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Bee collected pollen load (BCPL) as alternative culture media for bacterial and yeast growth

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Abstract

Background: The high cost of the microbial culture media paved a way for the production of alternative media using cheap local raw materials.

Objectives: The study aimed to prepare three liquid and four solid alternative culture media coded $BCPL_1$, $BCPL_2$, $BCPL_3$, and $BCPL_4$ for cultivation six strains of gram positive bacteria, nine strains of gram negative bacteria and three strains of yeast.

Materials and methods: Bee collected pollen load (BCPL) is used to formulate both solid and liquid media. The growth on formulated media was compared with growth on the conventional media (Nutrient broth {NB}, Nutrient agar {NA}, Sabouraud dextrose agar {SDA}, Sabouraud dextrose Broth {SDB} and Blood agar {BA}.

Results: Most organisms appeared growth on liquid and solid media. In gram positive bacteria *staphylococcus aureus* (Methicilin resistant *staphylococcus aureus*) showed better growth in $BCPL_1$ broth (0.52) as compared with NB (0.43). On the other hand *Listeria sp*. exhibited equal growth turbidity in both $BCPL_1$ broth and NB (0.61). In gram negative bacteria *Proteus mirabilis* recorded higher growth in $BCPL_1$ broth (0.39) as compared with NB (0.26). *Pseudomonas aeruginosa* showed significantly high converging growth in both $BCPL_2$ and $BCPL_1$ broth (0.67, 0.65) respectively followed by NB (0.45). *Shigella sp*. showed good growth in NB and $BCPL_1$ broth (0.37, 0.34) and less growth in $BCPL_2$ broth medium (0.25). The results showed that $BCPL_1$ broth medium was significantly better than $BCPL_2$ broth to most tested bacteria. All tested yeasts grow least in $BCPL_3$ broth medium. The solid alternative media $BCPL_1$ and $BCPL_2$ agar were more effective on G- bacteria and yeast than G+ bacteria while $BCPL_3$ agar was effective for most tested organisms.

Conclusion: For these results these media may be recommended as substitutes for the imported conventional media NB, NA, SDA, SDB and BA for the growth of these microorganisms locally.

Keywords: Culture media, bee collected pollen load, yeast, bacteria, NA, NB, BA, SDA, SDB.

INTRODUCTION

Pollens grains are the male reproductive cells of flowers (1). It is a fine, powder-like material gathered by bees. Whose composition can vary due to their botanical and geographic origin (2). It Contain carbohydrates, amino acids, proteins, lipids, vitamins, minerals, phenolic compounds, flavonoids, concentrations of phytosterols and are also rich in phytochemicals, it is the bee`s primary food source (3). Microorganisms need nutrients, a source of energy and certain environmental conditions in order to grow and reproduce. In the environment, microbes adapt to the habitats most suitable for their needs while in the laboratory, these requirements must be met by a culture medium (4).

The increasing cost of microbial culture media has necessitated the continuous search for more readily available alternative culture media using local raw materials (potatoes, cerals, cassava, etc..) at an affordable price.

Different types of media are used for the growth and isolation of microorganisms has been reported from different substrates (5). (6) Has worked on cowpea as a cost effective alternative culture media for the growth of bacteria, through the authors have used cooked (boiled) cowpea to increase the shelf life up to three months in their study. Further there are also reports using vegetables as an alternative source such as (potato, Groundnut, cereals, ect…) for preparing culture media for the growth of fungi, yeasts and bacteria (7). (8, 9) used legume seeds as alternative culture media for fungi and bacteria. (10) Used sweet potato agar as a medium to culture yeasts. In Iraq (11) prepared a water extract (juice) from the root of *Beta vulgaris* (beetroot) under sterile conditions, then used for the first time as experimental bacterial growth medium for the growth of the bacteria: *Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumonia*; the juice was used as alternative growth medium for the nutrient agar and nutrient broth for the growth of these genera in the laboratory. Another study in Iraq was carried out to devise a local nutrient media for primary mycelium growth of two species of fungi belonging to the genus Oyster mushroom: *pleurotu ostereatus* and *pleurotus eryngii* using different nautral material such as: cumin, onion, oatmeal, banan beel, date seed, garlic and alfalfa, the results showed that the media prepared from cummins and onion powders have achieved best growth rate for the studied fungi (12). (13) Used *Medicago sativa* (Alfalfa) extract as suitable medium for growing the different type of gram negative and positive bacteria. (14) Prepared a new medium using extract of (*Phragmites australis*) as a differential and enriched medium for identification of gram negative bacteria. **Amis:** The present study is aimed at replacing the nutrient in artificial culture media by nutrient in Bee collected Pollen Load extract to find an alternative media characterized by cheap cost and simple preparation could be used instead of the routinely prepared media that is used in the laboratories.

MATERIAL AND METHODS

Collection of sample:

The tested alternative nutrient sample such as (Bee collected load), purchased from Ibn-Hayyan (Al-Suwaira) apiaries, Iraq

Bee collected load Extract Preparation:

The pollen load was crushed into powder using electric blender and sieved as fine powder. The powder was stored in sterile containers until its use.

(20 gram) amount of BCPL powder was immersed in 500 ml of hot distilled water (100°C) and allowed to stand for 10 min on water bath. Each preparation was filtered through sterilized medical gauze and then centrifuged by a centrifuge, then the extract volume completed to 1000 mL with distilled water.

Formulation of alternative culture media:

The media termed (BCPL) Bee Collected Pollen Loads medium.

Liquid media formulation:

Three different liquid media were prepared as follows:

- $BCPL_1$ Broth: it consisted 100 ml of plant extract containing 0.5 g sodium chloride. PH adjusted to 7.1 then sterilized at 121°C for
- min used autoclave.
- $BCPL_2$ Broth: it was the same BCPL1 except that NaCl was not added, also PH adjusted 7.1 and sterilized by autoclave.
- $BCPL_3$ Broth: it consisted 100 ml of plant extract containing 2 g of D(+)-glucose PH adjusted to 5.6 then sterilized at 121°C for 15 min, and then the antibiotic chloramphenicol

(0.005g/100ml) and cyclohexamin (0.04g/100ml) was added to sterile and warm medium.

The media were decanted into sterile test tube.

Solid media formulation:

Four different solid media were prepared as follows:

- BCPL1 Agar: it consisted 100 ml of plant extract containing 0.5 g sodium chloride, and 1.5 g agar. PH adjusted to 7.1 then sterilized at 121°C for 15 min used autoclave.
- $BCPL_2$ Agar: it was the same $BCPL_1$ except that NaCl was not added, also PH adjusted 7.1 and sterilized by autoclave.
- $BCPL_3$ agar: it consisted 100 ml of plant extract containing 2 g of D(+)-glucose and 1.7 agar. PH adjusted to 5.6 then sterilized at 121°C for 15 min, and then the antibiotic chloramphenicol (0.005g/100ml) and cyclohexamin (0.04g/100ml) was added to sterile and warm medium and approximately 20 ml of the sterilized medium was distributed into each of the sterile petri dishes.
- $BCPL_4$ Agar: it consisted the same as $BCPL_1$ medium except that 5% sterile human blood was added after sterilization of medium).

Preparation of Artificial (Control) Media:

- **Nutrient agar, Nutrient Broth:** it is used for growing bacterial solid and liquid cultures.
- **Sabouraud dextrose agar and Sabouraud dextrose Broth**: it is used for growing yeasts cultures.
- **Blood agar:** it is used for growing bacteria and yeasts solid cultures.

The previous media are used for comparison the microbial growth rate with alternative media. They were prepared according to the instruction of manufactures (Fluka). After these media were brought to boiling on hot plate to dissolve the constituent completely, and sterilized in autoclave. Blood agar medium was prepared by the addition of 5% sterile blood to sterile and warm blood agar base from fluka.(15)

Microorganisms Tested

The following strains of bacteria (15 species) and yeasts (3 species) were used:

- Gram Positive bacteria: Methicillin resistant *Staphylococcus aureus* (MRSA), *Streptococcus fecalis, Streptococcus aglactia, streptococcus mutans, Listeria monocytogenes, Bacillus subtilis.*
- Gram Negative bacteria: *Escherichia coli., Enterobacter aerogense., Proteus mirabilis., Serratia marcesens, Pseudomonas aerogenosa, Acinetobacter baumanni, Shigella sp. Klebssiella pneumonia., Salmonella sp.*
- Yeasts: *Candida albicans, Candida tropicalis, Candida Kruzi.*

They were obtained from Central Public Health Laboratory and bacteriology lab of Al-Husain Hospital in Karbala city.

Microbial Inoculation:

Liquid media:

The various alternative and artificial media of bacterial and yeasts strains ($BCPL_1$, $BCPL_2$, $BCPL_3$ Broth) (Nutrient Broth, Sabouraud dextrose broth) were dispensed into many sterile plain tubes (5 ml) in each one and the suspensions prepared from selected colonies of 24 hrs. nutrient agar, Blood agar incubated cultures for the standard bacterial strain and from SDA for yeasts, the inoculums picked up by an inoculating loop (loopful) to prepare microbial suspensions, its turbidity equal to 0.5 MacFarland turbidity standard ($1.5 * 10^8$ CFU/Ml), (absorbance was 0.08 for bacteria and 0.1 for yeasts).

The plain tubes of the new media divided into 32 groups for bacterial strains and 3 groups for yeasts strains, in each group three tubes devoted for each type of the used microorganisms, three tubes of bacterial and yeasts alternative liquid media left without inoculation as a control.

On the other hand the design that used with artificial media was 16 groups for bacterial strain, 3 groups for yeasts strains, and three tubes for each of nutrient broth and Sabouraud dextrose broth, it serves as a control tubes.

After 24 hours of incubation at 37ºC. The microorganism's growth measured was done by the optical density measurement using spectrophotometer (Spectrum) at 600 nm.

Solid Media:

The various alternative solid media ($BCPL_1$ agar, $BCPL_2$ agar, $BCPL_3$ agar, $BCPL_4$ agar) and conventional solid (control) media (Nutrient agar, Sabouraud dextrose agar, Blood agar) were inoculated with the previous standard inoculum that prepared in the previous paragraph by **streak plate method** (15) into a sterile petri dishes, petri dishes which divided to the same design that was used with the above mentioned tubes, then the plates incubated at 37ºC for 24 hours. All the incubated plates were examined and the microbial growth (microbial colonies) were investigated.

Visual assessment and comparison with growth on conventional and artificial media was carried out.

Measurement of growth rate:

- *Liquid media:*
  The growth rate of bacteria and yeasts strains was measured at OD_{600nm} by spectrophotometer at (0, 24) hours.
- *Solid media:*
  Microbial growth was investigated by the presence of microbial colonies and density of growth after (24-48) hours.

The growth examination in the liquid medium and solid medium using gram stain and biochemical tests:

After incubation of liquid and solid new media, the gram stain and the necessary biochemical tests were done for the growing bacteria and yeast in the all type of alternative media to ensure that the growth in the new media was from the certain selected strain and not from any contamination.

Statistical Analysis:

The data obtained were analyzed by using SAS Software version 9.1, 2003, USA.

RESULTS

In the present study, the growth of bacteria and yeasts in alternative liquid and solid media was measured and compared with the artificial laboratory media.

In table 1 (**Figure 1, 2, 3,4,5)** the results demonstrated that all bacterial and yeasts strains showed growth turbidity in liquid media except the bacterium *Streptococcus agalactiae,* that did not grow in $BCPL_2$ broth. The growth turbidity of Gram positive bacteria in $BCPL_1$ broth was (0.52, 0.13, 0.32, 0.19, 0.61, 0.31) for (*Staphylococcus aureus (MRSA), Streptococcus fecalis, Streptococcus agalactiae, Streptococcus mutans, Listeria monocytogenes, Bacillus subtilis*) respectively compared with $BCPL_2$ broth (0.11.0.12, 0.0, 0,21, 0.23, 0.37) but for Nutrient broth it was (0.43, 0.28, 0.49, 0.93, 0.61, 0.48) respectively after 24 hr. of incubation at 37ºC. On the other hand the results showed that the growth turbidity of Gram negative bacteria in $BCPL_1$ broth medium was (0.41, 0.54, 0.39, 0.39, 0.65, 0.39, 0.34, 0.35, 0.28) for (*Escherichia coli, Enterobacter aerogense, Proteus mirabilis, Serratia marcesens, pseudomonas aeruginosa, Acinetobacter baumanni, Shigella sp., Klebssiella pneumonia, Salmonella sp.*) respectively, compared with $BCPL_2$ broth medium (0.23, 0.28, 0.17, 0.1, 0.67, 0.32, 0.25, 0.33, 0.14) sequentially, and was (0.77, 0.62, 0.26, 0.45, 0.45, 0.79, 0.37, 0.72, 0.78) in Nutrient broth.

Yeasts recorded growth turbidity in $BCPL_3$ broth medium (0.7, 0.3, 0.68) for (*Candida albicans, Candida tropicalis, Candida kruzi)* respectively, compared with (1.31, 1.3, 1.16) in artificial media Sabouraud dextrose broth.

In table 2 (Fig. 6,7,8,9.10,11) the growth on solid alternative media such as $BCPL_1$ agar, $BCPL_2$ agar, $BCPL_3$ agar and $BCPL_4$ agar was varied between strains. All gram positive bacteria showed moderate growth on both $BCPL_1$ agar, $BCPL_2$ agar media but appeared a good growth on $BCPL_4$ agar and blood agar media, expect the bacterium *Bacillus subtilis* which recorded moderate growth on the last media. On the other hand all gram negative bacteria showed a good growth in $BCPL_4$ agar and blood agar and recorded a good growth on $BCPL_1$ agar and $BCPL_2$ agar media, except the strains *Escherichia coli* and *Salmonella sp*. that appeared moderate growth on the last alternative media and so are most of the yeasts strain showed a good growth on $BCPL_3$ agar and $BCPL_4$ agar, except the strain *Candida tropicalis* that showed less growth on $BCPL_3$ agar.

Table 1: Optical density reading for test organisms on various alternative and conventional liquid media

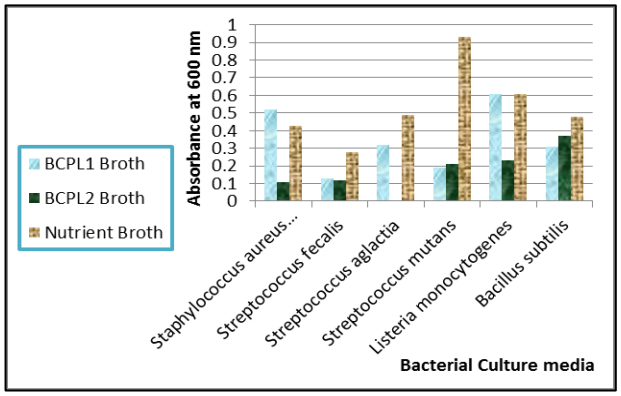
Microbes species Bacterial species		**Time of growth (hr)**	**Absorbance at 600 nm**			**LSD 0.05**
			Type of medium			
Gram Positive Bacteria			**$BCPL_1$ Broth**	**$BCPL_2$ Broth**	**Nutrient Broth**	
1	*Staphylococcus aureus (MRSA)*	0	0.09	0.09	0.09	0.05
		24	0.52	0.11	0.43	
2	*Streptococcus fecalis*	0	0.08	0.08	0.08	0.02
		24	0.13	0.12	0.28	
3	*Streptococcus aglactia*	0	0.09	0.09	0.09	0.01
		24	0.32	0.09*	0.49	
4	*Streptococcus mutans*	0	0.09	0.09	0.09	0.02
		24	0.19	0.21	0.93	
5	*Listeria monocytogenes*	0	0.09	0.09	0.09	0.01
		24	0.61	0.23	0.61	
6	*Bacillus subtilis*	0	0.09	0.09	0.09	0.02
		24	0.31	0.37	0.48	
Gram Negative Bacteria		**Time of growth (hr)**	**$BCPL_1$ Broth**	**$BCPL_2$ Broth**	**Nutrient Broth**	**LSD 0.05**
1	*Escherichia coli*	0	0.07	0.07	0.07	0.01
		24	0.41	0.23	0.77	
2	*Enterobacter aerogense*	0	0.07	0.07	0.07	0.02
		24	0.54	0.28	0.62	
3	*Proteus mirabilis*	0	0.09	0.09	0.09	0.01
		24	0.39	0.17	0.26	
4	*Serratia marcesens*	0	0.07	0.07	0.07	0.01
		24	0.39	0.1	0.45	
5	*pseudomonas aeruginosa*	0	0.09	0.09	0.09	0.01
		24	0.65	0.67	0.45	
6	*Acinetobacter baumanni*	0	0.08	0.08	0.08	0.01
		24	0.39	0.32	0.79	
7	*Shigella sp.*	0	0.09	0.09	0.09	0.04
		24	0.34	0.25	0.37	
8	*Klebssiella pneumonia*	0	0.09	0.09	0.09	0.02
		24	0.35	0.33	0.72	
9	*Salmonella sp.*	0	0.07	0.07	0.07	0.02
		24	0.28	0.14	0.78	
Yeasts Species		**Time of growth (hr)**	**$BCPL_3$ Broth**		**Sabouraud dextrose liquid medium**	**LSD 0.05**
1	*Candida albicans*	0	0.1		0.1	0.16
		24	0.7		1.31	
2	*Candida tropicalis*	0	0.1		0.1	0.22
		24	0.3		1.3	
3	*Candida Kruzi*	0	0.1		0.1	0.22
		24	0.68		1.16	

***: mean no growth.**

Table 2: Growth density for test organisms on various alternative and conventional solid media

Gram Positive Bacteria		$BCPL_1$	$BCPL_2$	Nutrient Agar	BCPL3	Blood Agar
1	*Staphylococcus aureus (MRSA)*	+	+	+*	+*	+*
2	*Streptococcus fecalis*	+	+	+	+*	+*
3	*Streptococcus aglactia*	+	+	+	+*	+*
4	*Streptococcus mutans*	+	+	+	+*	+*
5	*Listeria monocytogenes*	+	+	+	+*	+*
6	*Bacillus subtilus*	+	+	+	+	+
Gram Negative Bacteria		**$BCPL_1$**	**$BCPL_2$**	**Nutrient Agar**	**$BCPL_4$**	**Blood Agar**
1	*Escherichia coli*	+	+	+*	+*	+*
2	*Enterobacter aerogense*	+*	+*	+*	+*	+*
3	*Proteus mirabilis*	+*	+	+*	+*	+*
4	*Serratia marcesens*	+*	+*	+*	+*	+*
5	*Pseudomonas aerogenosa*	+*	+*	+*	+*	+*
6	*Acinetobacter baumanni*	+*	+*	+*	+*	+*
7	*Shigella sp.*	+*	+*	+*	+*	+*
8	*Klebssiella pneumonia*	+*	+*	+*	+*	+*
9	*Salmonella sp.*	+	+	+	+*	+*
Yeast		**$BCPL_3$**		**Sabouraud glucose agar**	**$BCPL_4$**	**Blood Agar**
1	*Candida albicans*	+*		+*	+*	+*
2	*Candida tropicalis*	+		+	+*	+*
3	*Candida Kruzi*	+*		+*	+*	+*

+*: mean a good growth; +: mean moderate growth.



Graph drawn using Microsoft Excel (2010)

Figure 1: Gram positive Bacterial growth in alternative and control liquid media

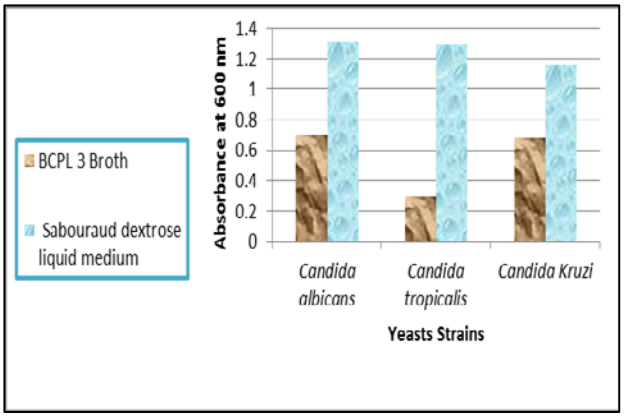


Figure 3: Yeasts growth in alternative and control liquid media

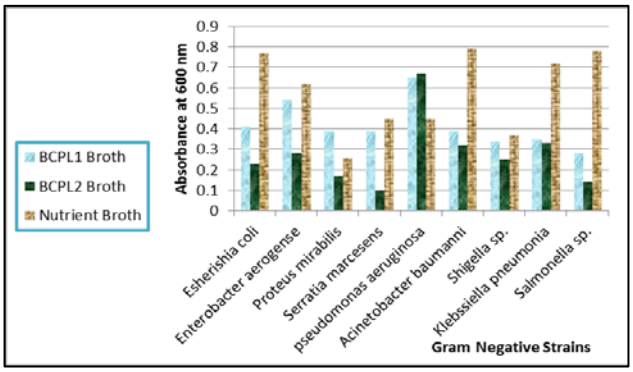


Figure 2: Gram Negative bacterial growth in alternative and control liquid media

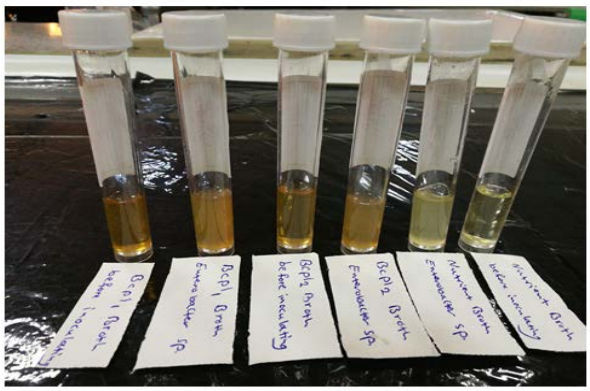


Fig. 4 *Enterobacter sp.* growth on $BCPL_1$, $BCPL_2$ broth and NB before and after inoculating

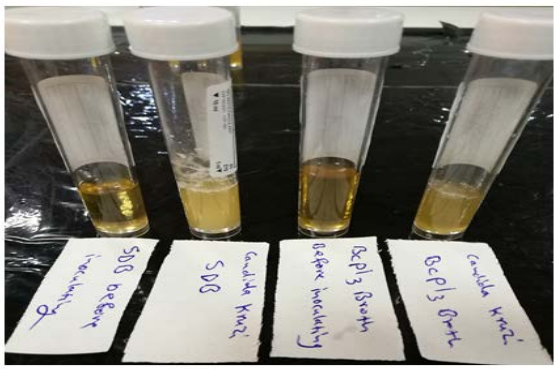


Fig. 5 *Candida kruzi* growth on $BCPL_3$ broth and SDB before and after inoculating

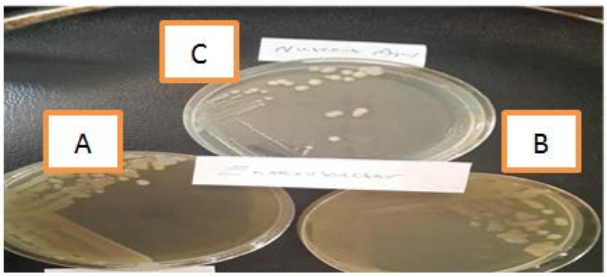


Fig. 6 *Escherichia coli* growth on (a) $BCPL_1$ agar (b) $BCPL_2$ agar (c) Nutrient agar

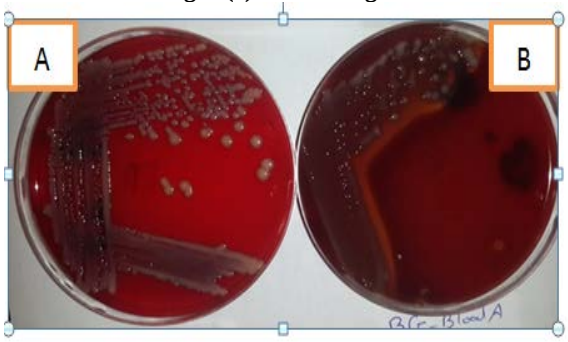


Fig. 7 *Enterobacter aerogense* growth on (a) Blood agar (b) $BCPL_4$ agar

Fig. 8 *Candida kruzi* on (a) Sabouraud dextrose agar (b) $BCPL_3$ agar medium

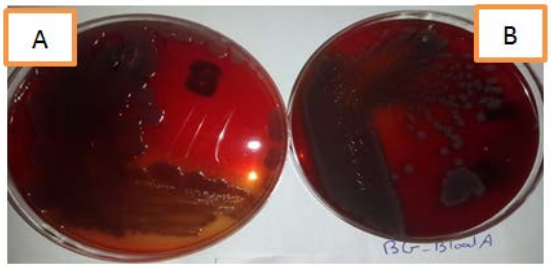


Fig. 9 Pseudomonas aeroginosa growth on (a) Blood agar (b) $BCPL_4$ agar

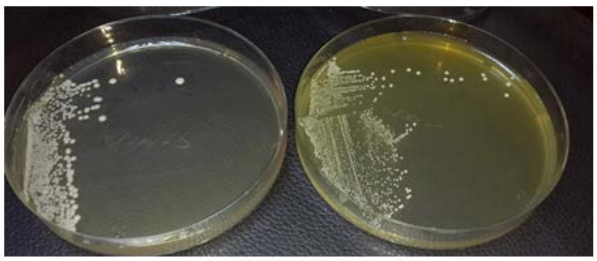


Fig. 10 *Candida albicans* growth on (a) Sabouraud dextrose agar (b) $BCPL_3$ agar

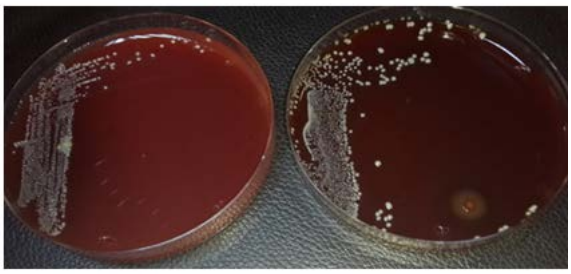


Fig. 11 *Candida albicans* growth on (a) Blood agar (b) $BCPL_4$ agar (b) $BCPL_4$ agar

DISCUSSION

In gram positive strain *staphylococcus aureus* (Methicilin resistant *staphylococcus aureus*) showed significantly higher growth (0.52) in $BCPL_1$ broth medium as compared with conventional media nutrient broth (0.43), but it showed significantly less growth in $BCPL_2$ broth medium (0.11). By another *listeria monocytogenece* exhibited equal growth turbidity in both $BCPL_1$ broth and Nutrient broth media (0.61), but demonstrate significantly less growth in $BCPL_2$ broth medium (0.23).that may be mean both media contain the same nutritional growth requirements for this strain.

Streptococcus fecalis, streptococcus agalactiae, streptococuus mutans and Bacillus subtilis showed high growth in nutrient broth (0.28, 0.49, 0.93,0.48) respectively, and moderate growth in other alternative media.

In gram negative bacteria *Proteus mirabilis* recorded higher significantly growth in $BCPL_1$ broth medium (0.39) as compared with nutrient broth (0.26) followed by $BCPL_2$ broth medium (0.17). On the other hand *pseudomonas aeruginosa* recorded significantly high converging growth in both alternative media $BCPL_2$ broth medium and $BCPL_1$ broth medium (0.67, 0.65) respectively followed by artificial medium nutrient broth (0.45). *Shigela sp* showed good growth in nutrient broth and $BCPL_1$ broth medium (0.37, 0.34) and less growth in $BCPL_2$ broth medium (0.25). The presence of growth due to the bee collected pollen load organic and inorganic component, (carbohydrates, amino acids, proteins, lipids, vitamins, minerals, phenolic compounds, flavonoids, concentrations of phytosterols and are also rich in phytochemicals) (3) and a satisfactory microbial culture media mast contain available source of carbon, nitrogen, inorganic salts and other growth substances (7).

Nutrient broth is efficient medium for other gram negative bacteria such *as Escherichia coli, Enterobacter aerogense, Serratia marcesens, Acinetobacter baumanni, Shigella sp., Klebssiella pneumonia, Salmonella sp.* , it showed significantly high turbidity (0.77, 0.62, 0.45, 0.79, 0.72, 0.78) followed by $BCPL_1$ broth medium (0.41, 0.54, 0.39, 0.39, 0.35, 0.28) respectively.

The results showed that $BCPL_1$ broth medium was significantly better than $BCPL_2$ broth to most tested bacteria, that is due to the presence of sodium chloride NaCl 0.5%, this gives the mixture proportions similar to those found in the cytoplasm of most organisms.

Finally yeasts growth level for three yeasts species *Candida albicans, Candida tropicalis, Candida kruzi* in alternative Sabouraud glucose broth $BCPL_3$ was decreased significantly (0.7, 0.3, 0.68) as compared with conventional medium Sabouraud dextrose broth (1.31, 1.3, 1.16).

In conclusion bee collected load pollen grain extract media that is the focus of this work have been shown to possess sufficient amounts of nutrients for support the growth of microorganisms such as *staphylococcus aureus* (Methicilin resistant *staphylococcus aureus*), *listeria monocytogenece, Proteus mirabilis,* and *pseudomonas aeruginosa*. It was also shown that, bee collected load pollen grain extracts are capable of insufficient the growth of other bacteria and yeasts. For example, the growth of gram positive bacteria *Streptococcus mutans*, and gram negative bacteria *Salmonella sp., Acinetobacter sp*. and the yeasts *Candida albicans, Candida tropicalis, Candida kruzi* were growth decreased compared to Nutrient broth and Sabouraud dextrose broth media. It is postulated that the decreased of the growth of some susceptible microorganisms may be due to compounds lacking in bee pollen grain extract components. This work has shown that bee collected pollen load extract products can be used efficaciously and economically for the cultivation of the bacteria that were reported in this work. Moreover, the equal effect of N. broth and $BCPL_1$, $BCPL_2$ broth medium on the growth of *staphylococcus aureus, listeria monocytogenece, Proteus mirabilis, pseudomonas aeruginosa,* is suggested that, bee collected load pollen grain extract media can be used for cultivation of bacteria in research laboratory.

In Table 2 the solid alternative media such as $BCPL_1$ agar, $BCPL_2$ agar, $BCPL_3$ agar, and $BCPL_4$ agar supported the growth of all the tested organisms by serving the nutrients essential for its growth, but growth was varied between strains. Hence, we do not deny the effectiveness of nutrient broth and Sabouraud dextrose broth which are artificial media for the cultivation of tested bacteria and yeasts, but the high cost of these media (500g) that ranging from 50-100 $, thus this study attempts to formulate media using local available natural cheap materials to make them available for use in laboratories. So the alternative media can be recommended for use in place of conventional N. Broth, N. agar, Sabouraud dextrose agar, and blood agar used in this study for culturing the organisms investigated.

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