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Isolation and diagnosis of *Acinetobacter baumannii* that produce protease and antimicrobial agents from the milk factory

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ABSTRACT: This study on the isolation and diagnosis of *Acinetobacter baumannii*, a bacterium known for its ability to produce protease and antimicrobial agents, has practical implications for the field of microbiology and food safety. A total of 50 samples were collected, including 30 swabs from the floors of various milk factory units and 20 samples from the lands adjacent to the laboratory. The samples were incubated in blood agar, MacConkey, and Mantol salt agar. Twenty bacterial isolates were obtained, including 10 Acinetobacter baumannii, 7 Staphylococcus aureus, and 3 E. coli isolates. All isolates were diagnosed using biochemical tests, API 20 E Kit, and Vitek 2 system. The ability of the isolates to produce the protease enzyme was tested using a skim milk agar medium. Seventeen isolates showed their ability to make the enzyme. *Acinetobacter baumannii* produced (10) 100%, *S. aureus* 5 (71.4%), while 2 (66.6%) isolates *E. coli*. By measuring the diameters of the hydrolyzate on the milk medium, it was found that *Acinetobacter baumannii* has a high efficiency in producing the protease enzyme with diameters ranging from 15 mm to 45 mm. After the most productive isolate was selected, its diagnosis was confirmed using a molecular technique using 16S rRNA. The crude extract of secondary metabolites was characterized by UV-visible and FTR. The antibacterial effectiveness of the extract was tested using primary and secondary screening protocols. The extract showed high effectiveness, with diameters of inhibition ranging from 20-25 mm against Gram-positive bacteria and 15-23 mm against Gram-negative bacteria.
Keywords: protease enzyme; skim milk agar; biochemical test.

Isolamento e diagnóstico de *Acinetobacter baumannii* como produtor de protease e agente antimicrobiano em fábrica de leite

ABSTRACT: Este estudo aborda o isolamento e diagnóstico de *Acinetobacter baumannii*, bactéria conhecida pela sua capacidade de produzir proteases e agentes antimicrobianos, e que tem implicações práticas para o campo da microbiologia e segurança alimentar. Foram coletadas 50 amostras, incluindo 30 amostras de pisos de diversas unidades fabris de leite na região de Babil, Iraque; e 20 amostras foram coletadas em terrenos adjacentes ao laboratório. As amostras foram incubadas em ágar sangue, ágar MacConkey e ágar sal Mantol. Foram obtidos 20 isolados bacterianos, incluindo 10 isolados de *Acinetobacter baumannii*, 7 de *Staphylococcus aureus* e 3 de *E. coli*. Todos os isolados foram diagnosticados por meio de testes bioquímicos, kit API 20E e sistema Vitek 2. A capacidade dos isolados em produzir a enzima protease foi testada utilizando meio ágar leite desnatado. Dezessete isolados mostraram capacidade de produzir a enzima. *Acinetobacter baumannii* produziu (10) 100%, *S. aureus* 5 (71,4%), enquanto *E. coli* produziu 2 (66,6%) isolados. Ao medir os diâmetros do hidrolisado no meio lácteo, constatou-se que *Acinetobacter baumannii* apresenta alta eficiência na produção da enzima protease, com diâmetros variando de 15 mm a 45 mm. Após a seleção do isolado mais produtivo, seu diagnóstico foi confirmado por técnica molecular utilizando 16S rRNA. O extrato bruto de metabólitos secundários foi caracterizado por UV-visível e FTR. A eficácia antibacteriana do extrato foi testada utilizando protocolos de triagem primária e secundária. O extrato apresentou alta eficácia, com diâmetros de inibição variando de 20-25 mm contra bactérias Gram-positivas e 15-23 mm contra bactérias Gram-negativas.
Palavras-chave: enzima protease; ágar leite desnatado; teste bioquímico.

1. INTRODUCTION

Milk factories are a suitable source and environment for the growth of various microorganisms, especially bacteria. Milk is an integrated nutritional environment that helps microorganisms grow and reproduce. The large number of workers in laboratories and the abundance of tools used may help in the spread of microorganisms and serve as a vector for them (HICKEY et al., 2015)

Microorganisms can be contaminated in milk laboratories from the moment milk is obtained from cows, when drops

of milk fall on the ground, on the equipment and tools used in the laboratory, or on the hands and clothes of workers. Various microorganisms can grow on the furniture and floors of the dairy factory, and Bacteria are the most abundant and widespread (YANG et al., 2012).

Gram-negative *Acinetobacter baumannii* is one of the most widespread bacteria in most environments. *It can secrete many enzymes, including lipase, oxidase, cellulase, and* protease. Its ability to produce protease enables it to grow in a dairy environment (JOHNSON; STEELE, 2013).

Four primary extracellular proteases are produced by S. aureus: metalloprotease (aureolysin; Aur), a second cysteine protease (Scp; also known as staphopain), serine protease (V8 protease; SspA), and a cysteine protease (SspB) expressed within the same operon (KARLSSON; ARVIDSON, 2002).

Protein degradation is crucial for controlling the amounts of proteins in E. coli and eliminating broken or aberrant proteins. Proteolytic enzymes are abundant in E. coli and are found in the periplasm, inner membrane, and cytoplasm (MAURIZI, 1992). During *Acinetobacter baumannii* growth, it produces primary and secondary metabolites. Most secondary metabolites are excreted in the medium, can be extracted, and their biological activity is monitored (KARLSSON; ARVIDSON, 2002). Secondary metabolites are toxic or lethal to competing organisms of bacteria in the environment in which they are present

Various bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes* and *E. coli*, are present in the soil and floors of milk factories. The present study aimed to isolate the bacteria from milk factories, test their ability to produce protease enzymes, and test the ability of some bacteria to produce secondary metabolites that kill other bacteria.

2. MATERIAL AND METHODS

2.1. Collection of samples

Samples were collected from the milk factory in Babil Governorate / Iraq, distributed among 30 samples from the floors of the factory units and 20 samples from the lands adjacent to and surrounding the factory. The samples were transferred to the laboratory to complete the study (SHEEHAN, 2013).

2.2. Sample handling

Soaps were distributed on the surface of the blood, MacConkey, and mannitol salt agar and incubated at 37 ° C for 24 hours. The soil samples were diluted and incubated in the same media (MACFADDIN, 2000).

2.3. Isolation and identification of bacteria

Three bacterial species were isolated according to morphological and biochemical tests, including the IMViC test, catalase, coagulase, API 20 E Kit (biomerix) and Vitek 2 system (MACFADDIN, 2000).

2.4. Skim milk agar Suspended

51.5 grams of skim milk agar in 1 liter of distilled water. Heated to boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates. Proteolytic bacteria hydrolyze casein to form soluble nitrogenous compounds, which are indicated as clear zones surrounding the colonies (WEHR; FRANK, 2004).

2.5. Molecular study

16S rRNA was identified using PCR in this manner for *A. baumannii*. Using the approach by Chapartegui-González et al. (2018) and a specific primer found in Table 1.

Table 1. 16S rRNA primer sequences.
Tabela 1. Sequências iniciadoras de 16S rRNA.

Characteristics	Description
Bacteria	*Acinetobacter baumannii*
Primer Name	16S rRNA
Sequences 5'-3'	GTTACTCGCAGAATAAGCACC
Size (bp)	191
Accession No.	LN611374.1

2.6. Extraction of raw secondary metabolites

A. baumannii were grown in LB medium for 28-72 hours, after which they were centrifuged at 6000 revolutions for 30 minutes to separate the metabolic materials from the rest of the cellular components. Then, they were filtered using paper filters. The extract was taken, and a volume of methanol was added. It was shaken using a shaking incubator for an hour until it separated into two layers: an aqueous layer and an alcohol layer containing the extract. The aqueous layer was removed, and the alcohol was evaporated using a rotary evaporator to obtain the extract in the form of crystals (WEHR; FRANK, 2004).

2.7. Primary screening

The extract's effectiveness as a killer or antibacterial was estimated using a perpendicular streak plate method (DHANASEKARAN; SELVAMANI, 2009; DHANANJEYAN et al., 2010).

2.8. Secondary screening

The positive results were obtained from the primary screening protocol, batch culture fermentation, and an agar well diffusion assay, which were used to determine the antimicrobial activities (PALLAVI et al., 2013).

2.9. Characterization of Produced Antibacterial

This study used two methods to characterize antibiotics extracted from *A. baumannii*: Ultraviolet (UV) and infrared (FT-IR) spectroscopy.

3. RESULTS

The samples were distributed among the different laboratory rooms; their percentage was 60% (30/50), while the percentage of soil samples was 40% (20/50), as shown in Figure 1.

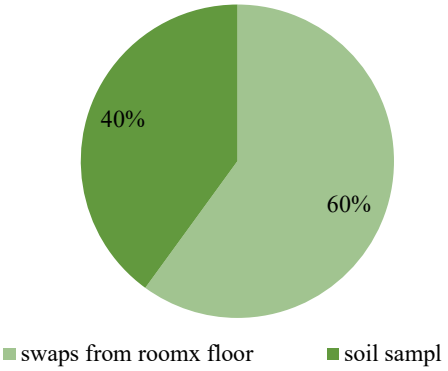

Figure 1. Distribution of study samples.
Figura 1. Distribuição das amostras do estudo.

3.1. Identification of Isolated Bacteria

From 50 samples, 20 bacterial isolates were obtained from 20 samples, and the other 30 samples were without growth. Twenty bacterial isolates were obtained and distributed as 10 *Acinetobacter baumannii* isolates, 7 Staphylococcus aureus isolates, and 3 *E. coli* isolates were obtained as clear in Table 2.

The bacteria were characterized based on their phenotypic, cultural, and biochemical characteristics and the Vitek 2 system. Depending on the Vitek 2 system, the bacteria were diagnosed as *A. baumannii* isolates, *S. aureus* isolates, and *E. coli* with a probability of 99%, 98%, and 98%, respectively, as shown in Table 3.

Table 2. Frequency distribution of the total isolated bacteria from the whole samples.
Tabela 2. Distribuição de frequência do total de bactérias isoladas das amostras inteiras.

Bacterial growth	Frequency	Percentage	
No bacterial growth	30	60%	
Bacterial growth	20	40%	
Type of isolated bacteria	Frequency	From total samples (n.=50)	From contaminated samples
A. baumannii	10	20%	50%
S. aureus	7	14%	35%
E. coli	3	6%	15%

Table 3. Diagnosing bacteria depends on the Vitek 2 system.
Tabela 3. O diagnóstico de bactérias depende do sistema Vitek 2.

Bacteria	Source	Probability (%)
A. baumannii	Floor swab	99%
S. aureus	soil	98%
E. coli	soil	98%

Table 4. Bacterial isolates produce protease.
Tabela 4. Isolados bacterianos produzem protease.

Bacteria	Total	N° producer protease	%
A. baumannii	10	10	100.0
S. aureus	7	5	71.4
E. coli	3	2	66.6

3.2. Protease enzyme

A Protease enzyme test was performed for all 20 isolates to determine the ability of these bacterial species isolated from the milk factory. The high percentage of production protease enzyme was for *A. baumannii,* while the isolates showed low production for each of *S. aureus* isolates and *E. coli*, as shown in Table 4 and Figure 2.

The ability of the bacteria to produce enzymes was determined by measuring the diameter of the lysis as a measure of production efficiency, as clear in Table 4. It was found that *A. baumannii* was the most efficient in production, and *A. baumannii* isolation showed the highest production capacity with an inhibition diameter of 45 mm (Table 5).

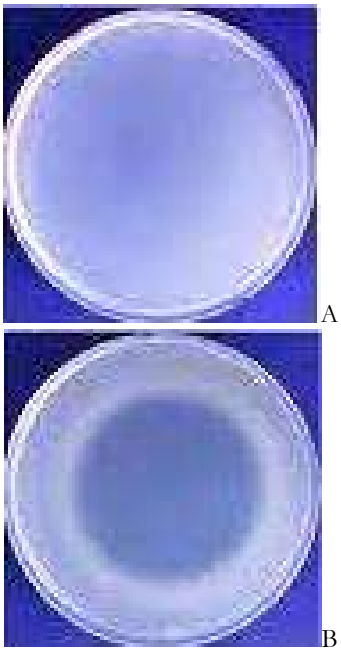

Figure 2. Protease enzyme test – (A) negative; (B) positive.
Figura 2. Teste da enzima protease – (A) negativo; (B) positivo.

Table 5. Diameters of milk lysis by bacteria produce protease.
Tabela 5. Diâmetros de lise do leite por bactérias produzem protease.

Bacteria isolates	Diameters of milk lysis (mm)
A. baumannii 1	20
A. baumannii 2	15
A. baumannii 3	25
A. baumannii 4	33
A. baumannii 5	45
A. baumannii 6	20
A. baumannii 7	24
A. baumannii 8	31
A. baumannii 9	30
A. baumannii 10	18
S. aureus 1	17
S. aureus 2	14
S. aureus 3	20
S. aureus 4	19
S. aureus 5	26
E. coli 1	12
E. coli 2	17

3.3. Molecular detection of 16S rRNA of *A. baumannii*

A. baumannii five was selected for genetic diagnosis. PCR diagnosis recorded A. *baumannii* as 10 (100%), as shown in Figure 3.

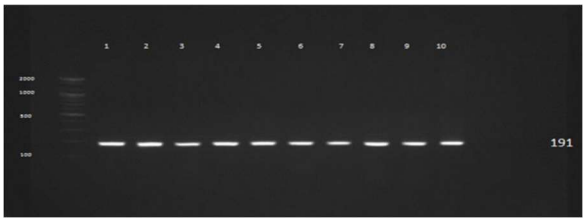

Figure 3. Agarose-gel-electrophoresis images were demonstrated in the PCR product. Investigation of 16S rRNA gene in *A. baumannii* isolates. Lane (M): marker ladder (100- 2000bp), lane (1-10): 16S rRNA gene positive at 191bp PCR product size.
Figura 3. Imagens de eletroforese em gel de agarose foram demonstradas no produto de PCR. Investigação do gene 16S rRNA em isolados de *A. baumannii*. Pista (M): escada de marcadores (100-

2000 pb), pista (1-10): gene 16S rRNA positivo no tamanho do produto PCR de 191 pb.

3.4. Primary screening results

The 10 *A. baumannii* isolates were tested by the perpendicular streak method against Gram-positive bacteria (*Staphylococcus albus*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative bacteria (*Escherichia coli*, *Klebsella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Aeromonas hydrophila*), which showed antibacterial activity as described in Figure 4.

3.5. Secondary screening methods

The secondary screening test was done for *A. baumannii* cultures extracellular extracts against pathogenic Gram-positive bacteria. Five isolates produced effective substances against pathogenic bacteria, as shown in Table 5 and Table 6.

Figure 4. Antibacterial activity of *A. baumannii*.
Figura 4. Atividade antibacteriana de *A. baumannii*.

Table 5. Antibacterial activity of external extract on pathogenic Gram-positive bacteria (secondary screening).
Tabela 5. Atividade antibacteriana do extrato externo em bactérias Gram-positivas patogênicas (triagem secundária).

Bacteria isolates	Inhibition diameter ± SD		
	S. aureus	*S. albus*	*S. pyogenes*
A. baumannii 2	20.5 ± 0.2	21.5 ± 0.03	23 ± 0.2
A. baumannii 3	20.3 ± 0.6	24.5 ± 0.4	25 ± 0.4
A. baumannii 5	22 ± 0.4	25 ± 0.4	25 ± 0.2
A. baumannii 7	20 ± 0.4	21 ± 0.1	20 ± 0.1
A. baumannii 9	223 ± 0.03	243 ± 0.02	213 ± 0.4

Table 6. Antibacterial activity of external extract on pathogenic Gram-negative bacteria (secondary screening).
Tabela 6. Atividade antibacteriana do extrato externo em bactérias Gram-negativas patogênicas (triagem secundária).

Bacteria isolates	Inhibition diameter ± SD (mm)				
	E. coli	*P .aeruginosa*	*K. Pneumoniae*	*S. typhia*	*A. hydrophila*
A. baumannii 2	16.5 ± 0.2	22.5 ± 0.03	17 ± 0.2	20.3 ± 0.6	20.8 ± 03
A. baumannii 3	20.3 ± 0.6	20.5 ± 0.3	23 ± 0.6	20.0 ± 0.4	21.0 ± 0.4
A. baumannii 5	22 ± 0.4	23 ± 0.1	23 ± 0.3	23 ± 0.1	23 ± 0.1
A. baumannii 7	15 ± 0.2	19,5 ± 0.1	20 ± 0.1	19.0 ± 0.4	18.3 ± 0.06
A. baumannii 9	22 ± 0.33	17.5 ± 0.05	15.2 ± 0.5	18 ± 0.3	15.0 ± 0.4

4. DISCUSSION

The most common bacteria were *A. baumannii*. The serious research point is that *A. baumannii* is found in the soil and milk factory units (HACKEL et al., 2015; THADEN et al., 2016; MCDANEL et al., 2017).

Acinetobacter baumannii is a Gram-negative opportunistic bacterium. Its ability to adapt to different environments, resist drought, and resist sterilizers increases the risk, as it can form biofilm.1 = Bacteria's ability to form biofilm is physiologically linked to their ability to produce the enzyme protease. The intracellular ATP-dependent Lon family of proteases is a group of evolutionarily conserved enzymes that specifically degrade misfolded proteins and several regulatory proteins (FU KG et al., 1997; DIERKSEN et al.,1994).

The current study's data showed a high infection rate in addition to the more prevalent contaminant environment sections of the factory room floor and wall (SHARMA et al., 2021). Gram-negative bacteria outnumbered Gram-positive bacteria in the current investigation. This outcome was consistent with previous research conducted in Malaysia, Brazil, and Morocco (NAJAFI et al., 2005; HANINA et al., 2015; BELEFQUIH et al., 2016).

Acinetobacter is almost exclusively isolated from hospital surroundings, even though it is frequently discovered in soil samples (which gives rise to the widespread misperception that *A. baumannii* is also a soil organism (Antunes et al., 2014). Though sporadic finds in environmental soil and water samples have been reported (Yeom et al., 2013), its native habitat remains unknown.

St. aureus causes a variety of infections. It is believed that the coordinated action of many released toxins, digestive enzymes, and proteins causes S. aureus to be virulent. *S. aureus* can produce protease in any environment it finds (ARVIDSON, 2000).

S. aureus produces four major extracellular proteases: staphylococcal serine protease (V8 protease; SspA), cysteine protease (SspB), metalloprotease (aureolysin; Aur), and staphopain (Scp) (KARLSSON et al., 2002)

Studies conducted in vitro have demonstrated that staphylococcal proteases are capable of cleaving and degrading several significant host proteins, such as elastin, plasma proteinase inhibitors, and the heavy chains of all human immunoglobulin classes (POTEMPA et al., 1986; PROKESOVA et al., 1992).

The presence of staphylococci that produce the protease enzyme indicates their virulence and the possibility of contamination affecting production in the milk factory (KULLIK et al., 1989).

The *E. coli* genome encodes at least 74 peptidases/proteases. Protease is secreted to rid the Escherichia coli of excess extracellular protein that affects the growth and reproduction of bacteria. Most intracellular proteolysis is initiated by energy-dependent proteases, including Lon, ClpXP, and HflB; HflB is the only essential

(GOTTESMAN, 1996).

The ability of bacteria isolated from the soil and floors of milk factory units indicates the possibility of contamination occurring in the factory. Their great ability to produce protease results from the abundant presence of the basic substance in their environment.

5. CONCLUSIONS

Soil can contain many types of microbes. The soil of laboratories and factories is contaminated with some raw materials or factory products. The soil of the milk factory is contaminated with protein and some sugars. The soil in milk factories contains multiple types of bacteria that can produce various kinds of protease, including glycoprotein. Bacteria that produce enzymes are characterized by their ability to produce secondary metabolic substances that have biological activity as an antibacterial agent against other bacteria, whether pathogenic or environmental.

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Authors' contribution: Y.A.J.: conceptualization, methodology, formal analysis, investigation, data curation, writing - original draft, writing - review and editing; Z.M.J.: conceptualization, methodology, formal analysis, investigation, data curation, writing - original draft; S.H.R.: conceptualization, methodology, formal analysis, investigation, data curation, visualization; A.S.A.: conceptualization, methodology, formal analysis, investigation, data curation, visualization. The authors read and agreed to the published version of the manuscript.

Data availability: Study data can be obtained by e-mail from the corresponding author or the second author upon request. It is not available on the website as the research project is still under development.

Conflict of Interest: The authors declare no conflict of interest. Supporting entities had no role in the study's design; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.