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بهینه‌سازی تولید اگزو پلی‌ساکارید سنتزشده توسط *Lacticaseibacillus paracasei* AS20(1) و پتانسیل آن به‌عنوان عامل ضدبیوفیلمی و ضدسرطانی

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چکیده

مقدمه: اگزوپلی‌ساکاریدهای (EPSs) سنتزشده توسط باکتری‌های اسیدلاکتیک، پلی‌ساکاریدهایی با فعالیت‌های درمانی متنوع‌اند. EPSها به‌دلیل ایمن‌بودن می‌توانند در تهیه غذاهای فراسودمند به کار گرفته شوند. مصرف این محصولات می‌تواند راهکاری نویدبخش به منظور جلوگیری از بیماری‌های مهلك و مقاوم به درمان همانند سرطان و عفونت‌های مرتبط با بیوفیلم‌های میکربی باشد؛ با این حال، راندمان پایین سنتز، کاربرد صنعتی آنها را بسیار دشوار می‌کند. مطالعه حاضر با هدف بررسی فعالیت ضدسرطانی و ضدبیوفیلمی اگزو پلی‌ساکارید به‌دست‌آمده از *Lacticaseibacillus paracasei* AS20(1) و بهینه‌سازی تولید آن انجام شد.

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مـواد و روش‌هـا: در مطالعـه حاضـر، سـنتز EPS توسـط *Lacticaseibacillus paracasei* AS20(1) بـا Central Composite Design بهینه‌سـازی شـد. همچنـین تـأثیر ایـن EPS بـر مهـار تشـکیل بیـوفیلم *Staphylococcus aureus* (۰/۷۵-۰/۲۵ میلی‌گرم بر میلی‌لیتر)، بقای رده سلولی سرطان کولون انسانی (HT-29) (۲-۰/۰۰۵ میلی‌گرم بر میلی‌لیتر) و القای آپوپتوز (۲ میلی‌گرم بر میلی‌لیتر) در آنها بررسی شـد؛ درنهایـت، سـمیت ایـن اگـزو پلـی‌سـاکارید میکربـی بـا استفاده از آزمون همولیز گلبول‌های قرمز انسانی (۵۰ و ۱۰۰ میلی‌گرم بر میلی‌لیتر) بررسی شد.

نتایج: با توجه به مدل پیش‌بینی‌شـده، بیشـترین تولیـد EPS در pH ۷/۲، غلظـت عصـاره مخمـر ۲/۳ درصـد (وزنـی / حجمی) و غلظت سوکروز ۳۳/۳ گرم بر لیتر به دست آمد. EPS مطالعه‌شده فعالیـت ضـدبیوفیلمی (۷۰-۱۱ درصـد) و فعالیت ضدسرطانی نویدبخشی را در برابر HT-29 نشـان داد (IC50=1451.42 میکروگـرم بـر میلـی‌لیتـر). تیمـار EPS درصد سلول‌ها را در حالت آپوپتوز اولیه (۲۸/۸ درصد) افزایش داد. این EPS میکربی، سمیت ناچیز (<۵ درصد) علیه گلبول‌های قرمز داشت.

بحث و نتیجه‌گیری: درمجموع، شرایط بهینه‌سازی‌شدهٔ ایدئال می‌تواند برای تولید EPS در مقیـاس بـزرگ توسـط *Lacticaseibacillus paracasei* AS20(1) مفید باشد که دارای فعالیت‌های زیستی ارزشمندی است.

واژه‌هـای کلیـدی: فعالیـت ضدسـرطانی، *Lacticaseibacillus paracasei* AS20(1)، بـاکتری‌هـای اسـید لاکتیـک، بهینه‌سازی

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Optimization of *Lacticaseibacillus paracasei* AS20(1)-Derived Exopolysaccharide and its Potential as an Anti-Biofilm and Anticancer Agent

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Abstract

Introduction: Bioactive exopolysaccharides (EPSs) synthesized by lactic acid bacteria are therapeutic polysaccharides. EPSs are safe, so they can be used to make functional foods. Consumption of these foods can be a promising approach to prevent life-threatening and hard-to-treat diseases such as cancer and biofilm-associated infections. However, the low efficiency of synthesis makes their industrial implementation much more difficult. The aim of this study was to evaluate the anticancer and anti-biofilm activities of *Lacticaseibacillus paracasei* AS20(1) EPS and to optimize its production.

Materials and Methods: In the present study, the EPS synthesis by *Lacticaseibacillus paracasei* AS20(1) was optimized by Central Composite Design. Also, the effects of this EPS on inhibition of biofilm formation of *Staphylococcus aureus* (0.78-25 mg/ml), the viability of human colon cancer cell line, HT-29 (0.005-2 mg/ml), and the induction of apoptosis (2 mg/ml) in them were evaluated. Finally, the toxicity of this microbial EPS (50 and 100 mg/ml) was evaluated through a hemolysis assay of human red blood cells.

Results: According to the predicted model, the maximal EPS production was achieved at pH 7.2, yeast extract, and sucrose concentrations of 2.3%(w/v) and 33.3 g/L, respectively. This EPS showed promising anti-biofilm (11-70%) and anticancer activity against HT-29 (IC50=1451.42 μg/ml). EPS treatment increased the percentage of cells in the early

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apoptotic state (28.8%). This microbial EPS showed negligible toxicity (<5%) on red blood cells.

Discussion and Conclusion: Collectively, the ideal optimized conditions can be useful in large-scale production of EPS by *Lacticaseibacillus paracasei* AS20(1) which possesses valuable bioactivities.

Key words: Anticancer Activity, *Lacticaseibacillus Paracasei* AS20(1), Lactic Acid Bacteria, Optimization

Introduction:

Currently, the market for organic food is continuously growing (16% yearly). Therefore, food industries intend to develop healthier products with therapeutic effects using natural ingredients (1). In this regard, probiotics, prebiotics, paraprobiotics, and postbiotics are considered promising sources of medicine to improve health. Nowadays, two hard-to-treat problems are cancers and biofilm-associated infections. Colorectal Cancer (CRC) is one of the most common cancers worldwide and its incidence has been increasing globally. Also, biofilm formation is considered the main problem in healthcare settings because a lot of nosocomial infections (75%) result from biofilm of residing bacteria. There is, therefore, an urgent need to find safe targeted therapies. Consuming functional foods containing probiotics or their derivatives seems to be a promising approach to enhance this life-threatening disease (2).

In this regard, Lactic Acid Bacteria (LAB) are nearly related to all of these beneficial terms. They play important roles in food, dairy, agricultural, pharmaceutical, and medical applications (3). Due to their health-improving effects, LAB have been exploited as probiotics (4).

LAB produce many functional substances, including short-chain fatty acids, bioactive peptides and metabolites, vitamins, cofactors, enzymes, immune-signaling compounds, and exopolysaccharides (EPSs) as postbiotics (5). EPSs are water-soluble long-chain biopolymers with sugar constituents or sugar derivatives. They can be synthesized as well-defined capsules or loosely organized slime. Loosely organized slime is of greater practical significance because its extraction is easier without protein or cell fragments contamination (1).

Similar to LAB, most of LAB derived EPSs have been considered the GRAS (generally recognized as a safe) status, which makes them attractive biopolymers for various industries (1).

These biopolymers due to having promising properties such as amphiphilic nature, gelling, biocompatibility, biodegradability, and bioactivity characteristics have numerous biomedical (as antimicrobial, anticancer, anti-biofilm, and antioxidants agents), environmental (as bioflocculants) and food applications (as gelling, stabilizer, thickener, and emulsifier substances) (6).

In comparison with plant or algal-derived polysaccharides, microbial EPSs have some advantages like lower production costs, and more efficient downstream processing as well as continuous EPS harvesting from the cell-free culture supernatant can be practical (7). Despite the various beneficial properties of LAB EPSs, their yields are still insufficient which limited their commercial applicability. Generally, the production of LAB heteropolysaccharide is markedly lower than that of homopolysaccharide (8, 9).

Collectively extensive bioactivities of LAB-derived EPSs, their stable chemical

and physical characteristics, and their potential applications have attracted the attention of researchers to study, understand, and explore opportunities for enhanced EPS production yield. It is known that the main determinants of EPS synthesis are incubation period, the downstream process for EPS extraction, and purification and culture conditions, including growth medium composition, temperature, pH, and aeration. The most influential factor is growth medium composition especially carbon and nitrogen sources as it directly affects EPS yield and chemical composition. Stable culture conditions should be ensured in the case of EPSs with industrial value to achieve consistent EPS structures with the same, proven biological properties (4).

Therefore, optimization procedures are requisite for the achievement of faster and maximum EPS production. One Factor at a Time, Response Surface Methodology (RSM), Orthogonal Matrix, Full Factorial design, and Central Composite Design (CCD) are some of the statistical tools to optimize EPS production (10).

In this regard, we aimed to statistically optimize the production of EPS produced by probiotic *Lacticaseibacillus paracasei* AS20(1) through optimizing culture composition and evaluating its anti-biofilm and anticancer activities.

Material and Methods

Microorganism: *Lacticaseibacillus paracasei* AS20(1), an EPS-producing lactic acid bacterium with the accession number OM884063, which had been previously isolated from cow butter, Rudbar-e-Qasran /Fasham, Tehran, Iran was employed. This strain was maintained at -80 ^{o}C as frozen stock cultures in MRS broth containing 20% (v/v) glycerol. This strain was initially revived in de Man, Rogosa, and Sharpe (MRS) (Merck) broth or MRS on agar plates, and the inoculated media were incubated at 37 °C for 24 h. *Lacticaseibacillus paracasei* AS20(1) was manifested as circular and flat colonies with entire margins.

Preparation Inoculum of *Lacticaseibacillus Paracasei* AS20(1): *Lacticaseibacillus paracasei* AS20(1) was inoculated in MRS broth consisting of peptone from casein (10.0 g/L), meat extract (8.0 g/L), yeast extract (4.0 g/ L), dipotassium hydrogen phosphate (2.0 g/L), tween 80 (1.0 g/L), di-ammonium hydrogen citrate (2.0 g/L), sodium acetate (5.0 g/L), magnesium sulfate (0.2 g/L), and manganese sulfate (0.04 g/L) which was supplemented by sucrose (5% w/v). The inoculated medium was incubated at 37 ^{o}C for 24 h under microaerophilic (in candle jar) and static conditions. The 24 h-old culture with an optical density ($OD_{600\ nm}$) of 0.8 was used as the inoculum in all experiments. The fermentation media, MRS broth, with various concentrations of sucrose, and yeast extract were inoculated with bacteria at a concentration of 10% (v/v) and incubated at 37 °C, under microaerophilic conditions (Table 1).

Experimental Design and Optimization of EPS Production: In the present study, the software Design Expert (Trial Version 11) was employed for experimental design and to predict the optimal conditions for maximum EPS production in a fermentation medium. A Central Composite Design (CCD) was used to determine the response pattern and then establish a model. The three variables used in this study were sucrose (X_1), yeast extract (X_2), and pH (X_3), with five levels of each variable. The value of EPS production (mg/L) of each trial was considered as the experimental response Y. A design of 20 experimental runs with six repetitive central points was formulated and experiments were

performed in a falcon containing different concentrations of sucrose and yeast extract at various pH. The results of the CCD were used to fit a quadratic equation.

The second-order polynomial regression equation is given as:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum_{i<j} \beta_{ij} X_i X_j$$

Where Y is the response (EPS production), K is the number of independent variables, β_0, β_i, β_{ii} and β_{ij} are the coefficients of intercept, linear, quadratic, and interactive terms, respectively, and X_i and X_j are levels of the independent variables.

Recovery of the EPS: At the end of incubation, the EPS-containing media was centrifuged at 10000×*g* for 30 min at 4 ^{o}C to remove bacterial cells. The supernatant containing the EPS was kept. Trichloroacetic acid at the final concentration of 4% (w/v) was added to the supernatant to remove proteins. The mixture was incubated for 30 min at room temperature. The proteins were removed via centrifugation (10000×*g* at 4°C for 30 min). Then, two volumes of cold 96% ethanol were added to the supernatant to precipitate the EPS. The mixture was left for 24 h at 4 °C. The precipitated EPS was collected by centrifugation at 10000×*g* for 20 min at 4 ^{o}C. After evaporation of ethanol, the resulting EPS pellet was dissolved in distilled water and dialyzed against distilled water (11). Finally, the mass of EPS produced was measured using a precision analytical balance in g. The yield was reported as the mass of EPS in mg per liter of the medium and denoted as the yield of EPS. The schematic diagram of the EPS extraction and recovery processes were shown in Figure 1.

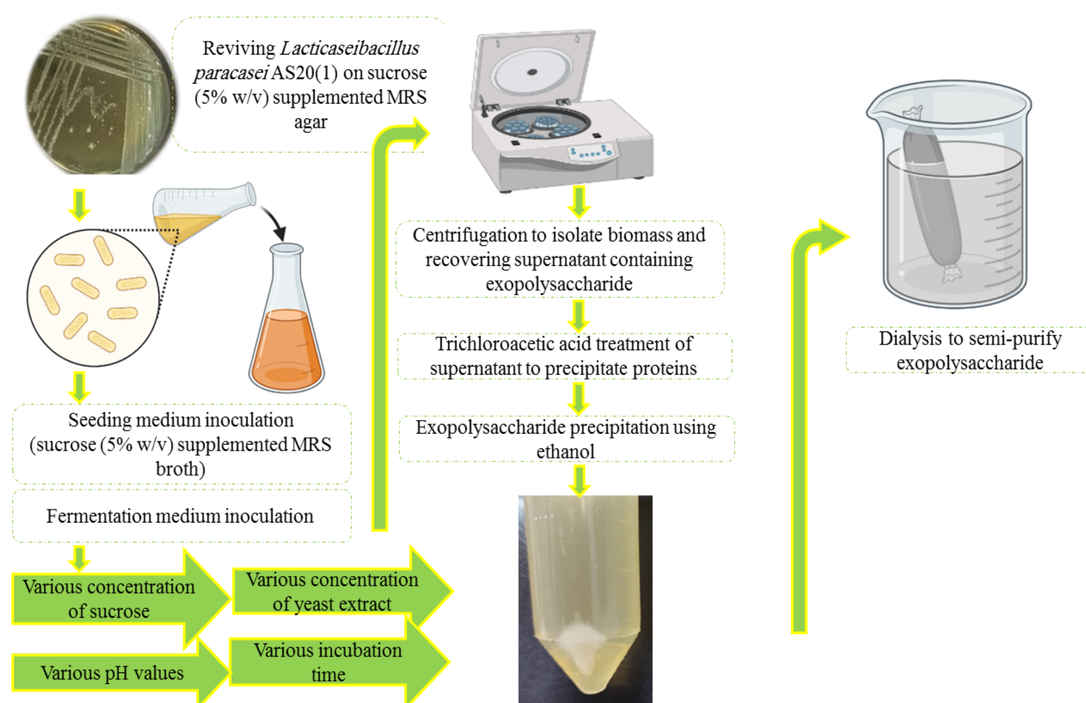

Fig. 1- The Schematic Diagram of the EPS Extraction and Recovery Process

Anti-biofilm Activity of EPS: The antibacterial effect of the EPS (5-50 mg/mL) on *Staphylococcus aureus* ATCC 6538 was evaluated using a microdilution

test and a concentration range in which EPS has no antibacterial effect on this bacterium, which was selected for anti-biofilm assay. To determine the anti-biofilm activity of EPS (0.78-25 mg/mL-200 µl), it was added to the wells of a sterile 96-well plate together with *S. aureus* ATCC 6538 suspension (10 µl, 10^6 CFU/ml) in tryptic soy broth containing yeast extract (0.1% w/v) plus glucose (2% w/v) (Ibresco, Iran) as a carbon source (37 °C for 24 h). The 96-well plate was incubated at 37 °C for 24 h. After incubation, the wells were washed (three times) with distilled water to remove non-adherent cells. Methanol (200 µL, 15 min) and crystal violet (100 µL,1% w/v, 20 min) were used to fix and stain the biofilm, respectively. Acetic acid (200 µL, 33% v/v) was then used to solubilize the attached crystal violet. The absorbance of the released crystal violet was measured at 595 nm. The wells containing inoculated tryptic soy broth-yeast extract/glucose in the absence of EPS served as the positive control (12). The inhibitory effect of EPS on biofilm formation was calculated as follows:

$$\text{Anti-biofilm activity} = 1 - \frac{Ac}{A0} \times 100$$

In the above formula, A_c is the optical density of the wells treated with EPS at a given concentration (c), and A_0 is the optical density of the wells with formed biofilm (positive control). Well-containing EPS dissolved in PBS was considered as the negative control. The tests were performed three times.

Evaluation of the Inhibitory Effect of EPS on Colon Cancer Cell Culture: The human colon cancer cell line, HT-29 was acquired from the Iranian Biological Resource Center (IBRC, Tehran, Iran). HT-29 cells were grown in an RPMI medium with 10% FBS, penicillin G (100 U/ mL), and streptomycin (100 U/ml) and incubated in a CO_2 incubator (37 °C, 5% CO_2, humidified atmosphere). The medium was changed daily to maintain the exponential growth of the cells. Cell counts were assessed by standard procedures of cell counting using a hemacytometer. To evaluate the inhibitory activity of *Lacticaseibacillus paracasei* AS20(1) EPS on HT-29 cells, its stock solution was prepared using PBS. Serial dilution of EPS was prepared using RPMI medium (0.005-2 mg/ml). The cells were seeded at a concentration of 1×10^5 in 96-well plates and incubated overnight for cell adhesion in a humidified atmosphere of 5 % (v/v) CO_2 at 37 °C. At the end of the incubation process, the cells were treated with different concentrations of EPS. Then treated cells were incubated at 37 °C for 72 hr. Cells with culture medium without EPS were also used as untreated growth control. After the incubation process, a freshly prepared MTT solution (10 µL, 5 mg/ml) was added to each well and allowed to incubate for an additional 4 hours. MTT is reduced by mitochondrial dehydrogenases in viable cells and forms purple-colored formazan. The crystals of formazan were subsequently dissolved in DMSO (100µL/well). Plates were shaken gently to facilitate formazan crystal solubilization. Absorbance (OD_{570nm}) of wells containing treated and untreated cells were measured by a microplate reader. Absorbance from untreated cells was considered as 100 % of growth and was used for toxicity calculation. Finally, the effect of EPS on the human colon cancer cell line and half-maximal inhibitory concentration (IC50) was calculated (13).

$$\text{Toxicity (\%)} = 1 - \frac{\text{Mean OD570 of treated cells}}{\text{Mean OD570 of untreated cells}} \times 100$$

Annexin-V/PI iodide apoptosis assay: Apoptosis-inducing effects of EPS were evaluated through differential counting of apoptotic, necrotic, and viable cells using

the fluorescein isothiocyanate (FITC)-Annexin-V/PI staining kit (Apoptosis detection kit, Roche, Germany). Untreated cells were used as control. Briefly, the HT-29 cells (3×10^5 cells/well) were incubated for 24 hr with 2 mg/ml of EPS or saline for control. At the end of the incubation period, the trypsinized cells were washed with PBS and suspended in Annexin-V binding buffer. In the following, FITC Annexin-V (5 µl) and PI solutions (10 µl) were added to 100 µl of cell suspension, respectively. Then, the cells were incubated at 25 ^oC for 10 minutes. Finally, apoptotic, necrotic, and viable cells were analyzed by a flow cytometry machine. Analysis of the data was carried out using FCS Express Flow Cytometry Software. Cell analysis by flow cytometry has shown cells in four states, including cells in healthy (Q4 zone, Annexin-V^+, and PI^-), early apoptotic (Q3 zone, Annexin-V^-, and PI^+), late apoptotic (Q2 zone, Annexin-V^+ and PI^+), and necrotic states (Q1 zone, Annexin-V^-, and PI^+). The used fluorochromes were fluorescein isothiocyanate (FITC) conjugated to Annexin-V in the FL1 channel and phycoerythrin (PE) conjugated to PI in the FL2 channel. In order to determine the effects of EPS on the induction of apoptosis or necrosis, the percentage of cells located in each area was calculated and reported by flow cytometry software (FCS Express).

Hemolytic Assay: The hemolytic activity of the antimicrobial EPS was studied using fresh human red blood cells (RBCs) (14). To inhibit blood coagulation, sodium citrate (Merck) (0.5 ml of 3.8%) was added to the blood (4.5 ml), and then plasma was removed by centrifugation. The RBCs were washed three times with PBS buffer (pH 7.2) and finally resuspended in PBS (10% v/v). The equal volume of RBCs was mixed with EPS solution at the final concentrations of (50 and 100 mg/ml), and the mixture was incubated at 37 °C for 1 h. At the end of incubation, the mixture was centrifuged at 2500 *g* for 6 min. Then, the absorbance of the supernatant was read at 540 nm. The PBS buffer and 0.2% Triton X-100 were used as negative and positive controls, respectively. The experiments were replicated three times, and the hemolysis percentage was measured by the following formula:

$$\text{Hemolysis}(\%) = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}} \times 100$$

Results

Optimization of the EPS Production: The results of optimization of the EPS production along with the uncoded values are shown in Table 1. The EPS yield ranged from 150 mg/L to 350 mg/L, and the experiments of 6 and 20 gave the minimum and maximum yields, respectively. According to the predicted model, the maximal EPS production was achieved at pH 7.2, yeast extract concentration 2.3% (w/v), and sucrose concentration 33.3 g/L.

The result of the analysis of variance is shown in Table 2. The results demonstrated that X_1 and X_2 ($p<0.05$) and all the quadratic parameters X_1^2, X_2^2 and X_3^2 ($P<0.05$) have a significant effect on the production of EPS. However, the interaction parameters X_1X_2 were insignificant ($P>0.05$). The Model F-value implies that the model is also significant. The sig-value of the model was <0.0001 indicating the significance of the model. The Lack of Fit F-value of 0.3976 implies the lack of fit is not significant ($P>0.05$).

Table 1- Experimental Designs of the Five-level and the Experimental Data with Actual Values of Variables

Run	Factor 1 A: Sucrose (%-w/v)	Factor 2 B: Yeast extract (%-w/v)	Factor 3 C: pH	Predicted Response 1 R1 (mg/L)	Observed Response 1 R1 (mg/L)

1	2.50	2.5	6.8	341.6	340.0
2	4.00	2.5	6.8	282.4	290.0
3	2.50	4.0	6.8	257.15	260.0
4	2.50	2.5	6.8	342.6	340.0
5	3.39	3.4	7.5	278.36	280.0
6	1.00	2.5	6.8	160.33	150.0
7	2.50	2.5	6.8	371.6	330.0
8	2.50	2.5	6.8	342.4	340.0
9	2.50	2.5	8.0	224.3	220.0
10	1.61	1.6	6.1	159.7	160.0
11	1.61	3.4	7.5	190.8	190.0
12	3.39	3.4	6.1	290.8	280.0
13	2.50	2.5	5.6	228.4	230.0
14	2.50	2.5	6.8	341.6	350.0
15	1.61	3.4	6.1	193.3	200.0
16	3.39	1.6	6.1	217.3	220.0
17	3.39	1.6	7.5	214.8	210.0
18	1.61	1.6	7.5	167.276	170.0
19	2.50	1.0	6.8	175.5	170.0
20	2.50	2.5	6.8	344.2	350.0

Table 2- ANOVA for Response Surface Quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	940.3	9	104.5	161.9	< 0.0001	significant
A-Sucrose	187.1	1	187.1	290.0	< 0.0001	
B-Yeast extract	85.33	1	85.33	132.27	< 0.0001	
C-pH	0.53	1	0.53	0.82	0.3875	
AB	6.13	1	6.13	9.50	0.0116	
AC	0.13	1	0.13	0.19	0.6692	
BC	0.13	1	0.13	0.19	0.6692	
A^2	263.71	1	263.71	408.80	< 0.0001	
B^2	285.96	1	285.96	443.29	< 0.0001	
C^2	242.36	1	242.36	375.72	< 0.0001	
Residual	6.45	10	0.65			
Lack of Fit	3.62	5	0.72	1.28	0.3976	not significant
Pure Error	2.83	5	0.57			

In conclusion, this model regression equation is as follows:

Production of EPS= $+341.63+37.01X_1+25X_2+8.75X_1X_2-42.78X_1^2-44.54X_2^2-41.01X_3^2$

The value of R-Squared was (R^2=0.9932), which indicated that only 0.68% of the total variations were not explained by the model. Fig. 2A shows the three-dimensional (3D) response surface plots (a) and contour plots (b). Fig. 2B shows the effects of the concentration of sucrose and yeast extract on the production of EPS when the pH is held at an optimum level. Based on the results, with increasing sucrose from 16 to 25 g/L, the production of EPS increased quickly. When the concentration of sucrose increased from 25 to 34 g/L, the EPS production increased slowly. Based on the results from other studies, the production of EPS is significant (P<0.05) with an increasing concentration of sucrose.

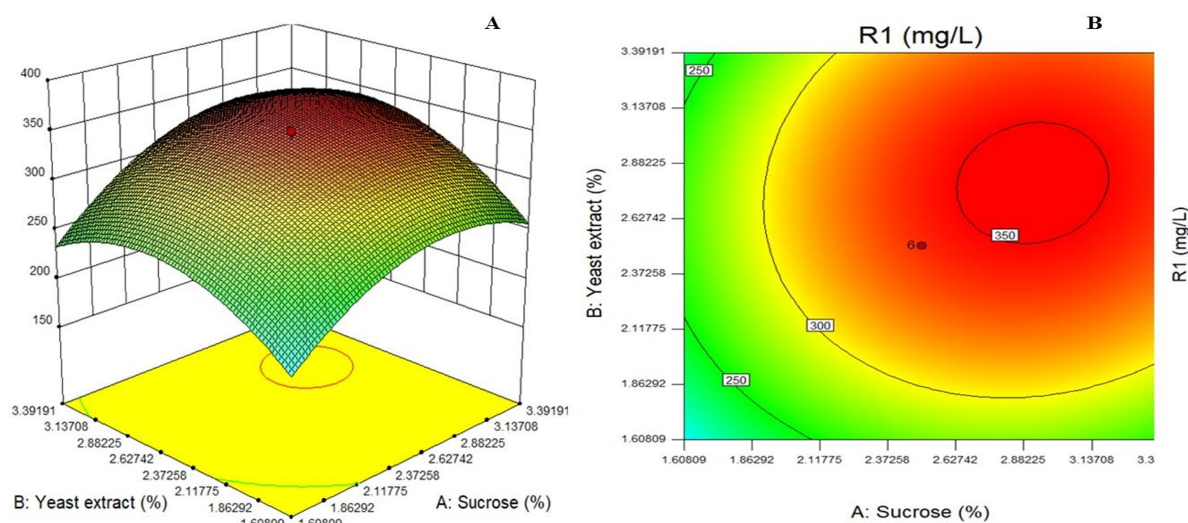

Fig. 2- Three-dimensional (3D) graph (a) and contour plot (b) achieved from RSM experimental design displaying the effects of the interaction of yeast extract vs sucrose on the EPS production

Inhibitory Effects of *Lacticaseibacillus paracasei* AS20 (1) EPS on the Formation of *S. aureus* Biofilm: The microdilution test showed that *Lacticaseibacillus paracasei* AS20(1) EPS possesses no antimicrobial effect on *S. aureus* in the concentration range of 5-50 mg/ml. Therefore, the inhibitory activity of *Lacticaseibacillus paracasei* AS20(1) EPS on biofilm formation of *S. aureus* was investigated at the concentration of 0.78-25 mg/ml. According to the results, the inhibitory effect of *Lacticaseibacillus paracasei* AS20(1) EPS against biofilm formation of *S. aureus* (11-70%) showed a dose-dependent state (Fig. 3A). There was no significant difference in the anti-biofilm activity of EPS in the concentration of 12.5 and 25 mg/ml. So, the concentration of 12.5 mg/ml is the most effective concentration for anti-biofilm application.

Anticancer Effects of *Lacticaseibacillus paracasei* AS20(1) EPS: As shown in Fig 3B, EPS treatment of HT-29 cells led to a reduction in cell viability. Therefore, *Lacticaseibacillus paracasei* AS20(1) EPS possesses an inhibitory effect on the human colon cancer cell line. This anticancer effect was dose-dependent. IC50 of *Lacticaseibacillus paracasei* AS20(1) EPS was 1451.42 µg/ml against the human colon cancer cell line. In the most applied concentration (2 mg/ml), more than half of the treated cells lost their viability.

Apoptosis-inducing Effects of EPS: The apoptosis rate of HT-29 cells treated with EPS for 24 h was examined by Annexin-V/PI iodide apoptosis assay. The results showed that the percentage of viable cells in EPS-treated HT-29 cells decreased to 63.2% compared to untreated cells (94.3%). Also, EPS treatment causes a significant increase in the percentage of HT-29 cells in the early apoptotic state (28.8%) in comparison with 1.98% in untreated HT-29 cells (Fig. 4).

Evaluation of toxicity *Lacticaseibacillus paracasei* AS20(1) EPS: The hemolysis test showed that EPS of *Lacticaseibacillus paracasei* AS20(1) exhibited negligible hemolysis (less than 5%) in concentrations of 50 and 100 mg L^{-1} (Fig. 5). There is no significant difference in hemolysis percent in these concentrations (P>0.05).

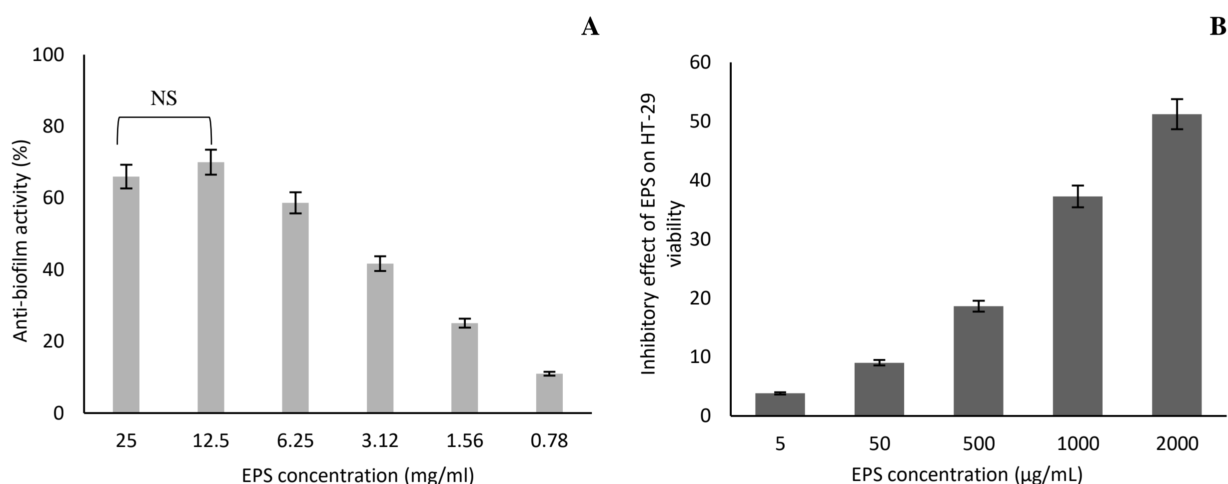

Fig. 3- Anti-biofilm activity of *Lacticaseibacillus paracasei* AS20(1) EPS against *Staphylococcus aureus* (A). Anticancer activity of *Lacticaseibacillus paracasei* AS20(1) EPS in various concentrations against HT-29 cell line after 72 h. exposure. All assays were performed in triplicate, and the mean±SD was illustrated. NS indicated that there is no significant difference among groups (P>0.05) at a confidence interval of 95% (B).

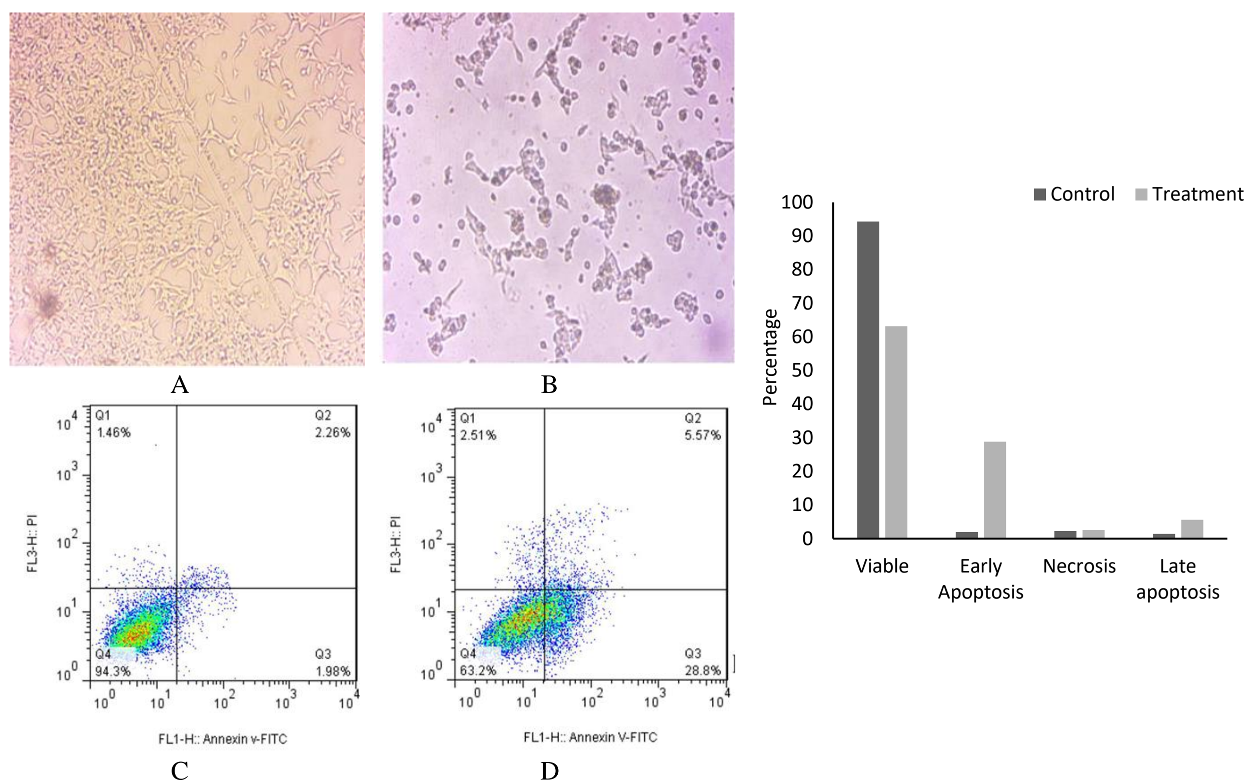

Fig. 4- Untreated HT-29 cells-control (A) and EPS-treated HT-29 cells (B). Flow cytometric analysis by Annexin V-FLUOS (FL1) in the x-axis and PI (FL3) in the y-axis double staining of HT-29 cells treated with EPS. Dot plots of Annexin-V/PI staining are shown in (C untreated HT-29 cells; (D) HT-29 cells treated with EPS (2000 μg/ml with 24 h incubation). The percentage of EPS-treated and untreated HT-29 cells in viable, early apoptotic, late apoptotic, and necrosis states (E)

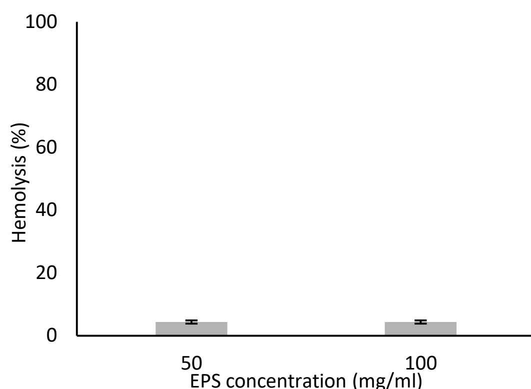

Fig. 5- Hemolysis Activity of *Lacticaseibacillus paracasei* AS20(1) EPS (50 and100 mg/ml)

Discussion

EPSs are considered one of the most important functional components of LAB metabolic products with several therapeutic activities, including antibacterial, anti-oxidant, and antitumor activities. They also exert immunoregulatory and anti-hypertensive effects. Among these bioactivities, antitumor activity has especially received intensive interest due to the increasing rate of cancer-related mortality (15).

One of these life-threatening cancers is colon cancer. Colon cancer is a serious cancer type with high incidence and mortality rates in developed countries. Colon cancer ranks third in the United States among cancers diagnosed in both men and women (16). It causes approximately 1.4 million new cases and more than 0.5 million deaths per year worldwide. Its onset is insidious, often without any obvious clinical manifestations (15).

Therefore, it seems that preventive strategies are critical. Most chemical antitumor agents are cytotoxic and have side effects which necessitate finding antitumor agents from natural sources (15). Consuming probiotics producing anticancer metabolites like EPS with inhibitory effects on these cancerous cells can be a safe and promising strategy.

So, the present study evaluated the anticancer and apoptosis-inducing effects of *Lacticaseibacillus paracasei* AS20(1) EPS on a mostly used colon cancer cell line called HT-29. *Lacticaseibacillus paracasei* AS20(1) was previously isolated from cow butter in a screening study. According to the results, *Lacticaseibacillus paracasei* AS20(1) EPS has acceptable inhibitory effects on the HT-29 cell line. The result of the current study (inhibitory effect of EPS in 500 µg/ml was 18.63%) is in agreement with the reported results of previous studies. For example, Di et al. showed that crude EPSs and acidic EPSs (500 µg/ml) produced by *L. casei* K11, *L. casei* M5, *L. casei* SB27, and *L. casei* have 12-18% and 16-31% toxicity toward HT-29 cell line, respectively (15). Also, Sun et al revealed that EPS of *L. plantarum* strains suppressed the proliferation of HT-29 cells by 12.3-31.3% in the concentration of 500 µg/ml (17). This inhibitory effect can be due to the apoptosis-inducing effects of the EPS.

Also, the inhibitory effect of *Lacticaseibacillus paracasei* AS20(1) EPS on *S. aureus* biofilm formation (11-70%) reveals another aspect of its therapeutic effect. Also, Xu et al. reported that EPS of *Lactobacillus casei* (0.3125-5 mg/mL) inhibited the biofilms of *B. cereus* (95.5%), *S. aureus* (30.2%), *S. typhimurium* (14.3%), and *E. coli* O157:H7 (16.9%) (18). It has been suggested that biofilms suppress phagocytosis of *S. aureus* strains and also make *S. aureus* resistant to antibiotics. Consequently, biofilm is an important virulence determinant in bacteria. So, anti-biofilm EPSs are necessary to overcome biofilm-related infections (18). According to anticancer and anti-biofilm activities of *Lacticaseibacillus paracasei* AS20(1) EPS, it can be a promising candidate to make functional safe foods. Consuming these functional foods may reduce colon cancer prevalence and *S. aureus* biofilm-associated infections without any side effects because *Lacticaseibacillus paracasei* AS20(1) EPS

had no toxicity effect on red blood cells even in high concentrations.

To commercialize these bioactive exopolysaccharides, their yield production should be acceptably increased. According to the previous studies, culture media composition especially carbon and nitrogen sources has major effects on the yield of EPS production by lactic acid bacteria. Sugars including, glucose, lactose, sucrose, and mannose are consumed by LAB strains as the carbon source for EPS production (10).

According to previous studies, sucrose is the best carbon source to optimize EPS production. For example, Joshi and Koijam reported that among fructose, glucose, galactose, mannose, lactose, sucrose, and ribose, sucrose is the best carbon source to maximize EPS production by *Leuconostoc lactis* (19). Xing et al. optimized EPS production of *Leuconostoc mesenteroides* DRP105 through RSM. They showed that the optimum condition was sucrose in the concentration of 86.83 g/L, tryptone in the concentration of 15.47 g/L, an initial pH of 7.18, and the maximum yield of 53.79±0.78 g/L in 36 h fermentation (20). Tilwani et al. optimized the production of *Enterococcus faecium* MC-5 EPS (16.48 g/L) by evaluating three different factors including sucrose, ammonium nitrate, and pH. Maximum EPS production could be attained by supplementing sucrose (30 g/L) and ammonium nitrate (15 g/L) in the MRS broth with an initial pH of 6.407 (21). Farinazzo et al. achieved the maximum amount of *Leuconostoc pseudomesenteroides* JF17 EPS (53.77mg/mL) under ideal conditions of MRS broth supplemented with sucrose at 18% w/v concentration, fermentation temperature of 20 °C, and an initial pH of 7.30 (22).

Also, Oleksy-Sobczak and Klewicka optimized the MRS medium for EPS synthesis by three *Lactobacillus rhamnosus* strains, namely ŁOCK 0943 (13-fold increase, 85→1138.2 mg/L), ŁOCK 0935 (9-fold increase, 103.67→ 900 mg/L), and OM-1 (7-fold increase, 133.67→ 987.84 mg/L). They found that the main medium-related determinant of EPS production by the studied *L. rhamnosus* strains is the carbon source (sucrose and fructose) (1). The EPS production of the *L. paracasei* AS20(1) isolated in this study was increased 3.8 times higher by optimizing the fermentation medium at pH 7.2, yeast extract, and sucrose concentrations of 2.3 %w/v and 33.3 g/L, respectively.

The carbon and nitrogen sources may affect the composition of EPSs. Therefore, we did not change them and just evaluate the effects of their various concentrations on EPS production. These sources have different effects on EPS production. It has been stated that a higher amount of carbon results in higher yields of EPS, while this does not occur with nitrogen sources. The reason is that large amounts of carbon can extend the growth phase of the producing microbial cells while large amounts of nitrogen can increase the production of EPS degrading enzymes. It was observed that 10:1 is the most favorable ratio of carbon/nitrogen (10).

Zhang et al. maximize the production of EPS by *Lactobacillus sanfranciscensis* (249.30 mg/L, 31% increase) by applying maltose, yeast peptone, and fresh yeast extractives in concentrations of 25 g/L, 10.24 g/L and 12.92 mL/L, respectively (11).

Another important parameter in EPS production is the pH of the culture media (19). The enzyme glycosyl-hydrolase is capable of degrading EPS after long fermentations, which reduces the molar mass. In this way, the optimum pH would be capable to balance the effects of production and degradation (10).

Oleksy-Sobczak et al. reported that the most EPS production of *Lactobacillus*

rhamnosus strains (namely 0943, 0935, and OM-1) was achieved in stationary cultures cultivated for 24 h at 25 °C with the initial pH of 5.7 and the inoculum of 5% v/v. Also, prolongation of the incubation time and application of mixing (80 rpm) were effective in the case of *L. rhamnosus* ŁOCK 0943 EPS production (23). Wang et al. reported maximum EPS production (599.52 mg/mL, 3 folds increase) of *Lactobacillus plantarum* KX041 EPS in the presence of soybean peptone concentration of (20 g/L), fermentation temperature of 35 ℃, and an initial pH of 6.38 (24). Mıdık et al. evaluate the effect of some culture conditions, including temperature, incubation time, pH, NaCl concentration, and carbon, nitrogen, and mineral sources on EPS production by *L. plantarum*, *L. namurensis*, and *Pediococcus ethanolidurans* species. The maximum EPS production was achieved at 30 °C and the pH of 6.0 after120 h of incubation, in the presence of glucose, bactopeptone, and calcium carbonate as carbon, nitrogen, and mineral sources, respectively. Also, NaCl did not stimulate EPS production, which indicates that EPS formation by the tested strains was not a stress response (25).

In conclusion, the current study revealed that *Lacticaseibacillus paracasei* AS20(1) EPS has significant anti-biofilm and anticancer bioactivities without toxicity against human red blood cells. Also, it has been shown that the production of this EPS can be improved by modifying physichochemical conditions of the fermentation medium and its composition.

Declaration of interest statement

The authors of the study deny any conflict of interest.

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Ethical Approval

Not applicable.

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