available materials.

This study is aimed to find a cheap solid culture media used in routine microbiological experiments derived from plant products once the commercial ones are expensive and in most cases (most of the times) are not available.

2. MATERIALS AND METHODS

2.1. Culture Media Preparation

The tested alternative nutrient samples such as cowpea (*Vigna*), lentil (*Lens culinaris),* split pea (*Pisum sativum*), Chickpea (*Cicer arietinum*), soy protein, mung beans (*Vigna radiata*) and rice (*Oryza sativa*) were obtained from Erbil local markets.

The samples were ground into powder using electric blender (Tefal) and filtered by sieve and stored dry in air tight containers. Three grams of each sample and 2 gm of agar were dissolved in 100 ml of distilled water. The pH of the media were measured, which was between 6.4-6.8. Culture media were sterilized by exposing to microwaves (Gosonic- Model No. GOM-423) for 3 minutes (Shareef *et al*., 2019) and poured into sterilized petri dish. The appearance and the color of each prepared alternative media were reported. Nutrient agar (NA), Mueller-Hinton, blood agar and potato dextrose agar (PDA) were prepared and used as a control.

2.2. Collection of Tested Microorganisms

The tested bacteria were: *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Pseudomonas aeruginosa*, while the tested fungi were: *Penicillium sp.* and *Aspergillus sp.* All tested bacteria and fungi were obtained from the Department of Biology / College of Science.

2.3. Inoculation of tested microorganisms

2.3.1. Bacteria

Serial dilution was done according to standard method to obtain a final concentration of $1.5x10^8$ cell/ml. Both NA and formulated media was inoculated in triplicate with 0.1 ml of a bacterial suspension broth. Solid media was inoculated on to the center and was spread uniformly by a sterile glass rod. All inoculated plates were incubated at 37^oC for 24h.

2.3.2. Fungi

Pure fungal cultures of *Penicillium sp. and Aspergillus sp.* were used. A fungal disc was cut by using sterile cork borer, placed upside down on the center surface of the formulated culture media. Another prepared fungal disc were inoculated on PDA media as control. All inoculated plates were incubated at 28±3 °C for 5 days.

After incubation fungal mycelia diameter was measured using a ruler in 3 directions and then average diameter was calculated for each fungus.

2.4. Blood Hemolysis test

Five ml of blood was added to 95 ml of alternative culture media to determine blood hemolysis. Blood base agar (LAB28) was used to prepare blood agar that used as control.

2.5. Antimicrobial susceptibility test

Antimicrobial susceptibility for four types of antibiotics (Amoxicillin, Amikacin, Gentamicin and Cloxacillin (Bioanalyse)) was tested for all species of tested bacteria on formulated culture media. The inoculum of each species of tested bacteria were, the suspensions broth were adjusted to an optical density of 0.5 McFarland. The antibiotic susceptibility testing was determined by using the Kirby–Bauer diffusion technique (Cheesbrough, 1987). Plates of formulated media and Mueller-Hinton were inoculated using

sterile cotton swabs. Tested antibiotic discs were placed on the inoculated media surfaces and incubated for 24 hours at 37^oC, the diameter of zone of inhibition were measured after incubation period and compared with the inhibition zone on Mueller-Hinton medium.

2.6. Statistical data analysis

A two way ANOVA was used to analyze the results.

3. RESULTS

Since the pH of different formulated culture media were ranged between 6.4-6.8, they were used without adjusting the pH.

The characteristics of formulated culture media were shown in the table (1), the color of most of the prepared media was white to creamy except for mung beans was light green and cowpea was light brown. The appearance of all prepared media was turbid and some contains particles, except that of protein soy agar was clear and transparent.

Table 1. Characteristics of formulated culture media

Formulated Medium	Color	Particles	Turbidity
Cowpea	Light brown	+	T
Lentil	Creamy	-	ST
Chickpea	Creamy	-	ST
Soy protein	Light amber	-	C
Mung beans	Light green	+	T
Split pea	Creamy	-	ST
Rice	White	-	ST

T=Turbid, ST=Slightly Turbid, C=Clear

All tested bacteria showed significantly ($p>0.05$) high growth rate on formulated media except rice. Among all tested bacteria; *B. cereus* showed the best growth on all formulated culture media (figure 1), there was no considerable differences in colony growth rates between the alternative media and NA. They exhibit swarming motility on chickpea and covered the surface of culture media, mean swarming was less on mung beans, cowpea, split pea and lentil. Swarming was controlled by increasing agar rate to 3% in the media (figure 2-4).

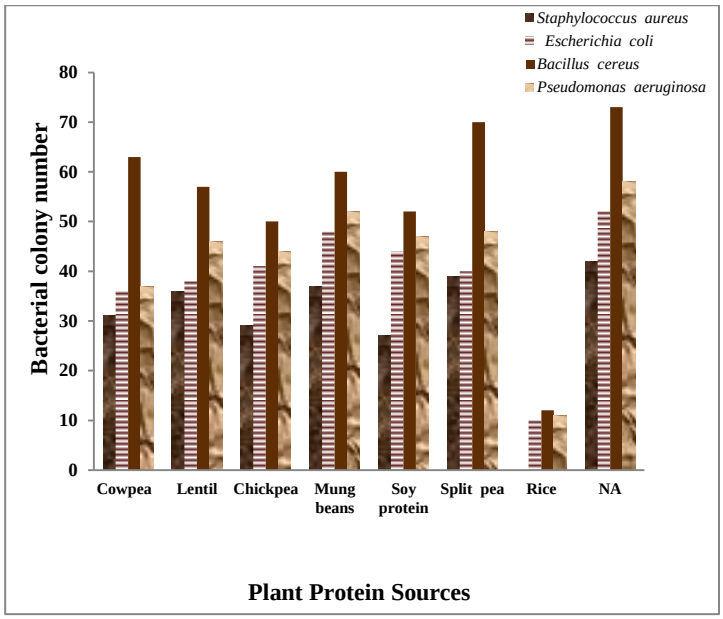

Fig 1. Bacterial growth on different formulated media

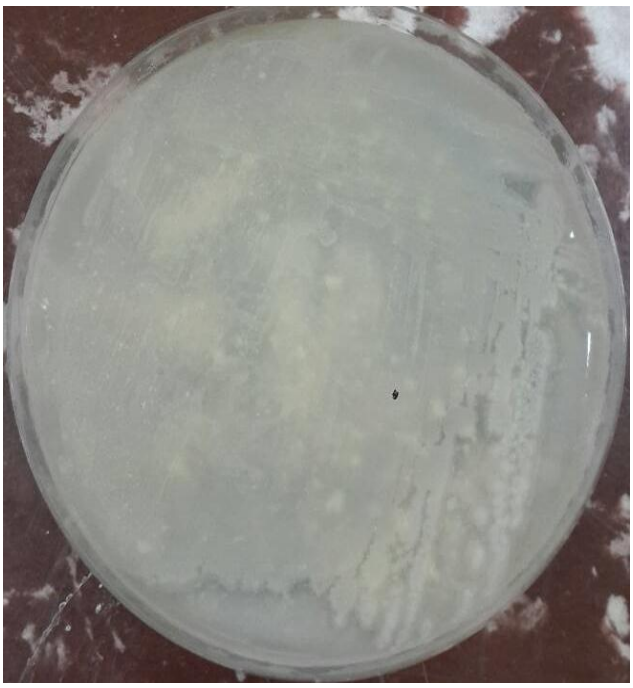

Fig 2. *B. cereus* colonies on Chickpea

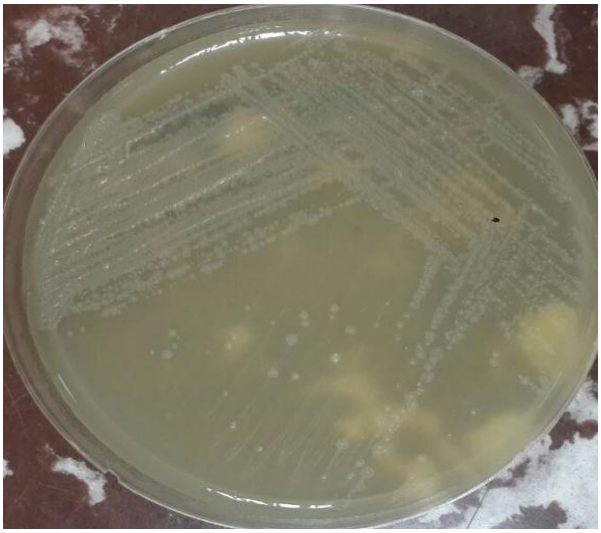

Fig 3. *B. cereus* growth on Soy protein agar

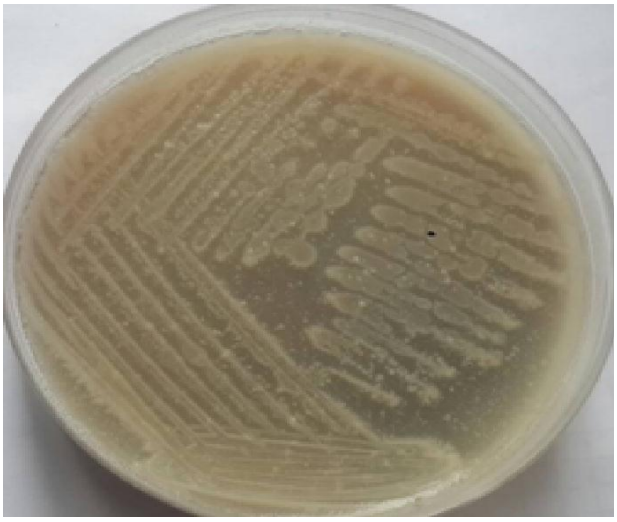

Fig 4. *B. cereus* growth on Cowpea agar

P. aeruginosa produced large opaque irregular colonies with sweet odor and characteristic pigments (figure 5). Pigmentation on formulated media was less obvious on soy protein agar. *E. coli* grew well on all formulated media except on rice, while *S. aureus* produced smaller colony size than on NA.

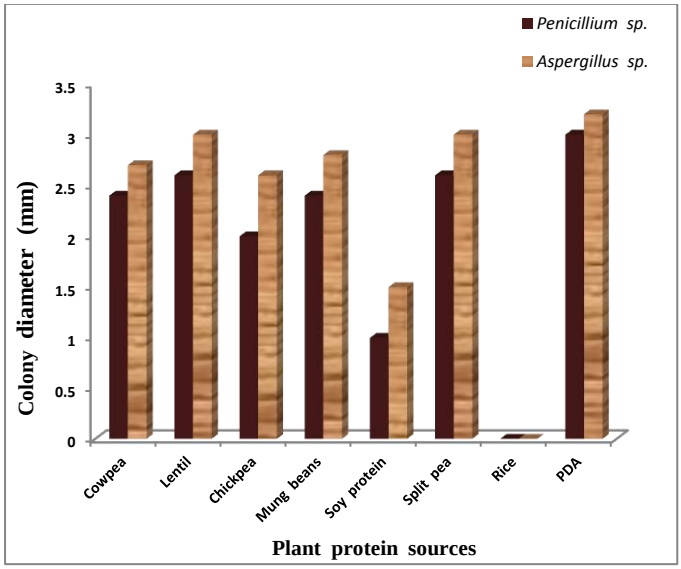

Fig 5. Fungal growth on formulated media

Both *Penicillium* and *Aspergillus* grew well on most prepared media except on rice (figure 6), while they produced small colonies on soy protein in comparison to their growth on PDA agar. Although these fungi showed better growth on PDA, the formulated media were suitable for routine culture of tested fungi (figure 7,8).

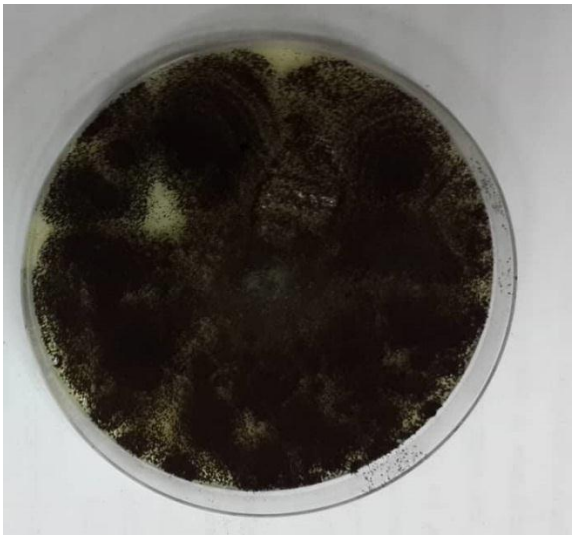

Fig 6. *Penicillium* colony on chickpeas

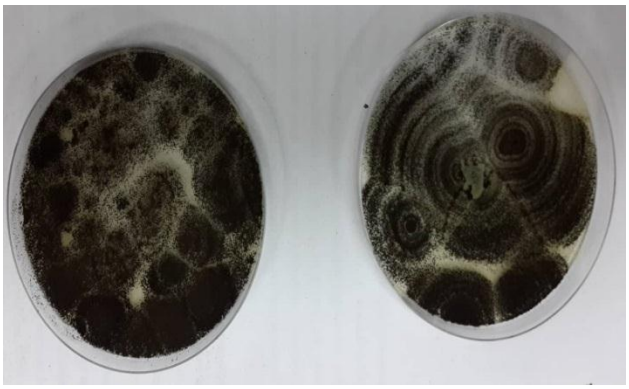

Fig. 7. *Aspergillus* and *Penicillium* colony on Split pea

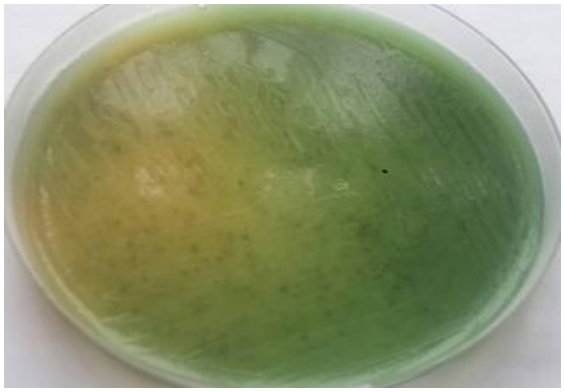

Fig 8. Pigmentation produced by *P. aeruginosa* on Lentil agar

taphyllococcus aureus, *B. cereus* and *P. aeruginosa* showed beta hemolysis on formulated blood agar. Hemolysis reactions of tested bacteria on formulated blood agar were shown in figure (9,10).

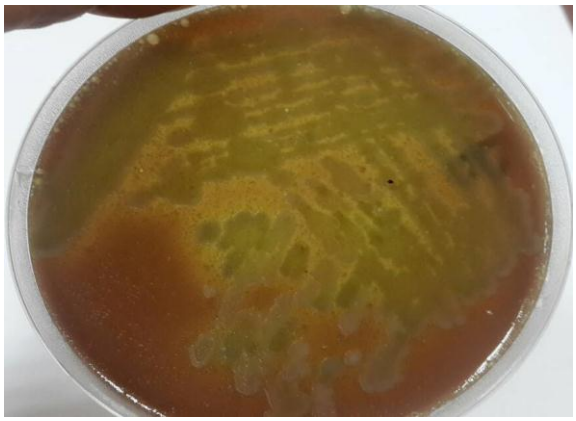

Fig 9. Beta hemolysis by *B. cereus* on Lentil agar

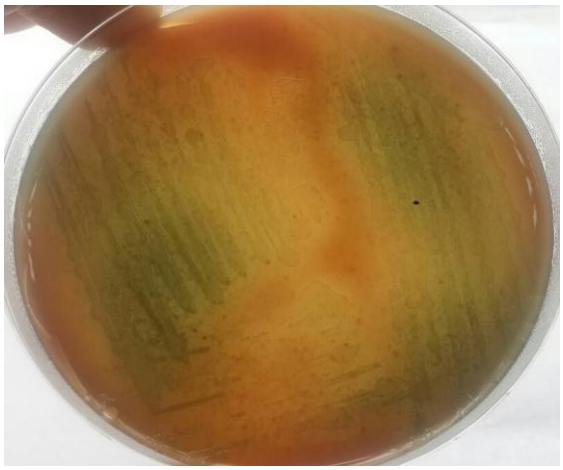

Fig 10. *Beta* hemolysis by *P. aeruginosa* on split pea agar

Staphyllococcus aureus hemolysis reaction on formulated blood agar media was less obvious than on blood agar while *B. cereus* and *P. aeruginosa* produced clear zone.

The susceptibility of bacteria against 4 antibiotics was tested on formulated media. Inhibition zone of each bacteria against antibiotics was measured and compared with inhibition zone of the same one on Mueller-Hinton agar as shown in table (2-4). All tested bacteria exhibited approximately the same inhibition zone against each antimicrobial agent on all formulated media. There were no significant differences between the inhibition zones on these media comparing with inhibition zones on Mueller-Hinton agar.

Table 2. Antibiotic activity against *S. aureus* on different formulated media (Inhibition zone (mm))

Antibiotic discs	Cowpea	Lentil	Chickpea	Soy protein	Mung beans	Mueller-Hinton
Amoxicillin	R	R	R	R	R	R
Amikacin	30	31	32	30	30	30
Gentamicin	33	34	36	36	40	36
Cloxacillin	R	R	R	R	R	R

R= resistant

Table 3. Antibiotic activity against *P. aeruginosa* on different formulated media (Inhibition zone (mm))

Antibiotic discs	Cowpea	Lentil	Chickpea	Soy protein	Mung beans	Mueller-Hinton
Amoxicillin	R	R	R	R	R	R
Amikacin	R	R	R	R	R	R
Gentamicin	R	R	R	R	R	R
Cloxacillin	R	R	R	R	R	R

Table 4. Antibiotic activity against *B. cereus* on different formulated media (Inhibition zone (mm))

Antibiotic discs	Cowpea	Lentil	Chickpea	Soy protein	Mung beans	Mueller-Hinton
Amoxicillin	16	15	14	13	13	14
Amikacin	34	33	36	35	36	36
Gentamicin	32	32	32	30	32	30
Cloxacillin	R	R	R	R	R	R

4. DISCUSSION

Because the pH of different formulated culture media were ranged between 6.4-6.8 they were used without adjusting the pH. This may be helpful in the laboratories that may not have pH meter especially in developing countries. In a study by Ravathie *et al* the pH of formulated culture media was between (6.12 - 6.89) (Ravathie *et al.*, 2012), they found no significant change in the number of colonies whether pH was adjusted to 7.0 or went on with the experiments as such.

The properties and appearance of all prepared media supported them to be suitable to use in microbiology laboratories.

The results showed that the formulated media contained sufficient amount of nutrients to support the growth of the tested bacteria (*S. aureus, E. coli, P. aeruginosa B. cereus*) as well as fungi (*Penicillium sp* and *Aspergillus sp.*) except for rice. The composition of culture media is an important factor for growth of microorganism. The alternative culture media such as cowpea, lentil, chickpea, soy protein, mung beans and split pea contain protein and other nutrient sources that microorganism need for growth. Agar has no nutrient value, it was used as solidifying agent to allow carrying out of common techniques such as streaking out cultures easy and then estimate microbial growth on solid media (Sambali and Mehrotra, 2010).

Many studies have been carried out to replace nutrient agar with alternative formulated culture media from natural protein source. In a study, Sago replaced nutrient agar for the growth of different bacteria (Kapilan and Thavaranjit, 2008). In another study by Annan Prah *et al* in 2010., cowpea agar was effectively replaced with NA. Berde and Berde (2015) used vegetable waste as alternative microbiological media. The legume seeds were also used as alternative culture media (Ravathie *et al.*, 2012).

Pseudomonas aeruginosa produced large opaque irregular colonies with sweet odor and characteristic pigments due to blue colored pyocyanin which spreads out over the formulated culture media. Pigmentation on formulated media was less obvious on soy protein agar. *Pseudomonas aeruginosa* produces many extracellular pigments that are excreted into the culture medium surrounding the colonies. Pigment production is depends on the composition of the medium (Garibaldi, 1967).

Pigment formation and elaboration rely on a dynamic metabolic equilibrium provided by medium constituents such as peptones, minerals, and various ions (Daly *et al*, 1984). Pigmentation by *P. aeruginosa* was not affected by the new media.

Although the tested fungi showed better growth on PDA, the formulated media were suitable for routine culture. In a study by Mekala *et al* (2016), when culture media were formulated from rice, chickpea, corn, dhal, thinai, natural soy flour and processed soy flour, they found that *Sclerotium* sp., *Aspergillus* sp., *Fusarium* sp. and *Penicillium sp*. showed significantly ($p>0.05$) high growth rate in PDA than other alternative culture media. Fungi require carbohydrate rich media to grow well and PDA contains large amount of carbohydrate that required for fungal growth and it also dextrose that serves as a growth stimulant (Beever and Bollard, 1970).

Hemolysis is the ability of bacteria to breakdown the red blood cells by the action of hemolysins, erythrocyte-lysing enzymes, that it produce. Colonies to induce hemolysis when grown on blood agar is used to classify certain microorganisms. Blood hemolysis detection requires a culture medium for bacterial growth and a source of blood as a culture medium supplement. In this study formulated media were used instead of blood base agar and supplemented with blood to obtain hemolysis.

There was no significant differences between the inhibition zone on these media in comparison to inhibition zone on Mueller-Hinton agar as shown in table (2-4). Based on this finding, formulated media can be used for antimicrobial susceptibility test for student experiment.

Results showed that all the plant natural protein sources used are an acceptable alternatives for the routine culture and subculture, hemolysis detection, antimicrobial

susceptibility test and fungi cultivation instead of NA, blood base agar, Mueller-Hinton agar and PDA, respectively for different species of bacteria and fungi specially for routine student experiments in practical microbiology.

In developing countries, with high costs of manufacturing dehydrated media, the use of protein formulated media is more practical and cheaper compared with commercial ones that are widely used in laboratories. Beside they can be prepared immediately.

There are a number of studies concentrated on alternative source of culture media to be used instead of NA, but there was no previous studies about using them for more purposes such as hemolysis detection and antimicrobial susceptibility test.

5. CONCLUSION

Many protein sources such as legume seeds were used in formulation of culture media for cultivation of different microorganisms including bacteria and fungi. Most of formulated media (except rice) were good and suitable for microbial growth. Nutrient agar, blood base agar, Mueller-Hinton agar and potato dextrose agar can be replaced with one of these cheap alternative media for routine experiments in microbiology schools specially in developing countries for culture, hemolysis detection and antimicrobial susceptibility tests in addition of fungi cultivation.

For this purpose only a bottle of agar that cost 30$ with 600g of one of the alternative nutrients that costs ~1.00$ is needed to prepare 20 litters of four important and essential culture media that used widely in microbiology laboratory.

Therefore the use of different natural plant protein sources as culture media in laboratories is very much possible and cheaper when compared to commercially prepared culture media. Although plant protein sources can be prepared easily, rapidly and they can be stored in air tight containers for a long time at room temperature.

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