PURIFICATION, CHARACTERIZATION AND EVALUATION OF BIOLOGICAL ACTIVITY OF MANNOPROTEIN PRODUCED FROM *SACCHAROMYCES CEREVISIAE* BY

H. GH. Rasheed N. H. Haydar
Researcher Prof.
Dept. of Biotechnology. College of Science University of Baghdad-Iraq
Email:h.g.biotechno@gmail.com

ABSTRACT

The current study was aimed to evaluate the biological activity of mannoprotein produce from local isolate *S. cerevisiae BY*. The results of purified mannoprotein using gel filtration chromatography revealed higher ratio of protein to mannose reached to 87.1%. FTIR indicated the presence of C-H and O-H bands could be due to the protein and carbohydrate components, respectively.C=N band due to acylated amino sugars frequently found in glycoproteins, HPLC indicated the presence of mannoprotein to 12 kinds of amino acid.Antibacterial, anti-adhesion and anti-biofilm activities of mannoprotein were evaluated against some pathogenic bacteria. Mannoprotein showed slightly inhibition zones ranged (10to14mm) and(9to 13mm) against *P.aeruginosa* and *S.aureus* respectively at concentration of mannoprotein from 50 to 200 mg/ml.The results showed higher anti-adhesive property of mannoprorein against *P.aeruginosa* (51.1%) and slightly antiadhesion against*S. aureus* 19% at 200 mg / ml of mannoprotein. Results of the anti-biofilm activity rises as the concentration of mannoprotein increases and reached to 51.8% against *P. aeruginosa* while slightly anti biofilm (19.7%) against S. *aureus*, at concentration of 200 mg/ml. The synergistic effect of mannoprotein with some antibiotics was studied.The combination of mannoprotein and antibiotics (Tetracycline, Gentamycin, Ampicillin and Ciprofloxacin)(1:1) at concentration 1mg/ml were studied. Results showed enhancement 26, 29, 33 and 36% of inhibition zone against *P. aeruginosa* while, enhancement was 20, 33, 40, and 14 % for *S. aureus*, respectively. The results demonstrated that mannoprotein possess antimicrobial activity in nature. Hence if co-administrated with another inhibitory agent, the mixture reduces the survival of pathogens.

KEYWORDS: biosurfactant,gel filtration, antimicrobial, antiadhesive.

تنقية, تشخيص وتقييم الفعالية البايولوجية للبروتين السكري المنتج من خميرة الخبز *Saccharomyces cerevisiae* BY

هند غازي رشيد ناظم حسن حيدر
باحث أستاذ
قسم التقنيات الاحيائية–كلية العلوم–جامعة بغداد

المستخلص

هدف الدراسة الحالية تقييم الفعالية الحيوية للبروتين السكري المنتج من العزلة المحلية.*S.cerevisiae*BYاظهرت نتائج التنقية باستخدام كروماتوكرافيا الترشيح الهلامي نسبة عالية للبروتين بالمقارنة مع سكرالمانوز حيث بلغت87.1 %.أشار FTIR إلى وجود مجاميعC–HوO–H مما يدل على وجود البروتين والسكر على التوالي في تركيب المستحلب. ظهور مجموعة C = Nيدل على الأرجح وجود السكريات الأمينية الأسيلية الموجودة بشكل متكرر في البروتينات السكرية.اظهر نتائج HPLC وجود 12 نوعًا من الاحماض الامنية المكونة للبروتين السكري.تم تقييم نشاط البروتين السكري كمضاد للبكتريا ومضاد الالتصاق ومضاد للغشاء الحيوي ضد بعض البكتيريا المرضية. أظهرت النتائج أقطار تثبيط تراوحت من (10 إلى 14 ملم)و (9 إلى 13 ملم) ضد *P.aerugenos*و*S. aureus*على التوالي بتركيزمانوبروتين يتراوح من 50 إلى 200 ملغم/مل.اظهرت نتائج فعاية عالية ضد الالتصاق للبروتين السكري ضد بكتريا *P. aeruginosa*بلغت 51.1 % وفعالية اقل ضد بكتريا*aureus* .S عند تركيز 200 ملغم/لتر.اظهرت النتائج بوضوح بان الفعاية ضد تكوين الغشاء الحيوي ازدادت بزيادة تركيز البروتين السكري وبلغت 51.8 % ضد بكتريا *P.aeruginosa* وبفعالية اقل بلغت 19.7 % ضد بكتريا*S. aureus* عند التركيز 200 ملغم/لتر من البروتين السكري.تم دراسة التأثيرالتآزري للبروتين السكري مع بعض المضادات الحيوية القياسية (تتراسيكلين، جنتاميسين، أمبيسيلين وسيبروفلوكساسين)(1:1)وبتركيز1mg/ml.اظهرت النتائج زيادة في فعالية التثبيط بنسبة 33،29،26و36٪ضد بكتريا*P aeruginosa* بينما اظهرت فعالية اقل بلغت 33،20 ،40و14٪ ضد بكتريا *S.aureus*. هذا يدل على امتلاك البروتين السكري فعالية ضد الميكروبات في الطبيعة، وعند خلطه مع عامل مثبط آخرفإن هذا الخليط يقلل من بقاء الممرضات.

الكلمات الرئيسية: السكريات، بروتين سكري، مضاد للميكروبات، مضاد للالتصاق.

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INTRODUCTION

Biosurfactants can be defined as amphiphilic compounds that are made via microorganisms that have significant emulsifying and surface properties (35). Different micro-organisms create biosurfactants. Their qualities of interest include: penetrating and wetting actions, reducing surfaceand interfacial tension, spreading, hydrophobicity and hydrophilicity actions (24) They are classified primarily based upon their microbial origins and chemical compositions. Biosurfactants are essential biotechnological products having numerous applications in a variety of industries, including cosmetics, food, and pharmaceuticals (11). Also, biosurfactants have substantial benefits compared with the chemical surfactants concerning ecological safety, bio-degradability, manufacturing from renewable resources, and functionality under harsh environments (6) Antiviral, antifungal and antibacterial effects of biosurfactants have been reported. As a result, they can be used instead of standard antibiotics to fight a variety of food-borne pathogens (33). Biosurfactants are produced by yeasts and filamentous fungus along with bacteria. In addition, hydrophilic moiety which consist of amino acids or peptides for cations or anions; di-,mono-, or polysaccharides; and a hydrophobic moiety comprising the saturated and unsaturated fatty acids make up their structure. The capability of *Saccharomyces* to create biosurfactants has been shown in a number of investigations (36). Saccharomyces cerevisiae is widespread and commonly found in nature, is a eukaryotic unicellular microbe characterized by round to oval multilateral budding yeast cells and short.More specifically, it is a globular-shaped belonging to the fungi kingdom. The cell wall of *Saccharomyces cerevisiae* is an elastic structure that provides osmotic and physical protection and determines the shape of the cell. The cell wall of *Saccharomyces cerevisiae* is an elastic structure that provides physical protection. The chemical composition regarding Saccharomyces biosurfactants has been investigate using various yeast isolates: the *Saccharomyces cerevisiae* derived biosurfactant is primarily composed of sugar and protein fractions; biosurfactants isolated from many Saccharo-myces have been categorized as multi-component mixtures containing polysaccharides and protein, while in other instance glycoprotein has been referred to as surface active compounds (14). Secondary metabolites, also known as microbial surfactants, play a vital role in the survival of microorganisms that produce biosurfactants through enhancing nutrition transport, microbe-host interactions, or acting as biocide agents (19,20), as well as biofilm development (10). The goal of this work is to investigate the generation, characterization, and biological activity of mannoprotein generated via *S. cerevisiae.*

MATERIALS AND METHODS

YEAST ISOLATION AND SAMPLE COLLECTION

Eleven samples from various sources were collected from (dry instant yeast, dairy products and fermented fruits. One gm of fermented fruits, dry instant yeast and one ml of dairy products were added to 9 ml 1% peptone water and mixed vigorously for 30 min at 30 °C. Then, the suspension was serially diluted up to 10^{-7} with peptone water1 %. A 0.1ml aliquot from the 10-4, 10-5, and 10-6 dilutions was placed to YPD agar plates and incubated at 30 °C for 48 hrs. The isolates have been purified by sub culturing on YPD agar (18), then the purified colonies were maintaining on the same media until using in the remaining studies.

identification of *S.cerevisiae* morphological and biochemical test:

In the current study, *S.cerevisiae* were primary identified according to the morphological tests includes, shape of colonies, size, and texture of colonies.Biochemical tests include ester production, urease and carbon source fermentation tests (13).

Screening of S.cerevisiae isolates for biosurfactant production:

S. cerevisiae isolates have been cultured in 50 mL YPD broth at 30 °C for 48 hrs. At the end of the experiments (48h), 10 ml of culture were centrifuged for dry biomass estimation. For the purpose of solubilizing the cell wall mannoprotein, cells have been harvested via centrifugation (10000, 5 min, 4 °C), washed two times demineralized water, suspended in 20ml of the phosphate buffered

saline (PBS:10Mm KH_2PO_4 and 150Mm NaCl with the level of the pH adjusted to 7) and heated for 5 min. The yeast cells were then centrifuged at (10000 rpm, 5 mins, 4 °C) and the rest of the supernatant has been tested for surface tension, Emulsification activity (E24%), and carbohydrate concentration (36).

Biosurfactant analysis using Emulsification Index (E24%):

Cell-free supernatant of 2ml was combined with toluene of 2ml in a vortex for a period of 2 mins, after that left at room temperature for 24 hrs. At 25 °C, the emulsifier layer's height was calculated. Also, the emulsification index (E24) was calculated by dividing the emulsion layer height by total height after 24 hours and multiplying by 100. The E24 is used to characterize the biosurfactant/Bioemusifier in emulsifying the hydrophobic phase in the hydrophilic phase (23).

Emulsion Index (E24) % = Height of emulsion layer /Total height of liquid × 100

Surface Tension (ST) Assay

The surface tension (ST) related to the aqueous solution was measured by the Wilhelm platinum plate with a QBZY-2 Tensiometer (China). Fifteen ml of supernatant has been placed on tensiometer platform and poured into 50 ml glass beaker. The measurement was conducted at 25±1℃ after dipping the plate in the solution, until monitoring the value of supernatant ST following the procedure of measurement written in the manual of the instrument. Between each one of the measurements, the Wilhelm plate has been rinsed with acetone and burned by alcohol burner to ensure no contaminant affect the recorded results.In addition to the standard weight of the instrument, ethanol (22 mN/m) and distilled-water (72 mN/m), were used for calibration. For more accurate value, the average of three records was used in the study (31).

Determining the carbohydrate concentration Carbohydrate concentration (mannose) has been determined with the use of Dubois approach 1956 (12) in the following way: 1ml of biosurfactant was mixed with 1 ml of Phenol (5%) with continuous shaking for 2-3 minutes, after that a concentrated sulfuric acid of 5 ml has been gradually added at the tube wall with shaking and directly placed in an ice bath for a period of (10-20) minutes, after that the absorbance has been measured at (490) nm with the use of a spectrophotometer. As shown in the Figure (1), a standard curve of mannose has been created utilizing various concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml). Each was pipetted in a sterile test tube in duplicate, and the absorbance was plotted against the corresponding mannose concentration.

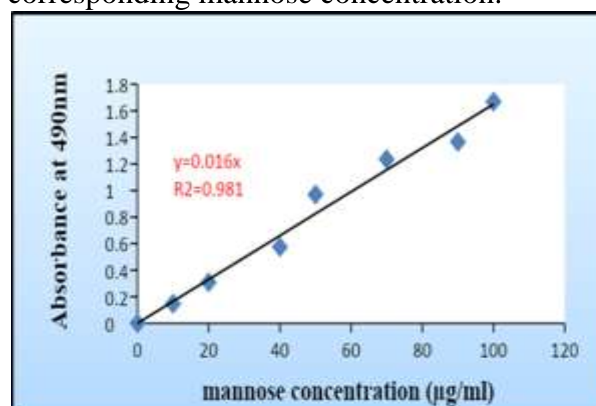

Fig.1. A standard curve of Mannose for determining the carbohydrate concentration.

Determining of protein concentration

The Bradford technique of 1976 (7) was used to determine protein concentration: with continuous shaking for a period of (2-3) min, biosurfactant (purified) of (20) µl was mixed with NaOH of (5o) µl was used to ensure soluble of mannoprotein in the color reagent and to allow the solubilization of membrane proteins and reduce the protein-to-protein variation in color yield , after that (1) ml of Coomassie Brilliant Blue G-250 has been added with shaking. After (2) min., the absorbance has been measured with the use of a spectrophotometer at 595nm against a reagent blank. Through preparing serial concentrations (0.1-1.0 mg/ml) from BSA stock solution, a standard curve of bovine serum albumin was created, as shown in (Figure 2). Each was pipetted in a sterile test tube in duplicate, and the absorbency was plotted versus the concentration of bovine serum albumin.

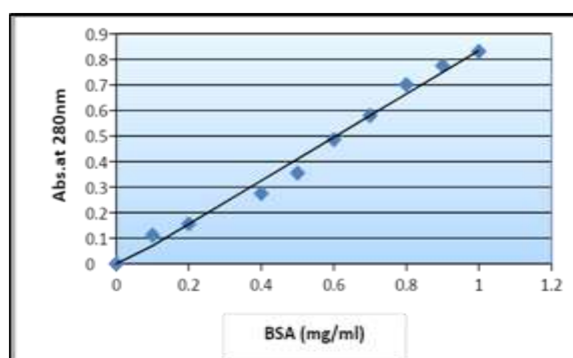

Figure 2. BSA standard curve performed via Bradford approach

Determination of Dry-weight

At the incubation period's end, culture of 10 ml was centrifuged for 15 mints at 8000 rpm to remove yeast cells. The collected cells were washed with phosphate buffer and allowed to dryness in oven (80°C) to obtain a constant dry weight, which is reported in terms of g/L (8).

Extraction of biosurfactant

The culture of *S. cerevisiae* collected after 72 h of incubation for biosurfactant production (3), at the end of the experiments, 10 ml of culture broth were centrifuged for dry biomass estimation. Cells have been also extracted using centrifugation (10000, 15min, 4 C). The yeast cells were washed two times in demineralized water and suspended in 20ml phosphate buffered saline (PBS: 10mM KH2PO4 and 150mM NaCl with pH adjusted to 7) and heated for 5mins for solubilizing the cell wall mannoprotein before centrifugation at (10000 rpm, 5 min, 4 C).The biosurfactant-containing supernatant has been transferred to a separation funnel as well as extracted with a variety of solvent systems, including a 1:2 mixture of methanol and chloroform, butanol, acetone, and ethanol (36). The aqueous layer at the bottom of separation funnel was removed, and the emulsion layer has been collected in a pre-weighted glass Petri dish and dried at (40°–45^oC) until powdered. The resulting powder was weighted and calculated to find the best solvent system for extraction of remaining biosurfactant in the supernatant. After drying the powder was stored in a clean vial at 4 ˚C for remaining research (23).

CHARACTERIZATION OF BIO-SURFACTANT FROM *S. cerevisiae*

Fourier transform infrared (FTIR) spectrum

To demonstrate the functional groups of the bioactive compound within the biosurfactant, the pellet was analyzed by FTIR. The functional groups and chemical bonds (post purification) were detected by mixing 1mg from biosurfactant with 100 mg of potassium bromide (KBr) matrix and pressed to form a pellet that is then analysed . The spectrum was limited at the range of 400-4000 cm^{-1} with resolution of 4 cm^{-1} (Ragavendran *et al*., 2011) (29).The experiment was done in the department of Chemical / College of science / University of Baghdad.

Biosurfactant analysis (HPLC):

The Rachana and Shalini approach was used to analyze the sample. (32). Biosurfactant was analyzed to their amino acids utilizing the High Performance Liquid Chromatography (HPLC). the system utilized in this investigation was a SYKAM- Amino acid analyzer as follows: 20ml of purified biosurfactant mixed with 20ml of Ortho Phthal aldehyde (OPA) act as a reagent in the analysis of amino acids and 10 ml of D.W was added with mixing ,then 20µl from mixed was injected into the sample loop, with the following running conditions: column type: C18, column dimension: 25×4.6mm, mobile phase: mix of buffer, flow rate:1.2 ml/min, and detection at 340 nm. Before injection into the sample loop, the samples have been filtered via an ultra-membrane filter (pore size 0.45m). The number of amino acids that have been utilized as standards. Retention times from authentic standards operated under identical conditions were used for determining the standards and sample.

Purification of biosurfactants using Gel filtration chromatography technique

A gel filtration employing Sephadex-G150 was used to further purify the biosurfactant. After partial purification with solvents, the biosurfactant was passed through a gel filtration column and eluted with 0.1 M phosphate buffer saline solution, pH 7.2, at a flow rate of 20 ml/h, 3 ml for each fraction. At wavelengths of 490 and 280 nm, the carbohydrate and protein fractions in each one of the fractions were assessed, and the activity regarding such fractions was evaluated. Furthermore, the effective fractions have been collected for concentration with the use of sucrose at 4 ˚C and distributed in tubes,

which were then frozen till used in following experiments. (26)

Pathogenic microorganisms used in the antibacterial activity: The pathogenic bacteria used in the current study were isolated from clinical samples obtained from Baghdad University, College of Science, Department of Biotechnology. The indicator bacteria used were *Pseudomonas aeruginosa* (isolated from burn) and *Staphylococcus aureus* (isolated from skin). Maintenance of pathogenic bacterial isolates were achieved by streaking on nutrient agar and subjected to incubation for 24 hrs at 37 ºC. The cultures were stored at 4ºC and then recultured every three weeks' interval time.

Determination of antibacterial activity of biosurfactant

The biosurfactant's antibacterial activity was determined against *P. aeruginosa and S. aureus,* using paper disc diffusion method (27,34) .Overnight growth (24h) culture of the test bacterium were adjusted to (1x10^8cfu/ml) equivalent to (OD= 0.5 on McFarland) were streaked on sterile Muller Hinton agar surface. Six millimeters diameter of Whatman filter paper discs (GF/C) were prepared by scissors and sterilized in a Petri dish at 121˚C for 15 min. After sterilization, each disc was impregnated with 100µl of different concentration of biosurfactant (50, 75,100, 150, and 200 mg/l). Then the discs were put on the surface of cultured plate with pathogenic bacteria separately. Phosphate buffer saline (PBS) has been used as a control, because it has no antimicrobial activity. Then,the plates are subjected to incubation for 24hr at 37˚C. Following the incubation, the diameter of inhibition zone has been assessed using electronic ruler in mm.

Determination of antiadhesive activity of produced biosurfactant

The anti-adhesive activity regarding the purified bio-surfactant from *S. cerevisiae* against target pathogens was followed based on the approach indicated via (24). For a short time, the wells of sterile 96-well micro titer plates were coated with 200 µl of different concentration of biosurfactant ranging from (50, 75,100, 150, and 200 mg/l). The plate was subjected to incubation for 24 hrs at 37 ºC. The biosurfactant solution was subsequently drained and the plate rinsed twice with 100 µl PBS pH 7.2 to reduce biosurfactant that was not adhesive. The next step was the addition of 150 µl of a washed bacterial suspension in PBS, adjusting to 0.5 McFarland standard turbidity (a final density of 10^8 CFU ml-1) to individual wells. Thereafter, the microtiter plate was again subjected to 24-hour incubation at 37˚C. By gently rinsing the wells twice with PBS pH 7.2 no adhering cells were removed. Quantification was carried out using violet crystal assay. After that 100 µl of 99 % methanol was applied to each well, mixed for 15 min and the plate was then air-dried. In the next step, 100 µl of crystal violet 2 %was added and retained before removing the superfluous crystal violet by pipetting for 20 min, and the residue in the wells was rinsed with tap water. The stain associated with the adherent pathogens was solubilized with 100µl of 33% glacial acetic acid for each well and the optical density readings of individual wells were recorded at 595nm using micro-Elisa auto reader (Model 680, Bio-Rad). This was followed by the preparation of bacterial suspension with no biosurfactant, as control. The percentage of adherence reduction was computed with the formula described by (17).

% microbial inhibition = $[1- (Ac/A_0)] \times 100$

Where: **Ac** = is the optical density regarding the well with different biosurfactant concentrations and pathogen, and A_0represents the optical density of the pathogen suspension without biosurfactant (control).Also, triplicate assays were conducted and the mean of optical density was taken. The microtiter plate anti-adhesion assay allows for the determination of biosurfactant concentrations which are efficient in reducing microorganism adhesion.

Determination of Anti biofilm activity of produced biosurfactant

Mannoprotein has been added to the wells following the bacterial lture reached confluent mass (24 h) to see if it could remove a preformed biofilm. Incubation of biofilms with mannoprotein causes dispersal of biofilm clumps. (14) The anti-biofilm activity regarding the biosurfactant fractions against target pathogens has been followed depending on the method described by (14) as below:

1. Pathogenic bacteria have been grown at 37 ◦C for 24 h in TSB of 5 ml that is

supplemented with 0.25% -glucose (TSBGlc),150µl of bacterial suspension after adjusting to 0.5 McFarland standard turbidity (a final density of 10^8 CFU ml-1)1.5 x 10^8 bacteria / ml was added to each well of a sterile 96-well microtiter plate along with 200 µl of biosurfactant fraction solutions prepared in PBS at different concentrations (50,75,100,150, and 200 mg/ml).

2. Control wells contained PBS and bacterial suspension without biosurfactant solution. Free microorganisms have been eliminated by rinsing the wells two times with PBS pH 7.2.

3.The microorganisms still adhering were fixed with 200 µl of 99 % methanol per well. Also, the plates were emptied following 15 minutes and allowed to dry.

4. The next step involved staining the plates for 20 mins with 100 µl of 2 % crystal violet (utilized for Gram staining) per well. After that, the wells were washed three times with PBS pH 7.2 for eliminating excess stain.

5. The plates were air-dried followed by re-solubilizing of the dye bound to adherent microorganisms with 200 µl of 33 % (v/v) glacial acetic acid per well and the optical density of each well was recorded at 595 nm in micro Elisa auto reader (Model 680, Bio-Rad).

6. Anti-biofilm activity of biosurfactant fractions was determined based on the following **formula:**

Anti-biofilm activity (%) = $[1 - (OD_c / OD_0)] \times 100$

Where:

ODc denotes optical density of the well with a biosurfactant concentrations and pathogen;

OD_0 denotes optical density of the pathogen suspension with no biosurfactant (control). Triplicate assays were conducted and the mean of optical density was taken.

The synergistic action of biosurfactant with some Antibiotics To test the synergistic effect of biosurfactant and standard antibiotics were utilized. Standard antibiotic has been freshly made. A 0.1gm of various antibiotic include (Ampicillin, Ciprofloxacin, Gentamycin and Tetracycline) with different concentration (0.25, 0.75 and 1mg/ml) The overnight growth of *S. aureus* and *P. aeruginosa* on the surface of Muller-Hinton agar has been adjusted to 1x 10^8 cell/ml (OD= 0.5 on McFarland) and depends on critical micelle concentration (CMC) of mannoprotein (200mg/ml). By using agar well diffusion method. Wells (6mm in diameter) have been prepared in each one of the agar plates and one well filled with 100µl of different concentrations of antibiotic (0.25,0.75 and 1) mg/ml, and it was used as control. A 50µl of biosurfactant was mixed with 50µl of different concentrations of antibiotic, then the mixture was transferred to the well made on agar plate. The plates have been incubated at 37°C for 24 hours. It was decided to measure the inhibitory zone. All of the experiments were repeated twice (2).

RESULTS AND DISCUSSION

Isolation and identification of *S. cerevisae*

Eleven samples were collected from(dry instant yeast, dairy products and fermented fruits).The samples were primarily grown onto YPD agar plates as growth media for isolation and incubated for 48 hr at 30 °C. Morphologically, the yeast isolates were smooth, flat, moist, glistening or dull, and cream in color. Microscopically, the yeast appeared under oil immersion lens (100x) as oval or round cell, arranged as single and grouping, as shown in (Figure 3).

Figure 3. The growth of *S. cerevisiae* BY on YPD agar after 48h at 30°C.

Biochemical tests are an important in identification of *S. cerevisiae* from other yeasts, where *S. cerevisiae* use the carbohydrates as energy and carbon source. The yeasts isolates differed in the speed of fermentation to the carbohydrate through change the indicator color from yellow to red at the test period. The results showed that ability of *S. cerevisiae* to ferment of the carbohydrate sources which included; Glucose, Sucrose, Maltose, Fructose, except Lactose due to they lack the enzyme lactase, also It was produced ester odor. This result similar to result obtained by (13). While it cannot have hydrolyzed the urea because they

do not possess urease enzyme therefore the indicator color did not change (22).

Screening of yeast isolates for biosurfactant production: Eleven isolates of yeast which belong to the genus *S. cerevisiae* were selected for screening of biosurfactant production. Among eleven isolates were screened, isolate *S. cerevisiae* (BY) revealed higher biosurfactant production. The isolates *S. cerevisiae* (BY) has demonstrated maximum development of biosurfactants compared with other isolates. The result showed higher Emulsification activity E24% (52%) and reduction in surface tension (43.24 mN/m) and biomass (0.25 g/l) after 48 h of incubation as show in Figure (4). Therefore, the isolate BY was selected for remaining studies.

Figure 4. Emulsification index (E24%) of *S. cerevisiae* BY in Toluene.

Extraction of produced biosurfactant

Optimum conditions for biosurfactant production by isolates *S. cerevisiae BY* were utilized for biosurfactant production. The isolate was grown of in YP medium (pH 7) containing 3% mannose as carbon source and 1% of yeast extract and peptone (1:1) as nitrogen source at 30 °C, with shaking (120 rpm) for 72 h. After that, the cells were boiled in phosphate buffer saline for 5 min and centrifuged for 15 min in 10000 rpm at 4°C. Supernatant was extracted using different solvent system to obtain partially purified biosurfactant. Equal amount of supernatant of culture and solvents were kept in separating funnel overnight for evaporation and dry weight of biosurfactant was obtained. It's obvious from the results in Figure (5) that the extraction of solvent system methanol: chloroform (1:2) has the highest yield reached 1 g/l, as well as higher emulsification activity reached to 72 %, next to methanol: chloroform extraction, Ethanol extraction showed E24 % reached to 70%.

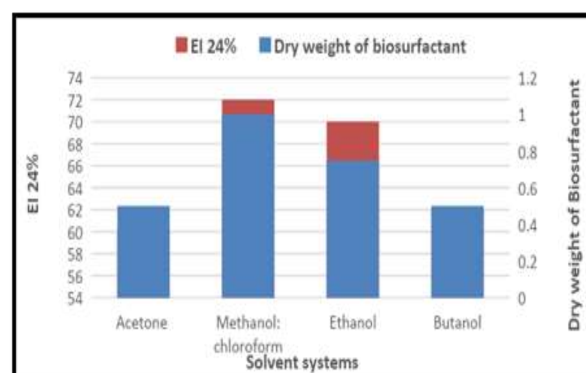

Figure 5. Extraction and emulsification activityof biosurfactant produced by *S. cerevisiae*

The yield of biosurfactant was highest using methanol: chloroform and formed white yellowish powder after drying Figure (6). Both ethanol and acetone precipitates formed a sticky, viscous, compact. In contrast, Virgie *et al,* 2010 observed highest emulsification index and biosurfactnt (mannoprotein) using acetone in extraction (36). Also Cold acetone was used for precipitation and for maximum recovery and purification of biosurfactant from three bacterial strains [*Bacillus* sp. m28, *Pseudomonas aeruginosa* PDKT-2, *Serratia marcescens* PDKT-1 and *B. licheniformis*PDKT-5 (28,30) respectively. However, the results in current study showed higher yield and recovery of biosurfactant using methanol: chloroform 1:2, therefore the solvent system is used for recovery of biosurfactan in the remaining supernatant.

Figure 6. biosurfactant extraction using Solvent system methanol: chloroform (1:2), after drying

Purification of biosurfactants using Gel filtration chromatography technique The white yellowish precipitate from methanol: chloroform precipitation was further purified using Sephadex G-150 gel filtration chromatography with the dimensions (32 x 1.5cm), and equilibrated with 0.1 M of phosphate buffer saline pH 7.2. Results in

(Figure 7) shows three active peaks, fractions 11 to 12 (33–36 mL), and fraction 25 (75 ml) with E24% ranging from 40 to 75%. The ratio of mannose to protein in the purified biosurfactant was approximately 12.9 %. The purification steps used to obtain the purified biosurfactant are summarized in Table 2. Similar results were observed by Nibras, (2018) for further purification of mannoprotein from yeast cell wall by gel filtration with the use of Sepharose-6B (26). Protein fractions from DEAE-cellulose have been pooled as well as passed via gel filtration column. In addition, the fractionation yielded 3 protein peaks at 280 nm and single peak as absorbance reading at 490 nm, with biosurfactant activity 83.18%. In contrast to results of our study, higher ratio of mannose to protein in the purified biosurfactant observed reached 60 %. Biochemical composition of the partially purified biosurfactant from *S. cerevisiae* 2031 revealed that it is composed of 74% protein and 21% carbohydrates; lipid was not present (36).

Table 1. Purification steps used to obtain the purified biosurfactant from *S. cerevisiae* BY

Purification step	Protein (mg/l)	Carbohydrate (mg/ml)	E 24%
- Crude extract by heat treatment	-	-	66.3
- Methanol: chloroform extraction	-	-	72
-SephadexG-150column chromatography	0.973	0.126	75

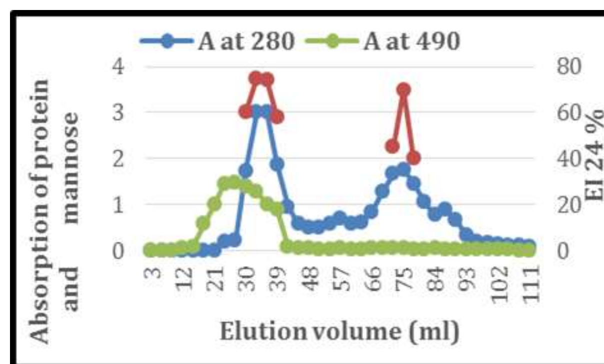

Figure 7. Gel filtration chromatography for mannoprotein purification from *S. cerevisiae* with the use of Sephadex –G150 column (32 x 1.5cm), eluted and equilibrated with phosphate buffer (0.1 M, pH 7.2), in flow rate 20 ml/h, 3ml for each fraction.

CHARACTERIZATION OF PRODUCED BIOSURFACTANT

FTIR Spectrum Analysis: The O-H valence vibrations appeared at 3390 cm-1 in FTIR examination of biosurfactant generated *via S. cerevisiae BY* (Figure 8). FTIR spectroscopy investigations have been used to determine the existence of mannoprotein. The C-H bond has been represented by the band at 2939.31 cm-1. The peaks at 1460.01 cm-1, 2893.02 cm-1, and 1633.59 cm-1 (C=N), reflect an aliphatic chain (CH3, -CH2-), indicating that mannoprotein is a glycoprotein. Because of the sugar contacts C=O, the sugar moiety structure is 1068.49 cm -1. The band at 865.98 cm-1 is extremely mannan-like; this matches Werner and Franziskus's findings (15). Virige *et.al,* found comparable results in their research (2010). They used FTIR spectroscopy to analyze molecular analyses and validated the existence of such macromolecules (36).

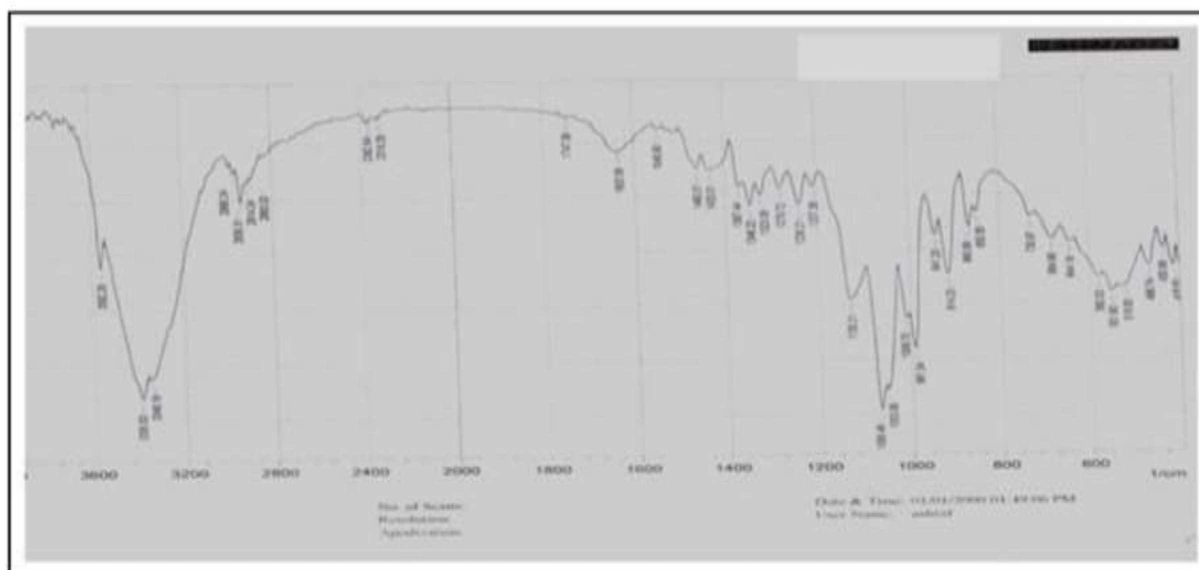

Figure 8:FTIR spectrums of the biosurfactant which has been produced by *S.cerevisiae.*

HPLC analysis of Produced Biosurfactant

In Figure 9, the results revealed the existence of 12 peaks in mannoprotein biosurfactant for various amino acids. Through comparing the retention times of the results to those of the authentic standards amino acids, the results were discovered. The ratios of cysteine, serine, and lysine were 14.6, 26, and 12.3%, respectively, with serine having a higher relative abundance of 100% compared to other amino acids as show in Table (2). Also, 2920 cm-1 (CH band: CH2-CH3 stretching), 3400 cm-1 (O-H band), and 1630 cm-1 (C=N) were the most relevans C-H bands. The carbohydrate and protein components may be responsible for the existence of O-H and C-H bands, respectively. C=N was most likely caused by acylated amino sugars, which are common in glycoproteins,as show in Figure (8). Mannoprotein has been identified as a glycoprotein based on its composition. The present findings were consistent with those of Liu *et.al.*, (2015), who found that mannoprotein had 17 different amino acids, with threonine, serine, and aspartic acid ratios of 20.08, 11.46, and 14.96%, respectively, which were greater compared to other amino acids(21). The results also were accordance with the study of Wang *et.al*, (37).

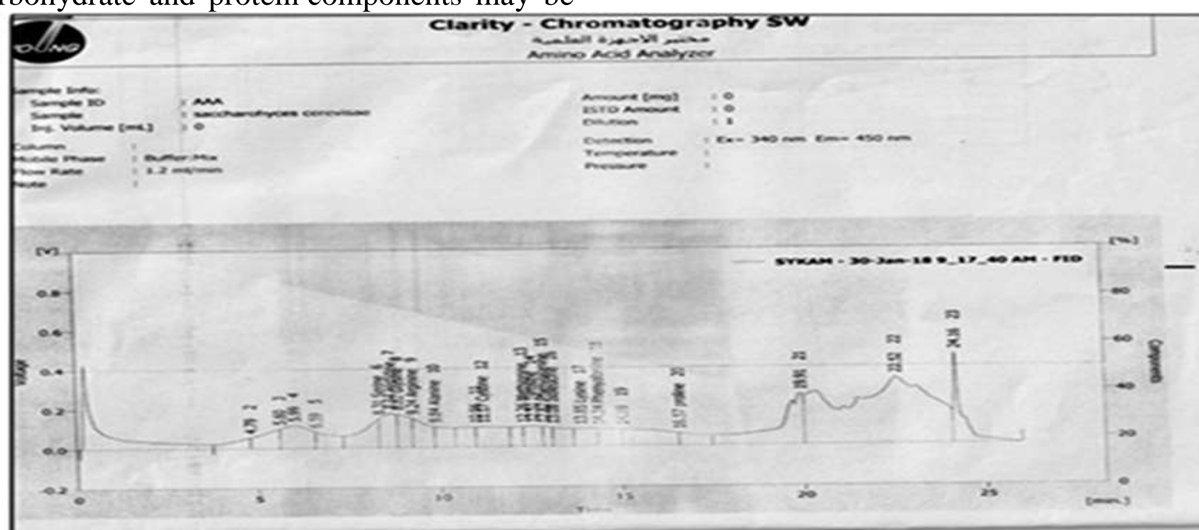

Figure (9): HPLC spectrums of the mannoprotein (amino acid) which has been produced by *S.cerevisiae*

Table (2): HPLC analysis profile of the Mannoprotein extract from *S. cerevisiae* BY

No.	Retention time (min)	Amino acid	Area %	Relative Abundance %
1	8.308	Serine	26.1	100
2	8.636	Histidine	10.8	41.37
3	8.824	Glycine	8.2	31.41
4	9.236	Arginine	4.7	18
5	9.844	Alanine	4	15.32
6	11.168	Cysteine	14.6	55.93
7	12.260	Methionine	6.9	26.43
8	12.512	Tyrosine	5.2	19.92
9	12.820	Phenylalanine	2.4	9.19
10	13.080	Isoleucine	4.7	18
11	13.852	Lysine	12.3	47.12
12	16.572	Proline	0.0	0

Antibacterial Activity of Mannoprotein Produced by *S. cerevisiae BY*

Biosurfactants interact with cytoplasmic membranes resulting in metabolite leakage and cell lysis, and disrupt protein conformation that eventually changes essential membrane functions (25). annoprotein are class of bio-surfactant with less antimicrobial effects. The antibacterial activity of produced mannoprotein by *S.cerevisiae* BY was tested using disc diffusion method on Muller-Hinton agar (MHA) plates against pathogenic bacteria *P. aeruginosa* and *S. aureus*. It has been observed from the results in Table 3 that the mannoprotein showed inhibition zones diameter ranged from (10) and (14) against *P. aeruginosa* and (9) to (13) for *S. aureus* respectively at concentration of mannoprotein ranged from 50 to 200 mg/ml. In addition, the mannoprotein fraction was found to be less efficient against gram positive and gram negative bacteria. The findings were agreed with the Nibras Study (2018) , illustrated that the effect of purified biosurfactant by using disc diffusion method against *C. urelyticum* isolates. The results showed that pure biosurfactant had lower inhibitory effect against all isolates under study with diameter of inhibition zone ranged from 8 to 18mm.

Table (3): Antibacterial activity of mannoprotein produced by *S. cerevisiae* BY against pathogenic bacteria using disc diffusion method

Concentration of Mannoprotein (mg/ml)	Inhibition zone of *P. aeruginosa* (mm)	Inhibition zone of *S. aureus* (mm)
50	10	9
75	10	9.5
100	10.5	10
150	12	12
200	14	13

Anti-adhesion activity of Mannoprotein produced by *S. cerevisiae* BY

One of the essential properties of biosurfactant is the shaping of a film which affects the wett ability regarding the original surface impacting pathogen adhesion properties (5).Biosurfactant developed out of *S.cerevisiae* BY has demon-strated anti adhesion activity against pathogens but the extent of activity has varied and depends also on the concentration of biosurfactants. The results in Figure (10) found the higher anti- adhesive property of mannoprorein against *P. aeruginosa* (51.1%) and slightly anti adhesion against *S. aureus* (19%) respectively at 200 mg / ml. Elzibeta et al.,(2007) revealed that mannoprotein created via *S. cerevisiae*, possesses surfactant activity, might be a little bit reduce the adhesion of *S. epidermidis* and *Staphylococcus aureus* when polystyrene wells have been coated with such agent before inoculation of bacteria, The antiadhesive effect depends on the concentration and the microorganism tested(14).

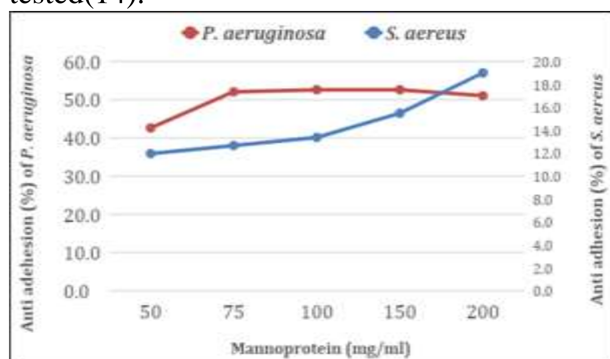

Figure10.Anti-adhesion activity of manno-protein against pathogenic bacteria.

Anti-biofilm activity of Mannoprotein produced by *S. cerevisiae* BY

Biofilms aid pathogens in avoiding host immune responses, will result in chronic infections (9). Results in Figure (11) clearly show that the anti-biofilm activity rises as the concentration of mannoprotein increases. This study showed that mannoprotein biourfactant was penetrate the biofilm and kill microorganisms with effective-ness against *P.aeruginosa* reached 51.8 % while slightly anti biofilm (19.7%)observed against S. *aureus*, at concentration of 200 mg/ml. Elzebita *et.al,* (2007) used surfactant (100 mg/ml) for coating the wells for the purpose of studying the influence of mannoprotein on biofilm formation they demonstrated that when mannoprotein was included in the culture medium, efficient inhibition of biofilm form-ation of S. *epidermidis* and *S. aureus* can be accomplished. Yet, this impact was dose and strain dependent: when mannoprotein was applied at a concentration of 100mg/ml, the greatest substantial drop in biofilm mass (12-87%) was found.

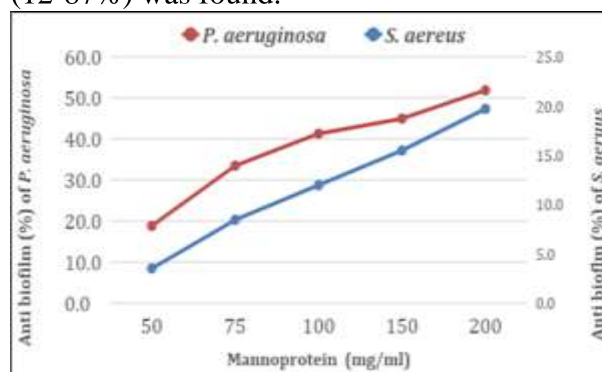

Figure 11. Anti-biofilm activity percentage of Biosurfactant against pathogens.

The Synergestic effects of biosurfactant with the standard Antibiotics

In current study, *P. aeruginosa* and *S. aureus* have been utilized for evaluating the synergistic activity of mannoprotein with four antibiotics. Those organisms are majorly occurring pathogens. Combination of mannoprotein (200mg/ml) (CMC of mannoprotein) with diff-erent concentrations of standard antibiotics (Tetracycline Gentamycin, Ampicillin and Ciprofloxacin) were prepared at three concentrations (0.25,0.75 and 1) mg/ml. The antibacterial activity of mannoprotein and antibiotics was indicated in Figure (12,13). The results specify that the antibiotics with concentration (0.25,0.75 and 1) mg/ml alone was effective against *P. aeruginosa* and *S. aureus* and showed total inhibition ranged from (13to 40mm)and(12 to 30 mm), respectively. However, when mannoprotein and antibiotics

have been utilized in combination, the total inhibition was increased to (15 to 35 mm) and (17 to 55 mm), for *S. aureus* and *P.aeruginosa*, respectively. The combination effect of mannoprotein and antibiotics (Tetracycline Gentamycin, Ampicillin and Ciprofloxacin) at concentration 1mg/ml enhanced 26,29, 33 and 36 % of inhibition zone for *P. aeruginosa* while, enhancement was 20, 33, 40, and 14 % for *S. aureus*, respectively as shown in table (4). In the bacterial infections' treatment, combined antibiotic therapy has been proven to slow the formation of bacterial resistance and create favorable synergistic effects (1). The synergistic activity of a crude biosurfactant made from *Bacillus subtilis* was tested against *S. aureus*. As previously stated, biosurfactants can create micelles, bilayer structures, and self-assembled structures that might contain water-soluble drugs, when both are administered at the same time. The results of this work showed the fact, mannoprotein biosurfactant alone could not completely inhibit the growth of *S. aureus* and *P.aeruginosa.*Yet, in case of cells treatment with combination of mannoprotein biosurfactant with antibiotics, enhancement of inhibition zones was observed as compared to the bacteria treated with antibiotics. A research conducted by Alshaikh *et.al*, (4) proved that Rhamnolipid produced by *Pseudomonas* aeruginosa A3 have synergistic effect against pathogenic bacteria when was combined with antibiotic and lead to enhancement of inhibition zone. Aleaa *et.al*, (2) also explain the synergistic effect of biosurfactant (lipopeptide) against pathogenic bacteria when combined with different antibiotics the inhibition zone range was increased and made sensitive. Another research Ghada *et.al* (16) proved that the combination effects of the biosurfactant and chloramphenicol at a 1000µg/ml concentration enhanced 35.50% and 22% of the inhibition zone for *S. aureus* and *E. coli*, respectively (16).

Table(4): Synergistic effect of mannoprotein (200mg/ml) produced from *S.cerevisiae* with different Antibiotics against pathogenic bacteria:

Antibiotic Concentration (mg/ml)	Inhibition zone (mm) of *P.aeruginosa*							
	TE	TE+Manno	CN	CN+Manno	AM	AM+Manno	Cip	Cip +Manno
0.25	15	17	25	27	20	28	40	45
0.75	20	35	13	25	25	30	35	40
1	30	42	25	35	20	33	35	55
Antibiotic Concentration (mg/ml)	**Inhibition zone (mm) of *S. aureus***							
	TE	TE+Manno	CN	CN+Manno	AM	AM+Manno	Cip	Cip +Manno
0.25	25	30	19	22	12	15	25	30
0.75	28	31	20	25	15	15	25	35
1	28	35	20	30	15	25	30	35

TE=Tetracycline, CN =Gentamycin, AM =Ampicillin, Cip =Ciprofloxacin
Mann=Mannoprotein (200mg/ml).

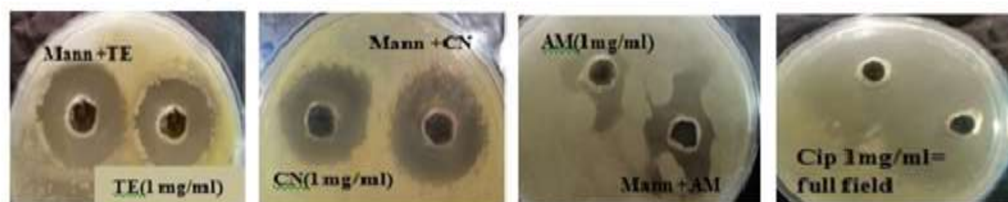

Figure 12. Synergistic action of antibiotics with mannoprotein against *P. aeruginosa*
Mann = Mannoprotein (200mg/ml), TE= Tetracycline, CN =Gentamycin, AM =Ampicillin, Cip =Ciprofloxacin

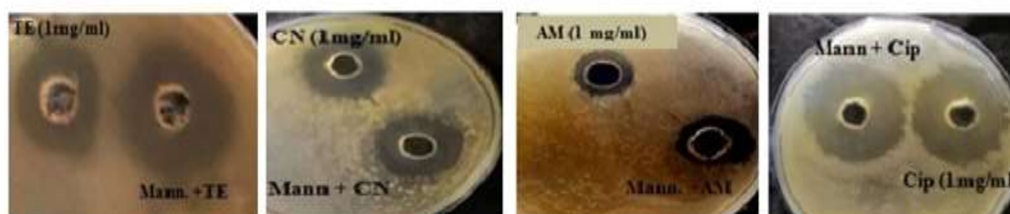

Figure 13. Synergistic action of antibiotics with mannoprotein against *S.aureus* Mann = Mannoprotein (200mg/ml), TE= Tetracycline, CN =Gentamycin, AM =Ampicillin, Cip =Ciprofloxacin

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