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Investigating the probiotic and antimicrobial effects of the predominant yeast isolated from sprouted soybean sourdough

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ABSTRACT

Recently, the investigation of probiotic yeasts isolated from fermented foods has received widespread attention due to their unique functional properties and health benefits. In the present study, the predominant yeast isolated from sprouted soybean sourdough was identified using PCR. Then the probiotic and antimicrobial properties of the yeast isolate were investigated. The survival of the isolated *Rhodotorula mucilaginosa* in the simulated conditions of the gastrointestinal tract was 49.61%. Moreover, its antibacterial and co-aggregation activity against *Escherichia coli* was significantly (p<0.05) higher than other studied foodborne bacteria, and in general, yeast isolate showed more inhibitory activity against Gram-negative bacteria than Gram-positive bacteria. The yeast isolate has no hemolytic activity and its auto-aggregation ability was 85.43%, and its hydrophobicity against xylene and hexane was equal to 93.69 and 56.32%, respectively. In addition, the yeast isolate showed sensitivity and relative sensitivity to fluconazole and natamycin, respectively, and it was resistant towards other antifungal compounds and antibiotics studied. Also, the yeast isolate prevented the growth and discoloration of Aspergillus flavus. Based on the mentioned findings, the predominant yeast isolated from the sprouted soybean sourdough has a suitable ability to be used as a protective probiotic culture in the food industry.

1.Introduction

Probiotics refer to live and active microorganisms which, when administered in adequate amounts, help to balance the microbial population of the gastrointestinal tract and improve its function. Probiotic microorganisms include lactic acid bacteria (LAB) and yeasts. While numerous reports have documented the probiotic capabilities of LAB, recently, the probiotic properties of certain yeasts have also been investigated. Probiotic yeasts are eukaryotic microorganisms that are resistant to antibacterial compounds and can exhibit remarkable functional applications such as tolerance to harsh gastrointestinal conditions, immunomodulatory effects, and antimicrobial activity. These characteristics make them promising alternatives to probiotic bacteria [1, 2].

Fermented products are rich sources of probiotics and microorganisms with antimicrobial capabilities, and yeasts are considered the dominant microbial flora in fermented foods, especially sourdough. The microorganisms present in fermented foods are often highly competitive against pathogens. They secrete inhibitory metabolites that exert antagonistic effects on pathogens and enhance food safety. Yeasts isolated from fermented substrates typically possess the ability to withstand stressful conditions such as those found in the gastrointestinal tract, due to their origin from environments with low pH caused by organic acid production [3, 4].

There are reports on the probiotic and antimicrobial effects of the predominant microbial flora of various sourdoughs, including barley [5], oak [6], sprouted clover [7], amaranth [8], buckwheat [9], rice bran [10], and oat [11] sourdoughs. Several studies have been conducted on the isolation of probiotic yeasts from different sourdoughs and their antimicrobial properties. For example, Rahimi et al. [12], in their study of 11 yeasts isolated from wheat germ sourdough, found that only the *Saccharomyces cerevisiae* strain RWGS07, with a survival rate of 95.74% under simulated gastrointestinal (SGI) conditions, exhibited strong antibacterial activity against *Listeria monocytogenes*. In addition to these properties, this yeast also demonstrated favorable aggregation and hydrophobicity capabilities.

Shruthi et al. [13] evaluated the probiotic properties of 73 yeasts isolated from different fermented substrates and ultimately identified 10 yeasts that were both safe and had desirable probiotic traits. Among these, *Meyerozyma guillermondii* strains MYSY23 and MYSY19, as well as *Meyerozyma caribbica* MYSY22, showed 65% survival under SGI conditions, over 50% inhibition against foodborne pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella paratyphi*, 90% hydrophobicity, 74.07% co-aggregation ability, and full resistance to all studied antibiotics, indicating their significant probiotic potential.

Muche et al. [14] also studied the microbial flora of Ethiopian injera sourdough and examined the probiotic properties of the isolated yeasts. They found that *S. cerevisiae* strains G1N1 and G2N4, *Candida humilis* strains G3N1 and B6N3, and *Pichia kudriavzevii* G8N1 all exhibited acceptable probiotic characteristics. The parameters studied included survival under simulated gastric and intestinal conditions, antibiotic resistance, and lack of hemolytic activity.

There are also reports on the antimicrobial effects of yeasts and their cell-wall components. For instance, five different products derived from the cell-wall of *S. cerevisiae* were assessed for their ability to inhibit the growth of *Clostridium perfringens* by analyzing pathogen growth kinetics [15]. All products inhibited the pathogen by reducing its growth rate, decreasing the maximum specific growth, and increasing the Lag phase duration. In another study, cell-wall fractions extracted from *Trichosporon mycotoxinivorans* were evaluated for their binding ability to Gram-negative pathogens such as *E. coli*,

Campylobacter, and *Salmonella*. Among these, only *Salmonella enteritidis* and *Salmonella Typhimurium* adhered to the yeast cell-wall, thereby interfering with bacterial flagella attachment to host intestinal surface cells [16].

Another study examined the effect of *Rhodotorula mucilaginosa* on *S. enterica Typhimurium*, where the yeast exhibited co-aggregation activity against the targeted bacterium [17]. The effects of three other yeasts including *Metschnikowia citriensis*, *Candida oleophila*, and *Pseudozyma antarctica* on *Penicillium digitatum* and *Penicillium italicum* were also studied. These selected yeasts inhibited fungal growth through severe cellular damage or lysis and controlled fungal bioactivity by forming biofilms and attaching to the mycelium [18]. In another study, Chen et al. [19] demonstrated the effect of *Saccharomyces boulardii* on *Clostridioides difficile*, a causative agent of gastrointestinal infection. This yeast neutralized the toxins via specific antibodies and protected the gastrointestinal tract against infection.

Based on a review of the literature, the probiotic and antimicrobial properties of the predominant yeast isolated from sprouted soybean sourdough have been less explored. Therefore, the present study investigates the probiotic and antimicrobial characteristics of the predominant yeast isolated from sprouted soybean sourdough.

2. Materials and Methods

2.1. Raw materials and flour analysis

The foodborne microorganisms used in this study included *E. coli* PTCC 1399, *S. aureus* PTCC 1112, *L. monocytogenes* PTCC 1298, *S. enterica* PTCC 1709, and *Aspergillus flavus* PTCC 5018, obtained from the Persian type culture collection of the Iranian research organization for science and technology. The microbial culture media and chemicals were purchased from Merck (Germany) and Chromagar (France). To prepare sprouted soybean, the seeds were first soaked in water at 25 °C for 24 h. Then, the soaked seeds were kept in a dark room at 25 °C for 3 days [20]. Subsequently, the seeds were dried in an oven at 50 °C for 48 h to reach a moisture content of 10%, and then ground into flour. The properties of the sprouted soybean flour were determined according to standard methods [21]. The flour used had the following composition: 13.4% fat, 34.7% protein, 11.3% moisture, 4.5% ash, and 36.1% total carbohydrates.

2.2. Spontaneous fermentation of sprouted soybean

For the fermentation process, a dough with a dough yield of 200 (dough/flour × 100) was prepared by mixing the flour with water and then incubated at 28 °C for 24 h [22].

2.3. Isolation of predominant yeast from sprouted soybean sourdough

To isolate the predominant yeast, serial dilutions of the fermented sprouted soybean were prepared in Ringer's solution and surface plated on yeast glucose chloramphenicol (YGC) agar. The plates were incubated at 28 °C for 24–48 h. To obtain pure single colonies of the predominant isolate, streak plating was performed. Microscopic morphology of the predominant yeast was also evaluated [12].

2.4. Molecular identification of the predominant yeast isolate

Genomic DNA was extracted from the predominant yeast using a commercial DNA extraction kit (Geneall, South Korea). Identification was performed via PCR using internal transcribed spacer (*ITS*) primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR products after gel electrophoresis were sequenced (Pishgam, Iran) and the obtained sequence was analyzed using the basic local alignment search tool (BLAST) against the national center for biotechnology information (NCBI) database [23].

2.5. Survival of the yeast under simulated gastrointestinal conditions

The yeast isolate was first incubated in a simulated gastric condition at pH 2 with 2 mg/mL pepsin at 37 °C for 2 h. Then, it was transferred to a simulated intestinal condition with pH 6.5 containing 0.3% bile salts and 0.5 mg/mL pancreatin and incubated at 37 °C for 3 h. Survival rate was assessed by comparing to an untreated control through serial dilution and surface plating on YGC agar [1].

2.6. Hemolytic activity

To assess hemolytic activity, the yeast isolate was streaked onto blood agar medium containing 5% defibrinated sheep blood and incubated at 28 °C for 48 h. Hemolysis types (α, β, or γ: no halo) were then evaluated [24].

2.7. Antibacterial activity

The antibacterial activity of the predominant yeast isolate was assessed using the co-culture method. Equal populations (10^6 CFU/mL) of the yeast isolate and each foodborne pathogen (*E. coli*, *S. aureus*, *L. monocytogenes*, *S. enterica*) were co-cultured in brain heart infusion (BHI) broth at 28 °C for 24 h. Serially ten-fold dilutions were plated on Chromagar specific media for each bacterium, and bacterial counts were compared with controls lacking yeast [25].

2.8. Antifungal activity

The overlay method was used against *A. flavus*. First, the yeast isolate was grown on YGC agar and incubated at 28 °C for 48 h. Then, fungal spores (10^4 spores/mL) were mixed with potato dextrose agar (PDA) and poured over the yeast-containing plates. After the second layer solidified, plates were incubated at 28 °C until the control (without yeast) fully covered the plate surface. The diameter of fungal growth inhibition zones was measured using ImageJ (version 1.4.3.67) software [26].

2.9. Auto-aggregation

Cells from a 24-hour yeast culture were centrifuged at 5000 g for 10 minutes at 4 °C (Sigma, Germany), washed twice with phosphate-buffered saline (PBS), and the initial absorbance (A_0) at 600 nm was recorded using a spectrophotometer (PGI, UK). The suspension was incubated at 37 °C for 3 h and the final absorbance (A_3) was measured. Auto-aggregation was calculated using the formula [27]:

$$\text{Auto-aggregation (\%)} = [1 - (A_3/A_0)] \times 100$$

2.10. Co-aggregation

To assess co-aggregation of the yeast isolate with *E. coli*, *S. aureus*, *S. enterica*, and *L. monocytogenes*, the yeast and bacteria (24-hour cultures) were centrifuged, washed with PBS, and mixed in equal volumes. After 24 h incubation at 37 °C, absorbance at 600 nm of each suspension (yeast alone: A_{yeast}, pathogen alone: $A_{pathogen}$, and the mixture: A_{mix}) was recorded and co-aggregation was calculated as [27]:

$$\text{Co-aggregation (\%)} = [(A_{yeast} + A_{pathogen})/2 - A_{mix}] / [(A_{yeast} + A_{pathogen})/2] \times 100$$

2.11. Hydrophobicity

The active yeast culture was centrifuged at 5000 g for 5 minutes at 4 °C, washed with PBS, and mixed with 1 mL of either hexane or xylene in 3 mL of the yeast suspension. After 3 h of incubation at 28 °C, the absorbance of the aqueous phase was measured at 600 nm. Hydrophobicity was calculated using [28]:

$$\text{Hydrophobicity (\%)} = [1 - (A_{final} / A_{initial})] \times 100$$

2.12. Sensitivity of yeast isolate to antimycotic and antibiotic agents

The disc diffusion method was used to evaluate sensitivity to common antimycotic and antibiotic agents. A 24-hour yeast culture (200 µL) was added to 4 mL of molten PDA (at 45 °C), poured into petri dishes, and antibiotic discs (streptomycin, ciprofloxacin, nalidixic acid, vancomycin, cefazolin, ceftriaxone, cephalothin, ampicillin) and antimycotic discs (fluconazole, ketoconazole, itraconazole, natamycin, potassium sorbate, calcium propionate) were placed on the surface. Plates were incubated at 28 °C for 24 h, and inhibition zone diameters were measured [29, 30].

2.13. Statistical analysis

All experiments were performed in a completely randomized design with three replications. Data were analyzed using one-way analysis of variance (ANOVA) in SPSS software (version 20). Mean comparisons were conducted using the least significant difference (LSD) test at a significance level of $p<0.05$. Microsoft Excel 2018 was also used to draw charts.

3. Results and Discussion

3.1. Molecular identification of the predominant yeast isolate

The gel electrophoresis of PCR products confirmed the presence of the targeted yeast DNA (Fig. 1). Based on sequencing results and comparison with NCBI database records, the isolated yeast was identified as *Rhodotorula mucilaginosa* with 96% sequence similarity, and was determined to be the predominant strain in the sourdough fermented sprouted soybean.

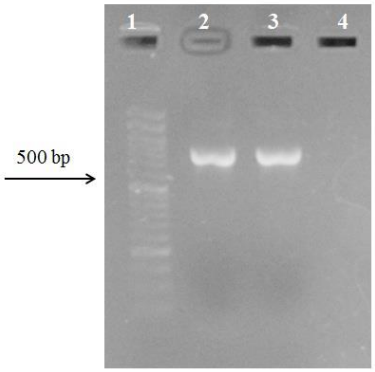

Fig. (1). Gel electrophoresis of PCR products to identify the predominant yeast isolated from sprouted soybean sourdough. Lane 1: 50 base pairs (bp) ladder, Lane 2: positive control from baker's yeast DNA amplification, Lane 3: amplification of isolated yeast DNA, Lane 4: negative control.

In a study by Agarbati et al. [31], 179 yeast strains were isolated from 13 sourdough samples, with *S. cerevisiae* and *Kazachstania unispora* being the predominant species. Similarly, Pahlavani et al. [32] identified *Wickerhamomyces anomalus* as the predominant yeast in sprouted barley sourdough. Fermentation environments act as stress ecosystems (due to pH, temperature, water activity, and osmotic pressure), which provide a niche for the isolation of potentially probiotic and antimicrobial yeasts.

3.2. Viability under simulated gastrointestinal conditions

The viability of the predominant yeast isolate under SGI conditions was recorded as 49.61 ± 1.87% compared to the control sample. In Agarbati et al. [31], the viability of *Debaryomyces hansenii*, *Kazachstania unispora*, and *S. cerevisiae* isolates under SGI conditions were 93.8%, 89.4%, and 91.6%, respectively. Another study reported that *Candida norvegensis* f2fp21 isolated from spontaneously fermented fura showed viability exceeding 100% under similar conditions [33]. Probiotic viability of at least 70% under gastrointestinal conditions is a critical requirement, as it enables microorganisms to survive and reach the target site in the gut. Key contributing factors include cell-wall integrity, stress response capabilities, and adaptability of the yeast. These features differ based on yeast strain and the fermentation substrate from which they are isolated [28].

3.3. Hemolytic activity

The isolated yeast exhibited γ-hemolysis, showing no hemolytic zones around colonies on blood agar. Hemolytic activity is categorized as α (green zone), β (clear zone), or γ (no zone). Muche et al. [14] found that all five selected yeast isolates showed γ-hemolysis, indicating safety for food applications. However, *S. unisporus* and *K. marxianus* showed α-hemolytic activity, and *Candida albicans* showed β-hemolysis in other studies [34]. Hemolytic

activity, particularly α and β types, can cause damage to intestinal epithelial cells and red blood cell lysis, highlighting the importance of γ-hemolytic or non-hemolytic strains for probiotic use.

3.4. Auto-aggregation and hydrophobicity

The auto-aggregation ability of the yeast was 85.43 ± 0.75%. Cell-surface hydrophobicity values in the presence of hexane and xylene were 56.32 ± 4.37% and 93.69 ± 1.2%, respectively.

In Menezes et al. [35], only 15 out of 115 yeast isolates showed gastrointestinal survival and high auto-aggregation. Among these, *Pichia membranifaciens*, *S. cerevisiae*, and *Candida quercitrusa* showed aggregation rates of 91.8%, 99.6%, and 95.5%, respectively. Similarly, *M. guillermondii* showed 92.75% hydrophobicity and 97.88% auto-aggregation [13]. Strains with more than 40% hydrophobicity or auto-aggregation are classified as hydrophobic and are considered capable of biofilm formation and epithelial adherence, helping to prevent pathogen colonization [36]. Yeast adhesion is a multi-step process involving hydrophobic interactions, electrostatic forces, and changes in cell-wall surface properties. Given the dynamic nature of the gastrointestinal tract, these traits are vital for colonization and survival. Yeasts often exhibit higher aggregation than bacteria, possibly due to their larger size and mass, facilitating sedimentation [2, 37].

3.5. Antibacterial and co-Aggregation activity

As shown in Fig. 2, the yeast isolate exhibited stronger antibacterial and co-aggregation activity against Gram-negative bacteria than Gram-positive strains. The highest inhibition (97.05 ± 0.58%) and co-aggregation (52.4 ± 2.51%) were also observed against *E. coli*, significantly higher (p<0.05) than against other pathogens. The lowest co-aggregation was recorded against *L. monocytogenes* (11.16 ± 3.57%) and the lowest antibacterial effect against *S. aureus* (20.71 ± 1.8%).

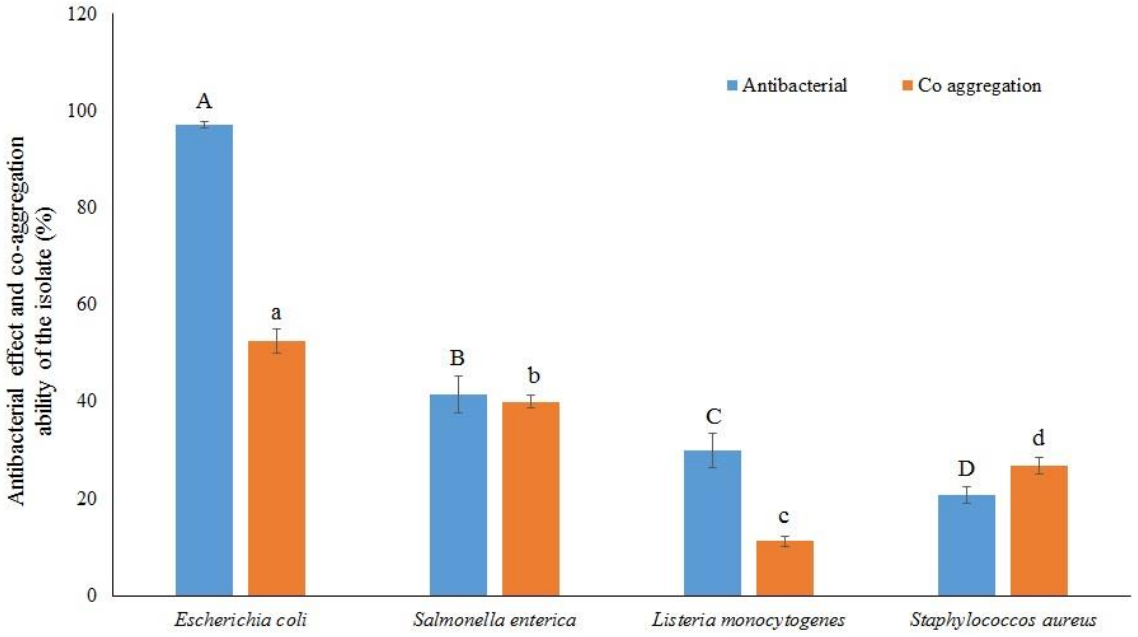

Fig. (2). The percentage of inhibition and the co-aggregation ability of the predominant yeast isolated from sprouted soybeans sourdough against some foodborne bacteria. Different lowercase and uppercase letters respectively indicate a significant difference at p<0.05 among the co-aggregation and antibacterial activities of the yeast isolate.

Agarbati et al. [31] found that 13 yeast isolates, including *S. cerevisiae* and *K. unispora*, were effective against *E. coli*, *S. enterica*, and *S. aureus*, but not *L. monocytogenes*. In another study, yeasts

including *S. cerevisiae* and *P. kudriavzii* inhibited various foodborne pathogens [28]. Antimicrobial activity in probiotics occurs through competitive elimination, secretion of inhibitory compounds, and enhancement of the intestinal barrier. Co-aggregation is another important probiotic mechanism that restricts pathogen colonization and aggregation, forming a competitive niche [2, 36]. Rahimi et al. [12] reported a maximum co-aggregation value of 75.71% against *S. enterica*, supporting the present study's observation of stronger interactions with Gram-negative bacteria.

3.6. Antibiotic and antimycotic resistance

The yeast isolate showed complete resistance to all tested antibiotics. According to Table 1, it was most sensitive to fluconazole, followed by natamycin, while it was resistant to other antimycotic agents.

Table (1). Comparison among sensitivity of yeast isolated from sprouted soybean sourdough towards the studied antimycotic compounds. Different letters indicate significant differences at $p<0.05$. The diameter of the inhibitory zone less than 10, between 10-14, and more than 20 mm respectively indicates the resistance, semi sensitivity and sensitivity of the yeast to the studied compounds.

Antifungal compounds (concentration)	Inhibition diameter zone (mm)	Sensitivity
Fluconazole (150 mg)	21.67 ± 0.74^{a}	**Sensitive**
Natamycin (50 mg)	14.75 ± 0.43^{b}	**Semi sensitive**
Ketoconazole (200 mg)	0^{c}	**Resistant**
Calcium propionate (60 mg)	0^{c}	**Resistant**
Potassium sorbate (60 mg)	0^{c}	**Resistant**
Itraconazole (100 mg)	0^{c}	**Resistant**

Shruthi et al. [13] observed resistance to all tested antibiotics in ten yeast isolates. Similarly, *K. marxianus*, *K. unispora*, and *P. fermentans* showed resistance, although *K. marxianus* showed relative sensitivity to streptomycin (1.75 mm zone). Qasim [38] found *Rhodotorula* species highly sensitive to natamycin, producing a 30 mm inhibition zone. Antibiotic resistance is a critical concern in probiotic applications. Yeasts naturally resist antibiotics and help prevent horizontal gene transfer of resistance genes to gut bacteria. Overuse of probiotic bacteria in food supplements can pose risks of transferring resistance genes to commensal gut microbes, with potential clinical consequences [2, 39].

3.7. Antifungal activity

The antifungal activity of the yeast against *A. flavus* is illustrated in Fig. 3, showing clear inhibition of fungal growth and prevention of color formation after 4 days of incubation.

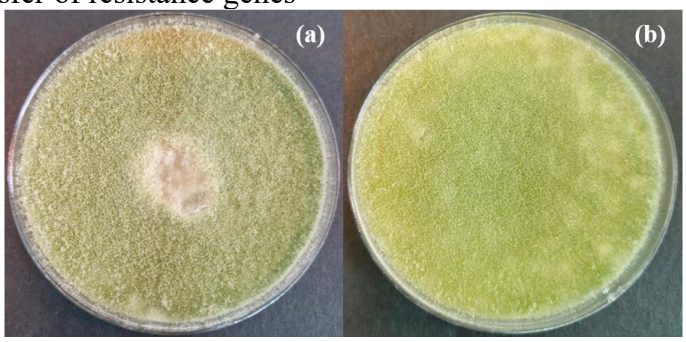

Fig. (3). Antifungal activity of *R. mucilaginosa* isolate against *A. flavus* using overlay assay (a) compared to the control sample (b).

Helmy et al. [40] reported moderate antifungal activity of *P. kudriavzevii* and *W. anomalus* against *A. flavus*, *A. niger*, and *A. fumigatus*, likely due to proteinaceous inhibitors such as mycocins. Yeasts such as *Heyerozyma guillermondii* and *H. caribbica* also reduced fungal growth by an average of 60.56% [13]. Antifungal mechanisms include competition for nutrients, secretion of antifungal metabolites (e.g., antioxidants, CO_2, methanol, low-molecular-weight peptides), chitinase activity, and mycocin production [2, 41].

4- Conclusion

Probiotic and antimicrobial properties of yeasts isolated from fermented substrates, especially as protective cultures, have recently attracted considerable attention in the food industry. In the present study, the predominant yeast isolated from sprouted soybean sourdough was first identified, and its survival under SGI conditions was evaluated. Additionally, its antibacterial effects against several important foodborne bacteria were investigated. The results showed that the *R. mucilaginosa* isolate had suitable survival rates, high auto-aggregation and co-aggregation abilities, and notable antimicrobial activity, particularly against *E. coli*. Moreover, the yeast lacked harmful hemolytic activity and demonstrated effective antifungal activity against *A. flavus*. Therefore, this probiotic yeast isolate can be used as a safe and effective bioprotective culture and natural preservative in the food industry.

5.Acknowledgments

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بررسی اثرات پروبیوتیکی و ضدمیکروبی مخمر غالب جدا شده از خمیرترش سویای جوانه‌زده

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چکیده

اخیرا بررسی مخمرهای با قابلیت پروبیوتیک جدا شده از بسترهای تخمیری به دلیل خواص عملکردی منحصر به فرد و فواید سلامتی‌بخش مورد توجه گسترده‌ای قرار گرفته است. در پژوهش حاضر، مخمر غالب جدا شده از خمیرترش سویای جوانه‌زده با استفاده از PCR شناسایی گردید. سپس ویژگی‌های پروبیوتیکی و ضدمیکروبی جدایه مخمری مورد بررسی قرار گرفت. زنده‌مانی مخمر *Rhodotorula mucilaginosa* جدا شده در شرایط شبیه‌سازی شده دستگاه گوارش، معادل ۴۹/۶۱ درصد بود. همچنین اثر ضدباکتریایی و دگراتصالی آن در برابر *Escherichia coli* نسبت به سایر عوامل غذازاد مورد مطالعه به شکل معنی‌داری ($p<0/05$) بیشتر بود و به طور کلی جدایه مخمری در برابر باکتری‌های گرم منفی خاصیت بازدارندگی بیشتری نسبت به باکتری‌های گرم مثبت نشان داد. این مخمر فاقد فعالیت همولیزی بود و قابلیت خوداتصالی آن ۸۵/۴۳ درصد و آبگریزی آن در مقابل زایلن و هگزان به ترتیب ۹۳/۶۹ و ۵۶/۳۲ درصد بود. علاوه بر این، جدایه مخمری در برابر فلوکونازول و ناتامایسین به ترتیب، حساس و حساسیت نسبی نشان داد و در برابر سایر ترکیبات ضد قارچ و آنتی‌بیوتیک‌های مورد مطالعه مقاوم بود. همچنین جدایه مخمری مانع از رشد و تغییر رنگ قارچ *Aspergillus flavus* شد. بر اساس یافته‌های مذکور، مخمر غالب جدا شده از خمیرترش سویای جوانه‌زده از قابلیت مناسبی برای استفاده به عنوان کشت پروبیوتیک محافظت کننده در صنایع غذایی برخوردار است.