ORIGINAL ARTICLE

Effect of the Phytase Enzyme Extracted from Saccharomyces Cerevisiae on Biofilm Formation by Pathogenic Bacteria

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ABSTRACT

Background: *Phytases are a large group of enzymes that hydrolyze phytate and its complexes. This most abundant organic phosphate in the world is commonly found in plant-based foods.* ***Objective***: *The study aimed to investigate the ability of S. cerevisiae. S. yeast extract to investigate the production of biofilm by bacterial isolates (E. coli and Staphylococcus aureus) and its relationship with the phytase enzyme.* ***Methodology***: *Staphylococcus aureus and Escherichia coli bacteria samples were obtained by isolation and identification from diarrhea samples. Congo Red Agar media and 96-well microplate titer method were used for biofilm detection. Phytase were extracted from Saccharomyces cerevisiae that found in local markets. Biofilm Genes expression (icaA and clfA gene for S. aureus) and (fimH and bcsA for E.coli) were studied.* ***Results***: *The study showed that the yeast-derived enzyme phytase has an inhibitory effect on biofilm formation in both Staphylococcus aureus and Escherichia coli. In S. aureus, phytase application resulted in a significant decrease in biofilm production and a decrease in the gene expression of both clfA and icaA. In E. coli, phytate exhibited a dose-dependent effect, with light values gradually decreasing with increasing concentration, accompanied by a significant decrease in the expression of fimH and bcsA genes. These results confirm the effectiveness of phytate as a natural biofilm inhibitor in both Gram-positive and Gram-negative bacteria.* ***Conclusion***: *The results showed that phytase, an enzyme extracted from yeast, has a clear ability to inhibit biofilm formation in both Staphylococcus aureus and E. coli, by reducing biofilm biomass and decreasing the expression of genes related to adhesion and extracellular matrix formation (clfA, icaA, fimH, and bcsA).*

INTRODUCTION

Saccharomyces cerevisiae, commonly known as baker's yeast, is a unicellular eukaryote that has been utilized for thousands of years in baking, brewing, and winemaking. This yeast is distinguished by its relatively simple genetic structure, containing approximately 6,000 genes across 16 chromosomes. Notably, it was the first eukaryotic organism to have its entire genome sequenced, making it a valuable model organism in scientific research[1].

S. cerevisiae reproduces through both asexual budding and sexual reproduction, allowing for genetic diversity. Researchers favor this yeast for its nonpathogenic nature, rapid growth rate, and the ability to investigate fundamental cellular processes, including cell division, gene expression, and protein interactions. Techniques such as auxotrophic markers and the two-hybrid system enhance its utility in genetic and biochemical studies [2]. Beyond its scientific relevance, S. cerevisiae plays a critical role in food and beverage production, serving as a key ingredient in leavening bread and fermenting alcoholic beverages [3].

Phytases are a large group of enzymes that hydrolyze phytate and its complexes. This most abundantorganic phosphate in the world is commonly found in plant-based foods. It can bind to essential minerals, making them less available for absorption. Enzymatic hydrolysis of phytates is the most beneficial method for reducing their content in foods and feeds[4]. Phytase supplementation enables more efficient utilization of phytate phosphorus. The enzyme is produced by prokaryotic and eukaryotic microorganisms, plants, and animals[5]. Several types of phytases, depending on certain structural and kinetic properties are described. Phytase activity is influenced by metal ions, surfactants, and various plant extracts[6]. Research has increased in recent years on the extraction of highly effective bioactives from S. cerevisiae yeast. Its metabolites can be used in medical and food applications due to their efficacy and activity[7]. Among them, mannoprotein is a compound with emulsifying properties and is therefore called Bioemulsifier activation, produced by yeast on the outer layer of the cell wall, is also known as a biosurfactant [8]. Biofilms are severe health concerns due to their multidrug

resistance abilities, host defense, and other stresses. Therefore, it leads to chronic bacterial infections worldwide, In addition to preventing infections and combating antibiotic resistance, preventing the creation of new biofilms or breaking up existing ones can enhance industrial processes, public health, and the operation of medical devices [9].

The fimH gene expresses the FimH protein, an adhesin protein. This adhesion paves the way for biofilm formation [9]. The bcsA gene is part of the gene cluster responsible for bacterial cellulose synthesis in E. coli[10]. The clfa and icaA genes are important genes in Staphylococcus bacteria. The clfa gene encodes a surface protein that acts as a major adhesion factor for bacteria [11]. The icaA gene encodes an enzyme responsible for the first step in the synthesis of polysaccharide intercellular adhesin (PIA)[12].

The study aimed to investigate the ability of *S. cerevisiae. S. yeast* extract to investigate the production of biofilm by bacterial isolates (*E. coli _ Staphylococcus aureus*) and its relationship with the phytase enzyme.

METHODOLOGY

Bacterial sampls collection

Staphylococcus and *Escherichia coli* bacteria samples were obtained by isolation and identification from diarrhea samples after culturing them on blood, MacConkey, and mannitol salt agar.

Investigation of Biofilm Production Qualitatively and Quantitatively

Congo Red Agar media was inoculated with young, pure colonies of bacterial isolates and incubated at 37°C for 24 hours. The results were then observed. A change in the color of the medium and colonies indicated a positive result. also, biofilm was detected using the 96-well microplate titer method, as described[13].

Extraction and Production of Phytase from *S. cerevisiae 's* Yeast

The method described by Ipata and Cerignani[14] was adopted.

One sample of dried *S. cerevisia* yeast from a well-known brand, supplied by foreign companies to the local market, was taken in one replicate to measure specific activity and protein concentration during the extraction and precipitation phase. The yeast was found to be the most efficient and effective for completing the subsequent purification steps to obtain a pure enzyme for biological applications.

Preparing local *S. cerevisiae 's* yeast: Local yeast was prepared using traditional methods using only flour and water, without any additives. A specific amount of flour, approximately 25 grams, is placed in a glass bottle with a tight lid. The same amount of distilled water (25 ml) was added to the flour and mixed well. The container was covered, leaving a small opening for air to enter for respiration. The mixture is left for 24 hours at room temperature (25°C). On the second day, 25 grams of flour and water were added, mixed, and left under the same conditions. This addition is important because the yeast needs nutrition to grow properly. This process is repeated for five days. Many bubbles are observed as a result of gas exchange, a clear indication of yeast formation in the dough. Additionally, the odor of the mixture changes to a smell resembling vinegar or acidic solutions, indicating the formation of methane gas. On the fifth day, after all the additions are complete, the container is tightly closed and placed in the refrigerator at 4°C for future use. The yeast is fed weekly to ensure its continuity.

Toluene solvent

Distilled water

Phytase enzyme extraction and production

100 g of prepared baker's yeast was weighed and mixed with 50 ml of organic toluene solvent. The mixture was placed in a water bath at 45°C for one hour. Leaved the mixture at room temperature at 25°C for 2-3 hours. Then, 100 ml of cold distilled water were added. The mixture was placed in a glass separating funnel and shake well for half an hour. Then, incubated at 4°C for 18 hours. The mixture was filtered, the separated aqueous phase was collected and centrifuged it in a refrigerated centrifuge at 10,000 rpm at 4°C for 20 minutes. The filtrate was taken and the precipitate discarded.

Measuring the activity of the phytase enzyme from baker's yeast

0.2 ml of the phytase crude extract was added to 0.3 ml of the basic buffer solution and 0.5 ml of the phytase solution. The final reaction volume was 1 ml. The mixture was left in an incubator for half an hour at 37°C. After the incubation period, the reaction was stopped by adding 4 ml of hydrochloric acid (HCl). The mixture was placed in a spectrophotometer at a wavelength of 286 nm, and readings were taken. The enzyme activity (unit/ml) was determined according to the following formula:

$$\text{Enzymatic activity (unit/ml)} = \frac{\text{Total volume of reaction medium x Absorbance}}{\text{Volume of enzyme added to the reaction x Time x Enzymatic coefficient}}$$

Investigating the effectiveness of *S. cerevisiae* yeast extract in neutralizing genes for biofilm production

After phenotypic screening of the bacterial isolates under study and their ability to produce biofilm, and after confirming their production of biofilm, the bacterial isolates were treated with *S. cerevisiae* yeast extract (phytase enzyme at concentrations of 100%, 75%, 50% and 25%, respectively, after culturing them on the aforementioned virulence factor media. The extract was then added to the virulence media. Phenotypic detection of biofilm was performed after treatment with the extract. The same bacterial isolates

that phenotypically produced biofilm before treatment with the yeast extract and those that did not produce biofilm after treatment were selected. The genes responsible for coding for biofilm production in these isolates were studied, which demonstrated an inability to produce the specific biofilm under study after treatment with the yeast extract, were then molecularly identified, and their ability to produce these factors was determined.

Detection of biofilm related genes by molecular techniques

Real time PCR (RT-PCR)

RNA Extraction from bacterial isolates

The RNA from *S. aureus* and *E. coli* cells was isolated by using genezol triRNA pure kit (Gene aid/Thailand). The isolates were cultured on microtiterplate, as mentioned before, for the purpose of having biofilm cells. Methanol was removed from the plates by washing with distilled water to remove all cells not adhering to the wells. Subsequently, biofilm cells were re-suspended in cold sterile normal saline by flushing the wells with this saline by using a pipette until no visible biofilm was left on the glass surface. Then, bacterial cells were transferred to a 1.5 ml microcentrifuge tubes and centrifuged for two minutes at14000g, followed by complete discard of the supernatant. RNA isolation from this lysed preparation was performed by following the instructions of the manufacturer (GENZOL TriRNA Pure Kit).

Complementary DNA (cDNA) synthesis

The cDNAs were used for the quantification of mRNA levels of biofilm encoding genes by utilizing the qRT-PCR, according to RT master mix (Hisenscript TMRH RT premix kit); 15µl of nuclease-free water was transferred to a specific tube of the kit, then 5 µl of total RNA was added. The mixture was mixed by vortexing, followed by brief centrifugation. The cycling protocol included 1 hour at 50°C for reverse transcription followed by 10 minutes at 85°Cfor RTase inactivation. The samples with synthesized cDNA were stored at -20°C until use.

Table 1: Primers used for real-time PCR for *S. aureus* and *E. coli* [15,16]

GENE (ORGANISM)	GENBANK ACCESS NUMBER	PRIMER SEQUENCE	TM (°C)	PCR PRODUCT SIZE (BP)
ICAA (S. AUREUS)	AF086783	F: 5′-GAGGTAAAGCCAACGCACTC-3′ R: 5′-CCTGTAACCGCACCAAGTTT-3′	60	151
CLFA (S. AUREUS)	Z18852.1	F: 5′-ACCCAGGTTCAGATTCTGGCAGCG-3′ R: 5′-TCGCTGAGTCGGAATCGCTTGCT-3′	60	161
16S RRNA (S. AUREUS)	L37597.1	F: 5′-GGGACCCGCACAAGCGGTGG-3′ R: 5′-GGGTTGCGCTCGTTGCGGGA-3′	60	191
FIMH (E. COLI)	GQ487191.1	F: 5′-CGCCAATGGTACCGCAATCCCTA-3′ R: 5′-CACGGCAATTAATGAGCCAGCT-3′	61.5	370
16S RRNA (E. COLI)	X80724.1	F: 5′-TCCTACGGGAGGCAGCAGT-3′ R: 5′-GACTACCAGGGTATCTAATCCTGTT-3′	60.8	466

Quantitative PCR protocol

First step of Quantitative PCR includes Initial denaturation for 3 minutes at 95C, followed by 40 cycles of denaturation (20 sec at 95C), annealing (20 sec at 55C) and extension (20 sec at 72C). the last step was melt curve at 55-95C.

RESULTS

Biofilm production in *Staph. aureus*

The figure (1) results showed that *Staph. aureus* produced the most biofilms in the control group, whereas the addition of phytase, an enzyme derived from yeast, at varying dosages resulted in a notable reduction. The intensity of biofilm development decresed at 25%(P=0.038) and 50%(P=0.001) concentrations in comparison to the control, suggesting that phytase inhibits the bacteria's capacity to produce biofilms.

The reduction in biofilm formation persisted at higher concentrations (75% and 100%) in comparison to the control group; however, the differences were not statistically significant when compared to the 50% concentration.

DISCUSSION

This might be an example of a saturation effect, which occurs when an enzyme's concentration rises above a particular point without causing more inhibition. This could be explained by the fact that phytase inhibits the capacity of bacterial cells to attach and aggregate by affecting essential elements involved in cell adhesion or the production of the extracellular matrix (EPS), which serves as the primary structure of

biofilms[17]. These findings are in line with earlier research that demonstrated that using enzymes with degradative activity for chemicals associated to adhesion or matrix might reduce the production of biofilms and make bacteria more susceptible to antibiotics[18]. Thus, phytase can be viewed as a promising component of biological approaches meant to counteract the biofilm linked to S. aureus infection [19].

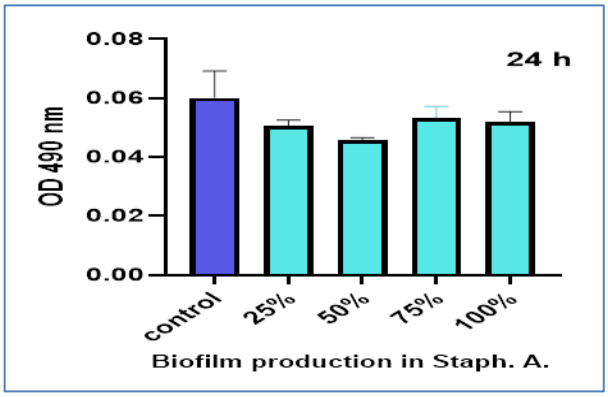

Fig. 1: Biofilm production in *Staph. aureus*

clfa gene expression

The results shown in Figure 2 showed the effect of different concentrations of the Phytase enzyme on the gene expression of the clfa gene, as it was shown that gene expression decreased as the enzyme concentration increased, which confirms the effectiveness of the enzyme at high concentrations (75%, P=0.017 and 100%, p=0.001) against biofilm gene expression in bacteria.

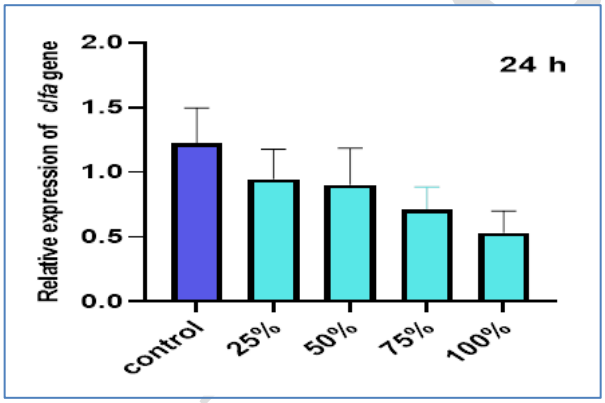

Fig. 2: Relative expression of clfa gene in *S. aureus*

icaA gene expression

The results shown in Figure 3 showed the effect of different concentrations of the Phytase enzyme on the gene expression of the icaA gene, as it was shown that gene expression decreased as the enzyme concentration increased, which confirms the effectiveness of the phytase enzyme at high concentrations against biofilm genes expression in bacteria.

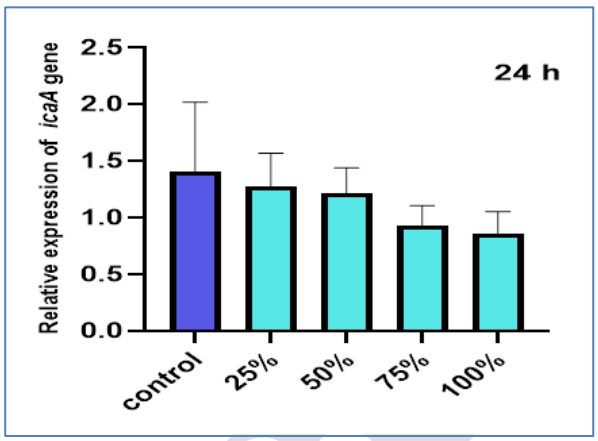

Fig. 3: Relative expression of icaA gene in *S. aureus*

Biofilm production in *E. coli*

The results showed that the yeast-derived enzyme phytase has an inhibitory effect on biofilm formation in *E. coli*. Photometric values (OD 490 nm) gradually decreased with increasing concentration compared to the control group. The inhibition was more pronounced at higher concentrations (75%, p=0.013 and 100%, p=0.002), indicating that phytase acts through a dose-dependent inhibition mechanism, figure (4).

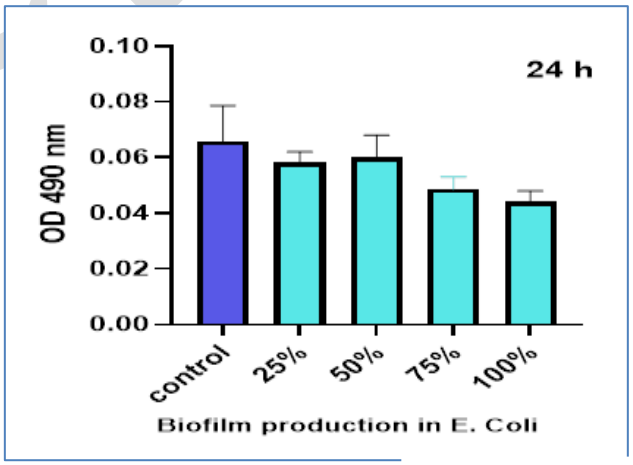

Fig. 4: Biofilm production

fimH gene expression

The results shown in Figure 5 showed the effect of different concentrations of the Phytase enzyme on the gene expression of the fimH gene, as it was shown that gene expression decreased as the enzyme concentration increased, which confirms the effectiveness of the phytase enzyme at high concentrations (70% and 100%, p<0.001) against biofilm genes expression in *E.coli.*

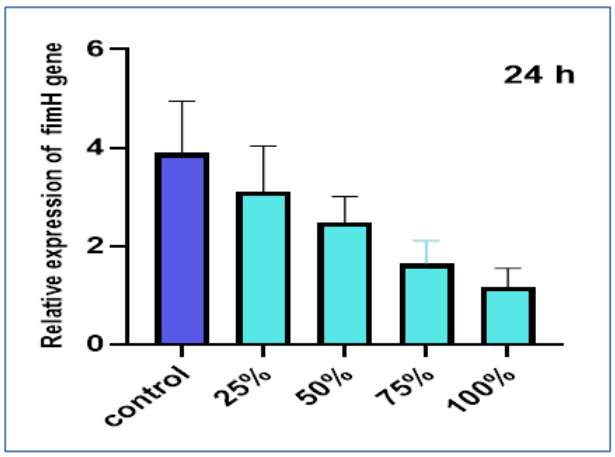

Fig. 5: Relative expression of fimH gene in *E. coli*

bcsA gene expression

The results shown in Figure 6 showed the effect of different concentrations of the Phytase enzyme on the gene expression of the bcsA gene, as it was shown that gene expression decreased as the enzyme concentration increased, which confirms the effectiveness of the phytase enzyme at high concentrations against biofilm genes expression in *E.coli*.

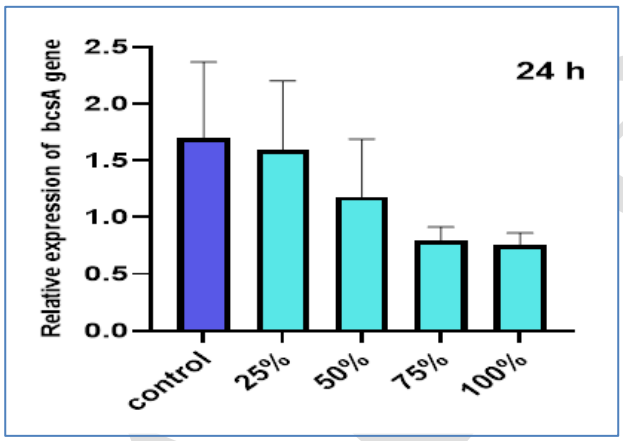

Fig. 6: Relative expression of bcsA gene in *E.coli*

DISCUSSION

The bacterial isolates studied responded better to high concentrations of *S. cerevisiae* yeast extract. Stronger effect with increased concentration. The 100% concentration worked for all isolates, unlike the others. This suggests that baker's yeast extract was hypertonic at high quantities but ineffective at low concentrations. Due to bacterial cellular structure and yeast extract composition differences. Despite host defences, pathogenic bacteria can penetrate human and animal organs by acquiring virulence factors, adhesion, and replication on host cells and tissues[20]. Mannose-containing glycoprotein residues in yeast cell walls can bind pathogenic bacteria expressing type 1 fimbriae, preventing them from penetrating the host[21].

Anna et al.[21] found that adding baker's yeast extract to bacteria inhibits growth and that mannoproteins from *S. cerevisiae* inhibit *E. coli*, *P. aureoginosa*, and *P. mirabilis* growth. They also support Saleh [22], who found that partially and fully pure S. cerevisiae mannoproteins affect selected bacterial isolates. Gel-filtered yeast mannoprotein inhibited eleven bacterial isolates, including *E. coli*, *P. aureoginosa*, *K. pneumoniae*, and *Staphylococcus aureus*. Rasheed and Haydar[23] found baker's yeast extract to be antibacterial, antiadhesive, and antibiofilm against several pathogenic microorganisms. They found that yeast extract inhibited *P. aerugenos* and *Staph aureus*. This shows that *S. cerevisiae* yeast extract inhibits harmful microorganisms. They also support Ghad]=//a et al.[24], who found that *S. cerevisiae* mannoprotein inhibited *Staphylococcus aureus* and *E. coli*. The current study confirms Elzbieta et al.[25], who found that *S. cerevisiae* mannoprotein inhibits *Staphylococcus aureus*. Mannoprotein speeds up the separation of mature *staphylococcal* biofilms generated under optimal conditions. Mannoprotein's anti-biofilm impact is due to its hydrophobic cell surface effect. In contrast, Walencka et al.[26] found no direct antibiotic action of *S. cerevisiae* mannoprotein against *Staph aureus* and *Staph epidermidis* cells. Mannoprotein from yeast reduced staphylococcal deposition and biofilm development and accelerated biofilm removal. The current study found that *S. cerevisiae* yeast extract inhibits pathogenic bacteria and limits undesired bacteria development.

Natural medicines and baker's yeast extracts can treat infections instead of antibiotics, which are no longer effective due to bacterial resistance [27]. Kadhem et al.[28] found that the yeast extract inhibits *E. coli* and *Staphylococcus aureus*.

The study found that the *S.cerevisiae* yeast extract encapsulates pathogenic bacteria, completely precipitates their DNA, and prevents the primer from completing the reaction by penetrating their cell membranes, causing ions to flow and cell death. Due to growth inhibition, the gene responsible for the biofilm virulence factor was lacking after treatment, preventing the bacteria from growing, adhering, and infiltrating the body and destroying it. The study's findings supported Hayder and Rasheed[23], who found that baker's yeast extract inhibits bacteria growth by preventing biofilm production.

Baker's yeast extract, produced by living organisms, reduced bacterium growth and virulence factor synthesis. Since the extract inhibits bacterial development, it can be used instead of antibiotics to kill infections from various sources. The yeast extract entirely encapsulates bacteria, preventing pathogenicity. The results of the study were consistent with Sharma and Saharan29, who found that the extract inhibits

pathogenic bacteria from producing virulence factors by affecting the cell membrane, which protects and sustains bacteria.

From the above, it was concluded that the *S. cerevisiae* yeast extract inhibited bacterial growth and precipitated bacterial DNA, preventing it from producing the virulence factors that allow bacteria to resist antibiotics and survive. Because precipitation of bacterial DNA hindered primer binding and interaction, the genes responsible for generating virulence factors did not present following treatment with baker's yeast extract. Thus, molecular detection did not reveal the genes. The treatment eliminated these genes, as desired. Due to bacterial growth suppression, yeast extract inhibits virulence factor synthesis. The work confirms Santovito and colleagues'[30] finding that yeast extract limits bacterial growth. Lactose utilisation and hydrocarbon breakdown are genes on bacterial plasmids for antibiotic, metal, and catabolic pathway resistance. Eliminating bacterial plasmids and identifying the antibiotic resistance mediator is possible. *S. cerevisiae* yeast extract neutralises and inhibits bacterial genes. Virulence factors relax the plasmid DNA and raise its melting point because interfering factors break the super-helical shape of the DNA, forming an open circular or linear shape that prevents bacteria from expressing the gene for virulence factors due to the *S.cerevisiae* yeast extract's gene neutralization[31].

Treating plasmids with inhibitory chemicals stops *E. coli* from encoding virulence genes. This is consistent with Liu and his group[32], who found that treating plasmids with chemicals or physical substances neutralises bacteria.

Phytase may degrade extracellular matrix (EPS) molecular bonds or lower biofilm cohesion-essential elements. Al-Madboly et al.[33] found that bioactive enzymes including DNase and protease break down matrix components and reduce bacterial cell cohesiveness, supporting the current investigation. Sharma et al.[34]found that microbial products and bioactive enzymes inhibit biofilm-regulating gene expression, making phytate a better treatment or prevention for persistent, antibiotic-resistant bacterial infections. Based on these findings, phytate may be a promising antibiotic enhancer or alternative treatment for *E. coli* biofilm infections.

CONCLUSION

The results showed that phytase, an enzyme extracted from yeast, has a clear ability to inhibit biofilm formation in both *Staphylococcus aureus* and *E. coli*, by reducing biofilm biomass and decreasing the expression of genes related to adhesion and extracellular matrix formation (clfA, icaA, fimH, and bcsA). These findings suggest that phytase is a promising natural antibiofilm agent that could contribute to reducing bacterial resistance to antibiotics and improving the efficacy of conventional treatments.

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Ethical Considerations: Ethical approval for this study was obtained from the Scientific Research Ethics Committee, Tikrit University, Iraq.

Conflict of Interest: The authors do not have financial competing interests

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