Improved Natural enrichment and differential medium for identification of Gram negative bacteria

Sawsan . H A. Korji
Dep. Biology, college of science, AL-Mustansiriya University

Abstract

Because of the upmost interest in isolation and diagnosis of pathogenic and non pathogenic bacterial species in all over the world especially in the development country effers were made to synthesize new natural media for enriched and diagnosis of gram negative bacterial species. The new medium was prepared using common reed extract (Phragmites australis) and named IPA (Iraqi phragmites australis). The media were prepared at six recepies (IPA_1, IPA_2, IPA_3, IPA_4, IPA_5, IPA_6,). The components of the media are available localy and found quite efficient in enriched and differentiate between gram negative bacteria, when compared with media know to be suitable for such uses (Nutrient broth and Macconkey agar and blood agar). The results showed non significant differences between improved media and the control media. The new medium was un expensive and easy to prepare and efficient to others. This will make it a good substitution for many commercial media.

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Introduction

Microorganisms require a suitable culture medium that can support their nutritional needs. Additionally the culturing of microorganisms in vitro requires careful control of various environmental factors, such as temperature, pH and oxygen levels (1). Atypical growth medium normally contains a source of nitrogens, inorganic jons (such as, P,S, Mg, Na, K, Cl, Fe , Zn….) (2). For the culture of heterotrophic microorganism such as *Pseudomonas*, *E.coli*, *Klebsiella*…..etc. specific organic carbon compound, such as glucose are included in the culture medium as growth substrate to meet the carbon and energy requirements for growth (3).

Different types of media are used for growing bacteria as pure culture many bacterial species can be grown in the laboratory on a define medium. Some microorganism require a complex medium (4).

Phargmites, the common reed, is a large perennial grass found in wetlands throughout temperate and tropical regions of the world. *Phragmites australis* is sometimes regarded as the sole species of the genus Pahragmites (5). *P. australis* may used for phytoremediation water treatment (6). Some other uses for in various cultures include baskets, mats, pen tips and a rough form of paper (7).

P. austuralis is used in many area for thatching roofs (8).In Australian Aboriginal cultures, reeds were used to make weapons like spears for hunting game (9). On the other hand *P.austuralis* is causing serious problems for many other North American hydrophyte wetland plants, Gallic acid released by Phragmites is degraded by UV light to produce mexoxalic acid, effectively hitting susceptible plants and seeding with two harmful toxins. Phragmites are so difficult to control that one of the most effective methods of eradicating the plant is to burn it over 2-3 seasons (10). The aim of this study to prepared anew natural medium using *P.austuralis* and evaluated its sensitivity for in vitro detection of gram negative bacteria isolated from suspected cases.

Materials and Methods

1. The plant extract.

   Mature and fresh *P.austuralis* leaves were collected from Baghdad city in 2010, and classified by Botany department, college of science, AL-Mustansiriya university as *P.austuralis* family Poaceae, tribe Arundineae, the local name reed.

   Plant samples were carefully washed under running tap water followed by air dried at room temperature (35°C) for two days and crushed into powder using asterilized electric blender and stored in air tight bottles.

   A eight gram amount of leaves powder was immersed in 100ml of hot distilled water (100°C) and allowed to stand for 5 min on a water bath. Each preparation was filtered through a sterilized whatman NO.1 filter paper. (1try to immersed: 4,8, 16, 20, g of power in 100ml of D.W but 8g in 100ml of D.W is the best concentration).

2. Preparation of culture medium.

   Various broths were compounded and tested. The medium named (IPA) Iraqi Phragmites Austuralis medium. A number of media were tested as the following:

   - Medium IPA_1 :it consisted 100ml of plant extract containing 0.5g sodium chloride, 0.2g glucose, 1g peptone. pH adjusted to 7.1, then sterilized at 121 C^o for 15 min used autoclave.
   - Medium IPA_2 .it consisted 100ml of plant extract without any edition. pH adjusted to 7.1 and sterilized as previously.
   - Medium IPA_3.it was the same as IPA_1 medium except that 1.8% agar was added. Also pH adjusted 7.1 and sterilized by auto clave.
   - Medium IPA_4 : it consisted the same as IPA_2 medium except that 1.8% agar was added (for solidification) pH adjusted 0.7 and sterilized in autoclave.
   - Medium IPA_5 : it consisted 100ml of plant extract containing 0.5 g sodium chloride, 1g lactose, 1.8g agar, 0.7ml neutral red (1% neutral red in ethand) and 0.5g Bile salt NO.3. also pH adjusted 0.7 and sterilized in autoclave.

- Medium IPA_6 (Blood IPA medium): it consisted the same as IPA_3 medium except that 5% sterile human blood was added after the sterilization of medium.

3. Preparation of control media

Nutrient agar, Nutrient broth, MacConkey agar were prepared according to the instruction of manufactures (Fluka). After these media were brought to boiling in water bath to dissolve the constituent completely except nutrient broth. The pH adjusted to 7 and sterilized in autoclave. Blood ager medium: it was prepared by the addition of 5% sterile blood to sterile and warm blood ager base from Fluka(11).

4. Test bacteria

A total of five bacterial species were tested: *Salmonella* typhi (one isolate) *Pseudomonas aeruginosa* (3 isolates) *Proteus spp*. (2 isolates), *E.coli*(3isolates) and *klebsiella spp*. (3 isolates). These species were originally isolated from. Clinical materials collected from patients. They were identified using standard biochemical tests(11) and detection by API - 20E strep (Biomeriux) was performed for all the isolates.

5. In vitro evaluation of IPA media for gram negative bacterial growth.

5-1: Bacterial isolates were inoculated in nutrient broth (Fluka) and incubated for 24hr . at 37C°, the bacterial growth and their turbidity were adjusted to approximately 0.5 Mcfarland tube (11). This suspension (10^8 CFU/ml) was then diluted serially in nutrient broth to prepare 10^6, 10^5, 10^4, CFU/ml.

5-2:evaluation of IPA broth media for gram negative bacterial growth. The IPA broth media (IPA_1, IPA_2) and nutrient broth (control medium) were inoculated with 0.1ml of each dilution described above (5.1) they were examined Macroscopically for turbidity change after 6,12, 24 hr. using spectrophotometer at 470nm, and the results were recorded.

5-3: Evaluation of IPA agar media for gram negative bacterial growth. 0.1ml of each bacterial suspension as describe previously (5-1) was added to each plate containing IPA agar media (IPA_3, IPA_4, IPA_5, IPA_6) by sterile cotton swab and allowed to remain in contact for 1 min. this technique repeated on MacConkey agar, Nutrient agar and blood agar media (control medium). Then the plates were incubated at 37C° for 24 hrs. then bacterial colony recorded using colony counter (12).

Results and Discussion

The identification of micro organism depends upon the ability to grow and maintain microorganisms in the laboratory and this is possible only if suitable culture media are available (13).

From the 12 bacterial isolates suspensions of five different gram negative species inoculated in IPA_1 broth and Nutrient broth. The results demonstrated that the bacterial growth in IPA_1 broth showed significant turbidity (1.26, 1.03, 1.02, 1.23 and 0.95) for *E.coli , Klebsiella , proteus, P.aeruginosa* and *S.typhi* respectively compared with 1.12 in nutrient broth after 24 hr of incubation at 37C°.(table 1) that due to the *Phragmitis australis* organic and inorganic component (6).

A satisfactory microbiological culture medium must contain available source of carbon , nitrogen , inorganic salts and other growth romoting substances (14). *P.australis* contain much protein (nitrogen source) and carbohydrates which enhanced the microbial growth (9). In another report Konderski (15) could isolated different bacterial species from *P.australis* leaves and stems and mentioned these plants supplied a good metabolic activity for the isolated bacteria.

On the other hand the results showed that the bacterial growth in IPA_2 broth exhibit a significant turbidity (1.18, 1.01, 0.99, 1.18 and 0.82) for *E.coli, klebsiella, proteus, P.aeruginosa* and *S.typhi* respectively compared with 1.21 in Nutrient broth after 24 hrs of incubation (table 1). Although the optical density (O.D) for bacterial growth in IPA_2 broth was less than in Nutrient broth and IPA_1, the statical analysis showed there is non significant ($p>0.05$).

The results in table -2 showed that the plant extract (*P.australis*) was effective and enriched the growth of test organisms, when the highest viable count was demonstrated for *E.coli* (165.8×10^8 CFU 1ml) on IPA_3 agar , while the lowest viable counts (62.9×10^8 CFU 1ml) was demonstrated for *S.typhi* on IPA_4 agar , compared with the viable count of nutrient agar which ranged between (91.6-95.8) $\times10^8$ CFU1ml.

that's may be due to the plant extract components and the addition of pepton which supply an available form of nitrogen since native proteins are not generally attacked by bacteria (14). Also the addition of glucose which play role as energy source (1) and although their were differences in avialble counts but the statistical analysis showed non significant differences ($P>0.05$) between the viable count on the three media.

Nutrient agar recommended as a general culture medium for the cultivation of the majority of the less fastidious microorganisms , as well as abase to which a variety of materials are added to give enriched media. Infusion of meat was employed together with peptone as nutrients in culture media (14). In this study the used of plant extract only (IPA_4) or added peptone and glucose and sodium chloride as in (IPA_3) with agar addition to give the solidity for the medium. The colonies of the five bacterial species grow will and give similar characteristics to that on nutrient agar (smooth, circular colony, convex). In table (3). On the other hand. Maconkey agar is a differential medium recommended for use in the detection and isolation of all types of dysentery, typhoid and paratyphoid bacteria form materials harboring these organisms (14). The addition of bile salts and neutral red lactose has added advantage of supporting excellent growth and give amore differential between enteric pathogens and the coli form groups (4). In this study the modification of IPA medium to be similar to Maclonkey agar was done and the fact that this medium promotes development of these organisms (*E.coli, Klebsiella, Proteus , P.aeruginosa , S.typhi)* and at the same time differentiates between lactose fermenting (*E.coli* and *Klebsiella*) and non lactose fermenting bacteria (*Proteus, S.typhi and p.aeruginosa*). On the other hand the modification of IPA medium to be similar to blood agar by the addition of 5% human blood. Colonies of bacteria upon IPA_6 admonstrated similar to that on blood agar (control medium) they grow luxuriantly and the *E.coli and P.aeruginosa* colonies (hemolytic type) exhibit clear distinct degrees of hemolysis.(B-hemolysis) (table-3).

The study concluded that IPA media (IPA_1, IPA_2, IPA_3, IPA_4,IPA_5,IPA_6) were found to encourage the growth of gram negative bacteria , and the medium also is easy to prepare and unexpensive and available and efficient to others and could uses as enrichment and differential medium.

Table (1): the comparative efficiency of IPA broth media and Nutrient broth for some gram-negative bacterial species according the measurement the optical density at 470nm.

Time of growth (hr)	Bacterial species /type of medium										
	E.coli		*Klebsiella*		*Proteus*		*P.aeruginosa*		*S.typhi*		Nutrient broth
	IPA1	IPA2	IPA1	IPA2	IPA1	IPA2	IPA1	IPA2	IPA1	IPA2	
0	0.07	0.08	0.09	0.08	0.06	0.08	0.07	0.08	0.06	0.07	0.07
6	0.41	0.38	0.26	0.19	0.28	0.25	0.32	0.25	0.18	0.12	0.31
12	0.95	0.85	0.78	0.65	0.85	0.73	0.97	0.75	0.55	0.51	0.84
24	1.26	1.18	1.03	1.01	1.02	0.99	1.23	1.18	0.95	0.82	1.21

*P>0.05 non signifieant.

Table (2): A comparison of the viable counts of some gram negative bacterial species on the IPA media and nutrient agar.

Medium	Bacterial species				
	E.coli	*Klebsiella*	*Proteus*	*S.typhi*	*P.aeruginosa*
	Viable count 10^8 CFU 1ml				
Nutrient agar	95.2	94.5	93.3	91.6	95.8
IPA_3 agar	165.8	90.6	88.2	77.3	154.2
IPA_4 agar	112.3	88.0	77.6	62.9	111.6

*(F=1.103, N.S, P>0.05)

Table (3): Characteristics of bacterial species grown on different types of IPA agar media.

Type of medium	Bacterial species				
	E.coli (3isolates)	*Klebsiella* (3 isolates)	*Proteus* (2 isolates)	*S.typhi* (1 isolate)	*P.aeruginosa* (3 isolates)
Nutrient agar	Circular, smooth colonies convex	Circular smooth convex	Smooth colony circular	Smooth, round colonies	Smooth round colony greenish color to the agar
IPA_3 agar	Circular and smooth convex colonies	Circular smooth convex	Circular smooth colonies	Circular smooth colonies	Smooth round colony without change the agar color
IPA_4 agar	Circular and smooth convex colonies	Circular smooth convex	Circular smooth colonies	Circular smooth colonies	Smooth round colony with change the agar color
MacConkey agar	Lactose fermented red colony	Lactose fermented red colony	Non lactose fermented pale colony	Lactose non fermented pale colony	Lactose non fermented colony
IPA_5 agar	Lactose fermented red colony	lactose fermented red colony	Lactose non fermented pale colony	Lactose non fermented pale colony	Lactose non fermented colony
Blood agar	B-haemolysis	No haemolysis	No-haemolysis with swarming	No – haemolysis	B-haemolysis
IPA_6 agar	B-haemolysis	No naemolysis	no haemolysis no swarming	No haemolysis	B-haemolysis

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تحضير وسط أغنائي وتفريقي لتشخيص البكتريا السالبة لصبغة كرام

سوسن حسن عثمان كورجي

الخلاصة

توجد حاجة لاستخدام اوساط زرعية كفوءة لعزل وتشخيص انواع البكتيريا المرضية وغير المرضية في كافة انحاء العالم و خاصة البلدان النامية. تم تصنيع وسط زرعي طبيعي جديد لتنمية وتشخيص انواع البكتيريا السالبة لصبغة كرام.
استخدم في تحضير الوسط والمسمى IPA من مواد اولية من خلاصة نبات القصب *(Phragmetis australis)* . حضر الوسط على هيئة تركيبات مختلفة فسمي,IPA_1, IPA_2, ,IPA_3 IPA_4,IPA_5 IPA_6تم التأكد من كفاءة الاوساط المحضرة بمقارنتها مع الاوساط الزرعية المعروفة لذات الاستخدام (المرق المغذي و ماكونكي الصلب ووسط اكار الدم) كما اظهرت النتائج عدم وجود فروق معنوية ($P<0.05$) بين الاوساط المحضرة وأوساط السيطرة وان هذا الوسط المحضر سهل التحضير وواطىء الكلفة ويمكن الاستفادة منه كبديل للاوساط الزرعية المختلفة المحضرة تجاريا.