Determining the effect of various physicochemical factors on inhibition activity of Lactobacillus acidophilus against Klebsiella pneumonia isolated from hospital-acquired infections by using optical density assay

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Abstract

This study aims to determine the effect of using *Lactobacillus acidophilus* in various physicochemical factors to inhibit the growth of *Klebsiella pneumoniae* isolated from patients of hospital-acquired infections by using optical density technique. The result revealed that different factors like pH, incubation temperature degree, incubation periods and concentrations, seemed to affect on inhibition activity of *L. acidophilus*. Maximum activity was noted at pH 3, temperature 45°C, incubation for 48 hr and three fold concentrations which clarified by values O.D reached to 0.111, 0.775, 0.660 and 0.040 respectively whereas the lowest effect appeared after the use of pH 9, temperature 15°C, incubation for24 hr and one fold concentrations where the inhibition values reached to 1.899, 1.180, 1.161 and 1.411respectively The results ensured that *L. acidophilus* sufficiency inhibited growth of *K. pneumoniae* with promising encourage to use the *L.acidophilus* as biotherapeutic agents against *K. pneumoniae* infections.

Key words: *Klebsiella pneumonia , Lactobacillus acidophilus*

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تحديد تأثير مختلف العوامل الفيزيوكيميائية على الفعالية التثبيطية لـ *Lactobacillus acidophilus* ضد *Klebsiella pneumonia* معزولة من مرضى الإصابات المكتسبة من المستشفيات بواسطة استخدام تقنية قياس الكثافة الضوئية

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الخلاصة

هدفت الدراسة الى تقدير فعالية استخدام *Lactobacillus acidophilus* لمختلف العوامل الفيزيوكيميائية لتثبيط نمو *Klebsiella pneumonia* المعزولة من مرضى الإصابات المكتسبة من المستشفيات بوساطة استخدام تقية قياس الكثافة الضوئية وأوضحت النتائج بأن مختلف العوامل مثل درجة حرارة الحضن، فترة الحضن، التركيز و الأس الهيدروجيني، تؤثر على القدرة التثبيطية لـ*L. acidophilus* اذ لوحظت أعلى قدرة تثبيطية عند الأس الهيدروحيني 3،درجة حرارة 45 مئوية، فترة حضن لمدة 48 ساعة والتركيز لثلاث مرات وهدا ما يتضح من خلال القيم التثبيطية التي تصل 0.775,0.111 ,0.040, 0.660 على التوالي في حين لوحظت اقل قيم تثبيطية عند أس هيدروجيني 9 درجة حرارة 15 مئوية، حضن لمدة 24 ساعة والتركيز مرة واحدة اذ انخفضت القيم التثبيطية لتصل إلى 1.411, 1.899, 1.180, 1.161 على التوالي وهذه النتائج تؤكد على كفاءة وثبات فعالية استخدام *L. acidophilus* وبمختلف الظروف كمثبط ضد نمو *K. pneumoniae* مما يشجع على استخدام هذه العصيات كعلاجات حيوية ضد هذه البكتريا الضارة.

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Introduction

The genus *Klebsiella* is generally classified into seven species and subspecies, some of them are regarded as "environmental" isolates were also isolated from human clinical specimens and animals (1,2). Pathogenic *Klebsiella* strains belonging to *K. pneumoniae* and *K. oxytoca* are known to cause hospital-acquired infections such as septicemia, pneumonia, and urinary tract and soft tissue infections (3). As a cause of nosocomial infection due to gram-negative bacteria, *Klebsiella* ranks next to *Escherichia coli*, accounting for 8% of endemic hospital infections and 3% of epidemic outbreaks (4). Meanwhile, the appearance of multiresistant strains among clinical *Klebsiella* isolates, especially those producing extended-spectrum betalactamases, which show resistance to extended-spectrum cephalosporins, has been increasing over the past several years., thus complicating the therapy of this infection (5,6) Therefore, *Klebsiella* nosocomial-infection surveillance is necessary to use the alternative treatment by using biotherapeutic agents in the prevention and control of *Klebsiella* infection (7)Several studies have assessed the potential of Lactobacilli particularly *Lactobacillus acidophilus* in prevention or treatment of certain infections by producing antimicrobial substances. In addition, *Lactobacill* or probiotic therapy is considered as "natural" and without side effects in contrast with conventional pharmaceutical treatments. The purpose of this study is to determine the effectiveness of using lactobacillus acidophilus to inhibit the growth of *K. pneumoniae* isolated from patients of hospital-acquired infections by using optical density technique.

Methods and Materials

Isolation and identification of *L. acidophilus*

A portion of 0.1 ml of yogurt samples were drawn aseptically to a previously sterilized test tubes containing 9.9 ml Manns-Regoz and Sharpe (MRS) broth, then incubated at 37 °C for 24 hr. under anaerobic conditions. After incubation, serial dilutions were made, and 0.1 ml from last dilution was streaked on the surface of MRS agar in Petri dishes, and then incubated for 24

hr. at 37 °C under anaerobic conditions (anaerobic jar). After that, part of the growth was transferred to MRS broth in a test tube and incubated under anaerobic condition.

The suspected *L. acidophilus* isolates were identified by microscopic examination, biochemical tests and carbohydrate fermentation test according to (8,9).

Isolation and identification of *K.pneumonia.*

K.pneumonia isolated from hospitalized patients whose blood culture yielded *Klebsiella* and identified by using standard microbiology techniques. The isolates were culture in Brain Heart Infusion (BHI) broth(Difco, USA) and routine susceptibility testing of some antibacterial agents(Trimethoprim- Sulphamethoxazole 30µg, Azithromcin 15µg , Cefotaxim 30µg, Chloramphenicol 30µg, Ciprofloxacin 5µg Lincomycin 2µg, Gentamycin 10µg, Tetracycline 30µg, Ampicillin 10µg, and Amikacin 30µg from Oxoid - England) was performed by disk diffusion method on Mullor-Hinton agar(Difco- USA) described in The Bergey's Manual of Determinative Bacteriology(10).

Performed *L.acidophilus* filtrate

L. acidophilus was inoculated in MRS - broth and incubated at 37^0C for 24 hrs in anaerobic condition. After incubation period, Cell - free supernatants were collected by centrifugation at 3000 rpm for 40 min. These supernatant was filtrated through Millipore filter unite (0.22 µm) as described in (11).

Detection of antagonistic a activity of *L. acidophilus* against the *K.pneumonia*

Optical density(O.D), The measure of the amount of light absorbed by a suspension of bacterial cells with the use of spectrophotometer As visible light passes through a cell suspension the light is scattered. Greater scatter indicates that more bacteria is present. The amount of light scatter can be measured in a spectrophotometer.

In present research we were inoculated *L.acidophilus* filtrate with *K. pneumonia* culture as a ratio (1:1) then incubated for overnight at 37° C. Typically, when working with a particular type of cell, you would determine the optical density at a particular wavelength that correlates with the different phases of bacterial growth. Generally we will want to use cells that are in their mid-log phase of growth, thus we estamated the antagonistic activity in the approach of optical density assay($O.D._{600}$) nm .

Determining Bacterial Concentration

To evaluate O.D, the following procedure was done according to (12).

1- with sterile pipette tips Place a 500 µl sample of the blank (BHI broth) in a cuvette using sterile technique to avoid contamination of the sterile media stock and also to protect the cultures from contamination. Place the cuvette in spectrophotometer**.**

2-After the spectrophotometer has read the blank, remove the cuvette, replace it with a cuvette containing 500µl of the *K. pneumonia* culture (control) and let the spectrophotometer read it.

3. Pipet 500 µl of each sample into a labeled cuvette and allow the spectrophotometer read them.

4-Record the O.D's. of each read.

The effect of different *L. acidophilus* incubation temperature on antagonistic a activity

Tubes containing 10 ml MRS broth were inoculated with 1% of *L. acidophilus* culture then incubated at 15 °C , 37 °C and 45 °C for 24 hr. After incubation, *L.acidophilus* filtrate was prepared and inoculated with *K.pneumonia* then incubated at 37 °C for 24 hr**.** then the antagonistic activity was estimated for each temperature. according to (13)

The effect of different *L. acidophilus* concentrations on antagonistic a activity

The filtrates of *L. acidophilus* which has equal volume 200 ml were concentrated by freeze - dryer to one fold (100 ml), two fold (50 ml), three fold (25 ml). Each concentrations was inoculated with *K. pneumonia* then incubated at 37 °C for 24 hr**.** then antagonistic activity was estimated as described in (14).

The effect of different incubation periods of *L. acidophilus* on antagonistic a activity

Tubes containing 10 ml MRS broth were inoculated with 1% of *L. acidophilus* culture then incubated at 37 °C for different incubation periods (24, 48 and 72) hr. After incubation, *Lactobacillus acidophilus* filtrate was prepare and inoculated with *K.pneumonia* then incubated at 37 °C for 24 hr**.** and the antagonistic activity was estimated for each period according to(15).

The effect of different pH value of *L. acidophilus* culture on antagonistic a activity.

Tubes containing 10 ml MRS broth were inoculated with 1% of *Lactobacillus acidophilus* culture, the pH of *Lactobacillus* culture was adjusted to 3, 6 and 9 respectively with 6 M HCl or 6M NaOH and autoclaved, then incubated at 37 °C for 24 hr**.** After incubation, *L. acidophilus* filtrate was prepare and inoculated with *K.pneumonia* then incubated at 37 °C for 24 hr**.** The antagonistic activity was estimated for each pH value of *L. acidophilus* culture as described in (16)

Results and discussion

The result showed that *K. pneumonia* in current investigate was associated with frequent adverse effects and resistance to antibiotics like Amikacin, Ampicillin, Chloramphenicol, Ciprofloxacin, Gentamycin, Tetracycline, Azithromcin and was sensitive to Lincomycin, Cefotaxim and Trimethoprim-sulphamethoxazol, therefore alternative methods to control this bacteria are needed (17).

The effect of different *L*actobacillus incubation temperature on antagonistic a activity

Our finding revealed that *L.acidophilus* was effective against *K. pneumonia* in different incubation temperature and had the best effect at 45 °C which reached to 0.775, while the less effect was observe when incubate *L.acidophilus* at 15 °C with inhibition values reached to 1.180, whereas the minimum values indicate less growth of *K. pneumonia* as shown in table (1).

Table 1 The effect of different *L.acidophilus* incubation temperature on antagonistic a activity

incubation temperature of *L.acidophilus*	O.D values of growth of treated *K. pneumonia*
	1.525 *K. pneumonia* only(control)
15 °C	1.180
37 °C	0.856
45 °C	0.775

*The less value indicate good inhibition activity

Thus indicate the temperature degree 45 °C that optimum incubation temperature for produce the inhibition agents like bactriocine , and this result accordance with (18)as which showed that the optimum degree to produce bactriocine was 45 °C.

The effect of different *L. acidophilus* concentrations on antagonistic a

The optimum inhibitory effect shown during use three fold of *L. acidophilus* filtrate where the inhibition values reached to 0.040 and the lowest effect appear with one fold *L.acidophilus* filtrate when the inhibition values reached to 1.411, as shown in table (2).

Table 2. The effect of different *L.acidophilus* concentrations on antagonistic a activity

L.acidophilus filtrate concentrations	O.D values of growth of treated *K. pneumonia*
	1.525 *K. pneumonia* only(control)
0ne fold	1.411
two fold	0.760
three fold	0.040

*The less value indicate good inhibition activity

Our result indicated that *L.acidophilus* filtrates concentrated to three fold have the highest inhibitory effect than the other folds because a variety of compounds with antimicrobial activity was concentrated therefore minimize the growth of *K. pneumonia* , an observation which come in accordance with other workers (19,20 and 21).

The effect of different incubation periods of *L.acidophilus* on antagonistic a activity

The target of this experiment was to test the ability of *L. acidophilus* for inhibition the growth of *K. pneumonia* with different incubation periods our result showed that incubation for48 hr. hade high effect reached to 0.660 as shown in table (3). Similar observation were recorded by other investigators like (22, 23 and 24) who found that inhibitory effect of LAB increased after 48hr of incubation, that indicated to many antimicrobial substance are produced in this period than other periods thus may be due to that the inhibitory materials (acidophilin) are secreted outside the cells after increasing the incubation time causing decrease in the inhibitory effect so the amount of antibacterial substance in the period after 24 hr. not enough

to reduce the growth of *K. pneumonia* and after 72 hr. the antimicrobial substance loss some of their inhibition activity.

Table 3 The effect of different incubation periods of *L. acidophilus* on antagonistic a activity

incubation periods of *L.acidophilus*	O.D values of growth of treated *K. pneumonia*
	1.525 *K. pneumonia* only(control)
24 hr.	1.161
48 hr.	0.660
72 hr.	0.960

*The less value indicate good inhibition activity

The effect of different pH value *L. acidophilus* culture on antagonistic a activity

Regarding pH the maximum inhibitory activity was observed at pH 3 which reached to 0.111 nm and minimum was observed at pH 9 which reached to 1.899, it was clear that the acidic pH was a better inhibitory effect than alkaloid and that confirmed by Aasen (25) who mention that acidic pH stimulated inhibitory effect against Gram positive (*Staph. aureus*, *Baccillus subtalis*) and Gram negative bacteria (*E.coli,klebsiella spp.,Proteus spp.)*. Tagg *et al*. (26) reported that *L. acidophilus* bacteriocin are resistant to acidic pH more than basic pH. Similar results were obtained by other investigator such as (27) when found that best inhibitory effect was gained during used acidic pH and *L. acidophilus* bacteriocins were active at as low as pH3.

Table 4 The effect of different pH value of *L.acidophilus* culture on antagonistic a activity

pHof *L.acidophilus* culture	O.D values of growth of treated *K. pneumonia*
	1.525 *K. pneumonia* only(control)
pH 3	0.111
pH 6	1.656
pH 9	1.899

*The less value indicate good inhibition activity

Our outcomes showed that *L.acidophilus* have the high inhibitory effect on *Klebsiella pneumonia* and this may due to bacteriocin (acidophilin) production from *L. acidophilus* furthermore antimicrobial activities of *L.acidophilus* were very stable under a series of different conditions with promising inhibitory spectrum but this activity strongly dependent on pH, incubation periods, concentrations and incubation temperature as claimed by (28, 29) From the results we can conclude that *L.acidophilus* could offer alternatives treatment and the studies should be aimed at opening new possibilities of bacteriocins synthesis to increase their production on an industrial scale.

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