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Comparison of methods of inoculation for antibacterial potential assay

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Abstract

Two different methods of inoculations (spreading and pouring) were compared to choose one for better results for antibacterial potential assay. For comparison, two different parameters were selected that includes inoculation method and quantity of supernatant. In spite of spreading variable quantities of indicator organisms, no remarkable difference was observed on growth bed on agar media after incubation. When indicator organisms were poured after mixing with luke warm media, significant difference in growth bed was observed. The quantity of supernatant of test isolate P15 (*Bacillus leicheniformis*) showed a direct relation to inhibition zone formed. Suggesting that pouring method is effective to test the antibacterial potential of isolated strains against indicator organisms by agar well diffusion method.

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Introduction

The pour and spread plate methods are extensively used for viable counts with pure cultures (Hoben and Somasegaran, 1982). During antibacterial potential assay, zone of inhibition (mm) formed against indicator organisms must be directly related to quantity of supernatant added in wells made with sterile borer. Agar well diffusion method is widely used to evaluate the anti-microbial activity of plants or microbial extracts (Magaldi *et al*., 2004; Valgas *et al*., 2007)**.** In this, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole is punched with a sterilized tip, and supernatant in a pre-decided volume will be introduced into the well. Then agar plates were incubated under proper conditions depending upon the indicator microorganism. The filled supernatant diffuses in the agar medium and inhibits the growth of the microbial strain tested (Balouiri *et al*., 2016).

The objective of this study was to optimize the conditions including inoculation method and quantity of supernatant filled in the wells, for antibacterial assay before further experimentation.

Materials and methods

Sample collection

Twenty different samples (about 50 ml) of commercially produced local curd (dahi) from different locations of Lahore city were collected at morning between 8.00 to 9.00 am in sterile tubes and brought to Food Technology Laboratory at Institute of Agricultural Sciences, University of the Punjab, Lahore for culturing.

The location of sample was noted and pH of dahi was recorded by pH meter. The samples were stored under refrigeration conditions for further isolation and subsequent experimentation.

Preparation of serial dilutions of dahi samples

Ten milliliters of each dahi sample was aseptically added into 90 ml of sterile 0.9% NaCl solution and mixed thoroughly. Then suspensions were serially diluted in proportion of 1:10, upto 10^8 (Harrigan, 1998).

Purification of bacteria

The single and isolated colonies were picked from plates and streaked on MRS and M17 agar plates respectively. These plates were kept at 37 °C for 24 hours. The process of streaking was repeated till pure cultures of isolates were obtained.

Bacterial characterization

After characterization, P15 (*Bacillus leicheniformis*) was selected for further experimentation during optimization of conditions.

Optimization of conditions

Preparation of supernatant

Bacterial isolate (P15) was grown in respective MRS medium by inoculating loop full of pure culture in tubes containing 7 ml of MRS broth at 37 °C for 24 hours. Supernatant was separated by centrifugation at 13000 rpm for 10 minutes.

Preparation of supernatant

All purified bacterial isolates were grown in respective MRS and M17 medium by inoculating loop full of pure culture in tubes containing 7 ml of MRS and M17 broth 37 °C for 24 hours. Supernatant was separated by centrifugation at 13000 rpm for 10 minutes. One bacterial supernatant P15 was selected for optimization supernatant quantity.

Screening of Bacterial Strains for Antibacterial Activity

Indicator organisms

The two indicator organisms (Table 1) were bought from First Fungal culture bank at Institute of Agricultural Sciences, University of the Punjab, Lahore. These indicator organisms were grown in tubes containing LB broth medium by inoculating loop full sample and incubating at 37 °C for 24 hours.

Inoculating methods

Spreading on agar media

Varying quantities of *Klebsiella pneumoniae* and

Listeria monocytogenes (30 µl, 50 µl, 70 µl and 100 µl) were dropped on agar plate with micropipette. Itwas properly spreaded with sterile glass spreader till all supernatant get absorbed.

Pouring in agar media

100 µl of *Klebsiella pneumoniae* and *Listeria monocytogenes* containing $2x10^8$ and $2x10^4$ cfu/mL were added with micropipette in 250 ml of luke warm sterile nutrient agar media separately. It was properly mixed before pouring on petri plate.

Antibacterial Assay

The antibacterial spectrum was tested against indicator organisms by agar well diffusion method (Kang and Lee, 2005). After solidification 5mm wells were bored with sterile borer and different quantities of P15 supernatant (30 µl, 50 µl, 70 µl and 100 µl) was filled in wells.

Agar plates were kept at room temperature for about 6 hours for proper absorption of supernatant into media before incubation at 37 °C for 48 hours. The zone of inhibition was measured in mm.

Results

Optimization of conditions

The conditions for antibacterial potential assay get optimized by comparison among spreading and pouring on nutrient agar plates.

Table 1. Indicator Organisms with their accession numbers.

	Indicator Organisms	Gram +ve/-ve	Accession number
1.	*Klebsiella pneumoniae*	*-ve*	FCBP-LB-0068
2.	*Listeria monocytogenes*	*+ve*	FCBP-LB-0144

Inoculation methods

Spreading

Spreading of varying quantities of *Klebsiella pneumoniae* and *Listeria monocytogenes* (containing $2x10^8$ cfu/mL and $2x10^4$ cfu/mL) (30 µl, 50 µl, 70 µl and 100 µl) on agar plates showed that very thick layer of growth was observed on agar surface and no remarkable difference was observed in growth thickness of indicator organisms as quantity of bacterial culture was increased (100 µl) or decreased (30 µl). (Fig. 1. And Fig. 2). When plates were observed after incubation it was found that there was no inhibition zone formed by variable quantities of supernatant of P15 (Table 2).

Table 2. Comparison of methods of inoculation.

Klebsiella pneumoniae			*Listeria monocytogenes*		
Method of inoculation	Quantity of Supernatant filled	Zone of inhibition (mm)	Method of inoculation	Quantity of Supernatant filled	Zone of inhibition (mm)
Spreading	30µl	0	Spreading	30µl	0
	50µl	0		50µl	0
	70µl	0		70µl	0
	100µl	0		100µl	0
Pouring	30µl	10mm	Pouring	30µl	8mm
	50µl	14mm		50µl	12mm
	70µl	17mm		70µl	15mm
	100µl	21mm		100µl	20mm

Pouring

Pouring of 100 µl *Klebsiella pneumoniae* and *Listeria monocytogenes* (containing $2x10^8$ cfu/ml and $2x10^4$ cfu/ml) after mixing in agar media showed proper growth and distribution of indicator organisms in agar (Fig. 3). After incubation, when plates were observed it was found that supernatant showed inhibitory potential against both indicator organisms *Klebsiella pneumoniae* and *Listeria monocytogenes* (Fig. 3). As quantity of supernatant was increased from 30µl to 100µl the inhibitory zone formed also increases as shown in Table 2. The maximum

inhibitory zone formed against *Klebsiella pneumoniae* was 21 mm when quantity of 100µl supernatant was filled in well. While 20 mm inhibition zone was observed against *Listeria monocytogenes* after filling 100µl of supernatant**.**

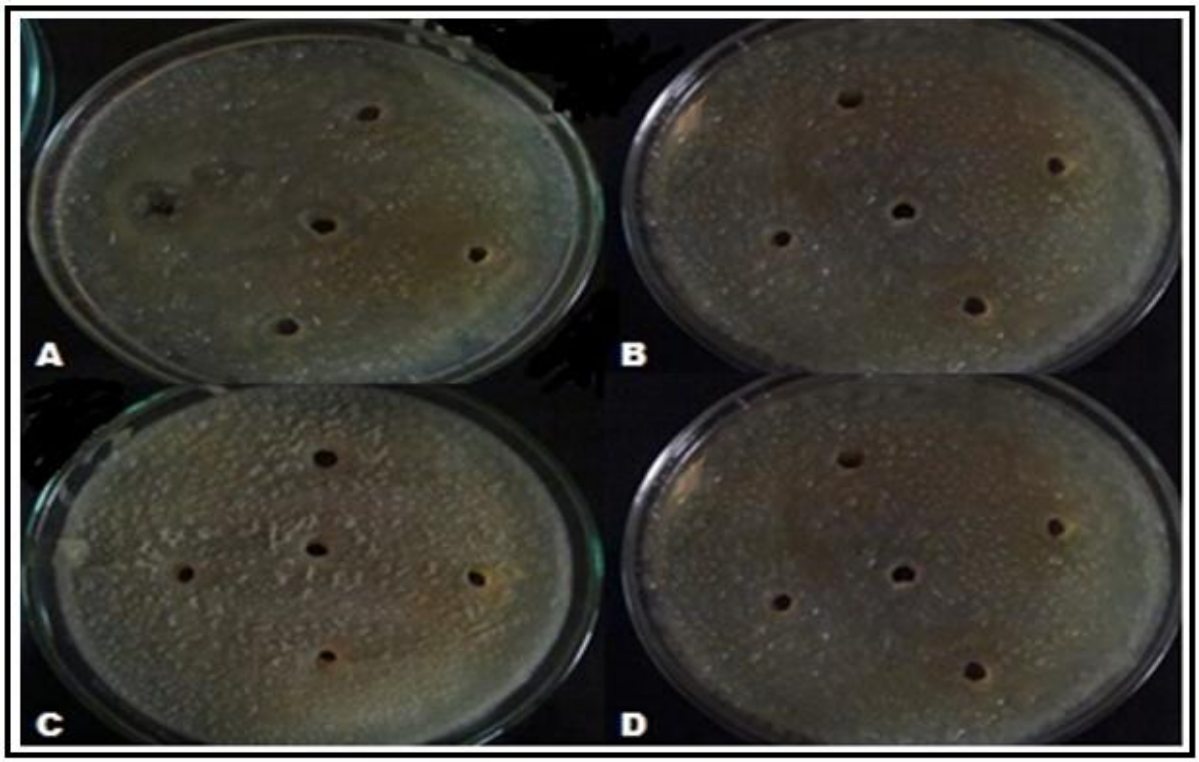

Fig. 1. Spreading of different quantities of *Klebsiella pneumoniae* on agar media; (A) 30 µl, (B) 50 µl, (C) 70 µl, (D) 100 µl.

Supernatant Quantity

As variable quantities of (30 µl, 50 µl, 70 µl and 100 µl) of bacterial supernatant were filled in wells, it showed variation in the inhibition zone formed around well. And it was observed that while adopting pour plate method, area of zones formed was found directly proportional to the quantity of supernatant filled (Fig. 3).

Discussion

For antibacterial potential assay, pouring is good as compared to spreading if agar well diffusion method is to be adopted.

Spreading

As during spreading of variable quantities of *K. pneumoniae* and *L. monocytogenes* on agar plate showed layer of thick growth on agar and when P15 bacterial supernatant was filled in it. It shows zero inhibitory potential against test bacterial supernatant. This might be due to reason as bacterial growth on agar was not embedded, it was just on surface and even maximum supernatant quantity 100µl showed no inhibition.

Pouring

During pouring the *K. pneumoniae* and *L. monocytogenes* were evenly distributed in agar media and so bacterial supernatant showed potential by creating inhibitory zones. This might be due to fact that during pouring method, as indicator organisms are mixed with luke warm agar media before pouring in petri plate so this result in uniform distribution of indicator organisms in media.

Variable amount of supernatant was filled in each well and this was allowed to absorb in agar media by keeping at room temperature before shifting in incubator.

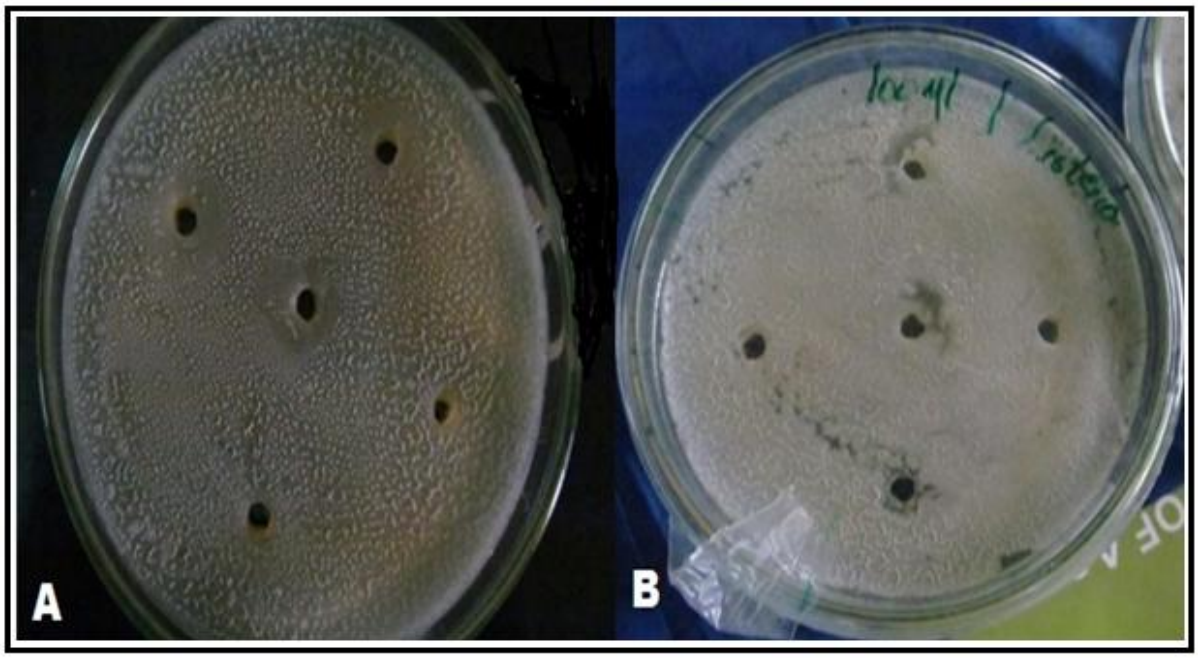

Fig. 2. Spreading of different quantities of *Listeria monocytogenes* on agar media; A showing 70 µl, B showing 100µl.

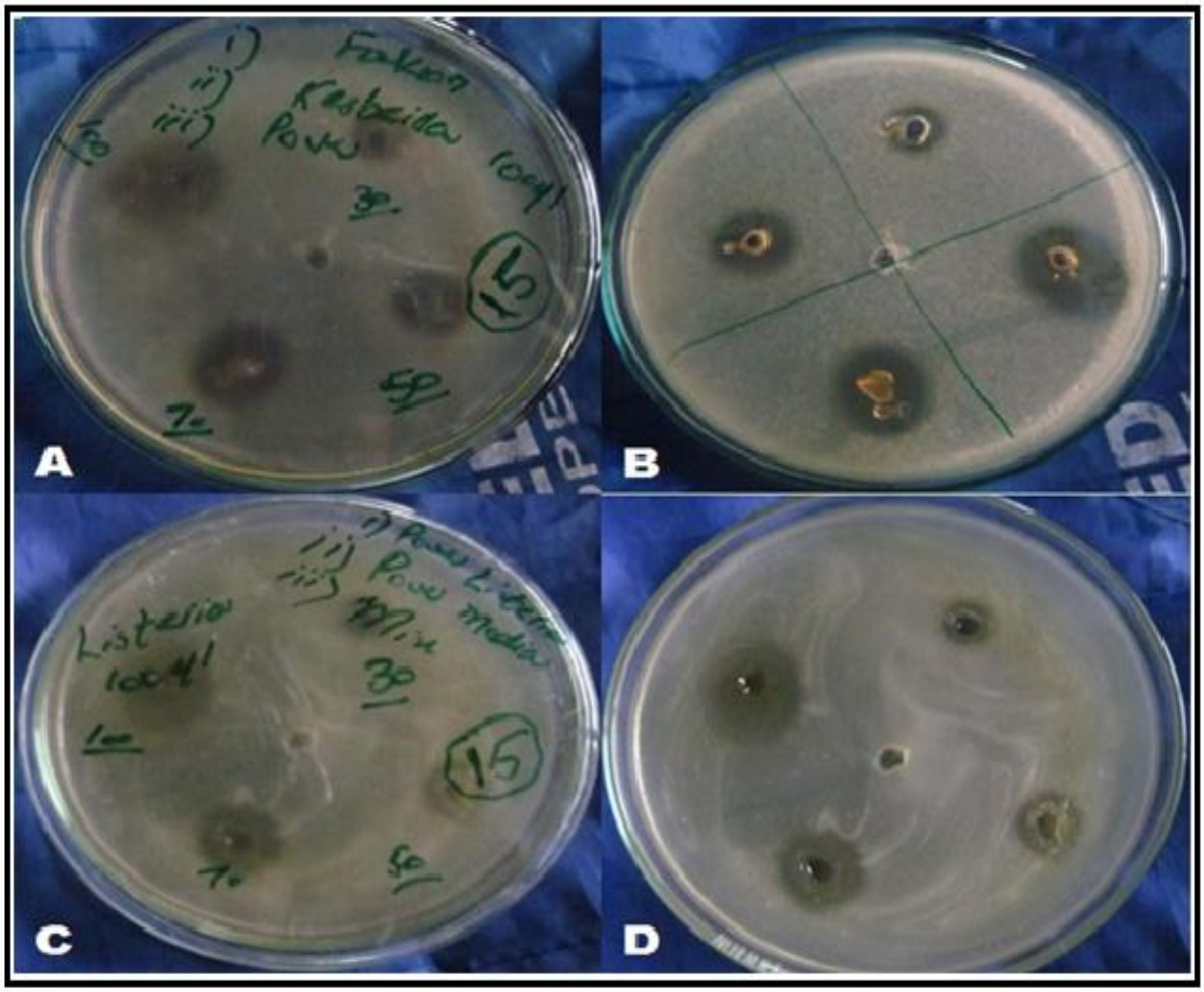

Fig. 3. Pouring of indicator organisms on agar media; (A) & (B) 100 µl *Klebsiella pneumoniae,* (C) & (D) 100 µl *Listeria monocytogenes.*

Conclusion

In conclusion, pour plate method is better method of inoculation as compared to spreading during antibacterial potential assay by adopting agar well diffusion method.

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