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Isolation and Characterization of *Saccharomyces cerevisiae* with Antagonistic Activity against *Salmonella pullorum*

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Abstract

Saccharomyces cerevisiae is considered to be an alternative to antibiotics in reducing the growth of pathogenic bacteria and enhancing the mucosal immune system. In countries with developed livestock industries, antibiotic resistance of *Salmonella pullorum* is still causing difficulties in controlling Pullorum disease. This study aimed to isolate and select *S. cerevisiae* strains with antagonistic activity against *S. pullorum* for potential application in probiotics production. A total of eight *S. cerevisiae* strains were isolated from 50 ripe mango samples. All the isolated strains exhibited inhibitory effects against *S. pullorum*. Among them, strains SC1, SC6, and SC8 showed strong antagonism, with inhibition zone diameters of 16 ± 0.87mm, 16 ± 0.50mm, and 17 ± 1.80mm, respectively. These isolates exhibited high stability under various conditions. These findings suggest that *S. cerevisiae* strains SC1, SC6, and SC8 are potential candidates for producing a probiotic to control poultry diseases.

Keywords

Probiotics, *Saccharomyces cerevisiae*, *Salmonella pullorum*, poultry, antibiotic resistance

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Introduction

Pullorum disease is an acute infectious disease caused by *Salmonella enterica* subspecies *enterica* serovar Gallinarum biovar Pullorum (*S. pullorum*), which can result in mortality rates of up to 100% in chickens between two and four weeks of age (Yeakel, 2024). Additionally, *S. pullorum* can persist for more than 40 weeks in the ovaries of infected chickens, leading to reduced egg production, decreased hatchability, and vertical transmission through eggs

(Wigley *et al.*, 2002). Although appropriate antibiotics can treat and reduce mortality in chickens with Pullorum disease, they cannot eliminate the pathogen from the flock, and recovered individuals remain susceptible to reinfection and may serve as reservoirs for the dissemination of *S. pullorum* (Shen *et al.*, 2022). Furthermore, antibiotic therapy is becoming increasingly ineffective due to the rapid emergence of antibiotic-resistant bacterial strains (Eng *et al.*, 2015; Sun *et al.*, 2021). Circulation of multidrug-resistant *S. pullorum* has also been reported in many countries, posing a serious threat not only to the poultry industry but also to public health (Parvej *et al.*, 2016; Penha Filho *et al.*, 2016).

Saccharomyces cerevisiae has a long history of use in food and beverage fermentations and is increasingly being acknowledged for its multifaceted application in improving livestock health and disease prevention (Ballet *et al.*, 2023). In recent years, *S. cerevisiae* has been identified as a potential probiotic capable of effectively controlling various pathogenic bacteria in the digestive tract, such as *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* (Pontier-Bres *et al.*, 2014; Feye *et al.*, 2019; Latif *et al.*, 2023). Several mechanisms by which *S. cerevisiae* controls pathogenic microorganisms have been investigated, including enhancement of the immune response, competition for nutrients and intestinal adhesion at sites in the intestine with harmful bacteria, and producing substances with antibacterial and toxin-neutralizing properties (Stier & Bischoff, 2016; Lin *et al.*, 2020). In addition, during the research process, yeast was also evaluated to have a superior survival ability compared to probiotics when exposed to digestive enzymes, bile salts, and digestive juices (Gut *et al.*, 2019). Incorporating yeast into animal diets has been found to enhance metabolism and nutrient absorption while also serving as a source of essential vitamins and amino acids. Consequently, *S. cerevisiae* supplementation can improve growth performance, enhance egg and meat quality in poultry, and increase overall production efficiency (Zhang *et al.*, 2005; Invernizzi *et al.*, 2013; Elghandour *et al.*, 2020).

It can be observed that biological products derived from the yeast *S. cerevisiae* represent a promising solution to address the problems caused by *S. pullorum*, including multidrug-resistant strains. Therefore, this study was conducted to isolate *S. cerevisiae* strains with antagonistic activity against *S. pullorum* in order to reduce damage caused by Pullorum disease and minimize the use of antibiotics in poultry farming.

Materials and Methods

Isolation and identification of *S. cerevisiae* from ripe mango

Fifty ripe mango samples were randomly collected from local markets in Gia Lam district, Hanoi. The samples were immediately transported to the laboratory for *S. cerevisiae* isolation. The isolation process was conducted following the method described by Moradi *et al.* (2018) with some modifications. Each ripe mango sample was cut into small pieces. Twenty-five grams of each sample were then homogenized in 225mL of phosphate buffered saline (PBS). Next, each sample was serially diluted in PBS, plated on Sabouraud dextrose agar (SDA; Becton, Dickinson and Company, NJ, USA), and incubated at 37°C for 24-48h. Presumptive colonies of *S. cerevisiae* (white, round, and convex) were picked up for Gram staining and morphological examination under a microscope (Kurtzman *et al.*, 2011). The isolates were preserved in Sabouraud dextrose broth (SDB; Becton, Dickinson and Company, NJ, USA) containing 20% glycerol and stored at -80°C. The putative *S. cerevisiae* strains were identified using the MALDI-TOF-MS technique. Briefly, colonies were smeared onto the target plate (MBT Biotarget 96). Subsequently, 70% formic acid (1µL) was applied to the smeared spot and allowed to air-dry before adding the matrix (1µL). After 5 minutes of drying at room temperature, the plate was then loaded into the system for identification. A score value ≥ 2.0 was considered reliable for species-level identification.

Antagonistic activity of *S. cerevisiae* isolates against *S. pullorum*

The antagonistic activity of *S. cerevisiae* against multidrug-resistant *S. pullorum* was evaluated according to the method described by Lopes and Sangorrín (2010) with some modifications. *S. pullorum* was previously isolated from a Salmonellosis outbreak at a chicken farm and stored in our laboratory. The bacterial suspension of *S. pullorum* (10^8 CFU/mL) was evenly spread on the surface of Mueller–Hinton agar (MHA) plates using a sterile swab to obtain a uniform lawn. Subsequently, the *S. cerevisiae* culture (10µL) was dropped onto the agar surface and incubated at 37°C for 24-48h. Antagonistic activity was determined by observing the formation of inhibition zones around the *S. cerevisiae* colonies. The diameter of the inhibition zone was measured in millimeters (mm) and categorized as follows: 0-5mm: no antagonism, 6-10mm: weak antagonism, 11-15mm: moderate antagonism, 16-20mm: strong antagonism, and 21-25mm: very strong antagonism. The whole experiment was replicated three times.

Survival of *S. cerevisiae* isolates at different temperatures

S. cerevisiae was grown in SDB for 48 h at 37°C. The culture was then diluted to reach a concentration of 10^8 CFU mL^{-1}. The effect of temperature on the survival of *S. cerevisiae* was assessed by incubating 5mL of the suspension (10^8 CFU mL^{-1}) at 24°C, 30°C, 37°C, and 42°C for 7 days. On days 1, 3, and 7 after incubation, a portion of each sample was withdrawn and serially diluted with PBS. The diluted suspensions (100µL) were then plated on SDA. The next day, viable cells on SDA were used to determine the survival rate under the different temperature conditions.

Stability of *S. cerevisiae* isolates in acidic and bile salt conditions

The acid and bile salt tolerances of the *S. cerevisiae* isolates were determined according to the methods described by Syal & Vohra (2013) with slight modifications. Briefly, *S. cerevisiae* cultures were transferred into PBS adjusted to pH 3.0 and PBS supplemented with 0.3% bile salts. After incubation at 37°C for 3h, 0.1mL of each sample was spread onto SDA and incubated at 37°C for 48h. Following incubation, the viability of *S. cerevisiae* was determined.

Statistical analysis

Statistical analyses were performed using SPSS software (version 30.0, IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test were used to determine the statistically significant differences of the means at $P < 0.05$.

Results and Discussion

Isolation and identification of S. cerevisiae

A total of eight *S. cerevisiae* strains were isolated from the 50 ripe mango samples. On SDA, *S. cerevisiae* colonies showed white, round, and convex morphology (**Figure 1**). Microscopic examination of the *S. cerevisiae* cells revealed an ellipsoidal to ovoid morphology (**Figure 2**). The morphology of the isolates in this study was consistent with findings reported in previous studies (Pham Thi Thu Thao *et al.*, 2019; Sulmiyati *et al.*, 2019; Le *et al.*, 2023; Chavez *et al.*, 2024), indicating that *S. cerevisiae* cells are generally spherical, ellipsoidal, or oval in shape. However, variations in cell morphology and size may occur depending on several factors, including observation time, culture conditions, and medium composition (Vopálenská *et al.*, 2005; Aon *et al.*, 2018).

The isolates were identified by MALDI-TOF-MS, confirming their identity as *S. cerevisiae*, which is known for its remarkable adaptability to survive in diverse environments and is widely distributed in nature, including soil, milk, fruit, and tree bark (Goddard & Greig, 2015). However, it is most frequently isolated from ripe fruits, which serve as a favorable habitat and a rich source of fermentable sugars. Similar findings have been reported in previous studies. Hou *et al.* (2022) conducted a study screening yeast strains from fruit for wine production; three isolates were identified as *S. cerevisiae*. A study by Guimarães *et al.* (2006) on yeast isolation from grapes also reported that 14 out of 61 isolates were *S. cerevisiae*. In Vietnam, research by Ho *et al.* (2023) identified 30 yeast strains from

local fruit samples, of which seven were confirmed as *S. cerevisiae*.

Antagonistic activity of *S. cerevisiae* isolates against *S. pullorum*

The antagonistic activities of the *S. cerevisiae* isolates against antibiotic-resistant *S. pullorum* are shown in **Table 1**.

The results in **Table 1** show that all eight isolates were able to inhibit the growth of *S. pullorum*. Among them, SC1, SC6, and SC8 exhibited the strongest antagonistic activity, forming inhibition zones of 16 ± 0.87mm, 16 ± 0.50mm, and 17 ± 1.80mm in diameter, respectively. While SC4, SC2, and SC5 showed moderate antagonistic activity, with inhibition zones ranging from 11 ± 1.32 to 15 ± 0.50 mm. In contrast, SC7 (6 ± 1.00mm) and SC3 (9 ± 0.87mm) had relatively weak antagonistic activity. Based on these findings, SC1, SC6, and SC8 were then selected for further characterization.

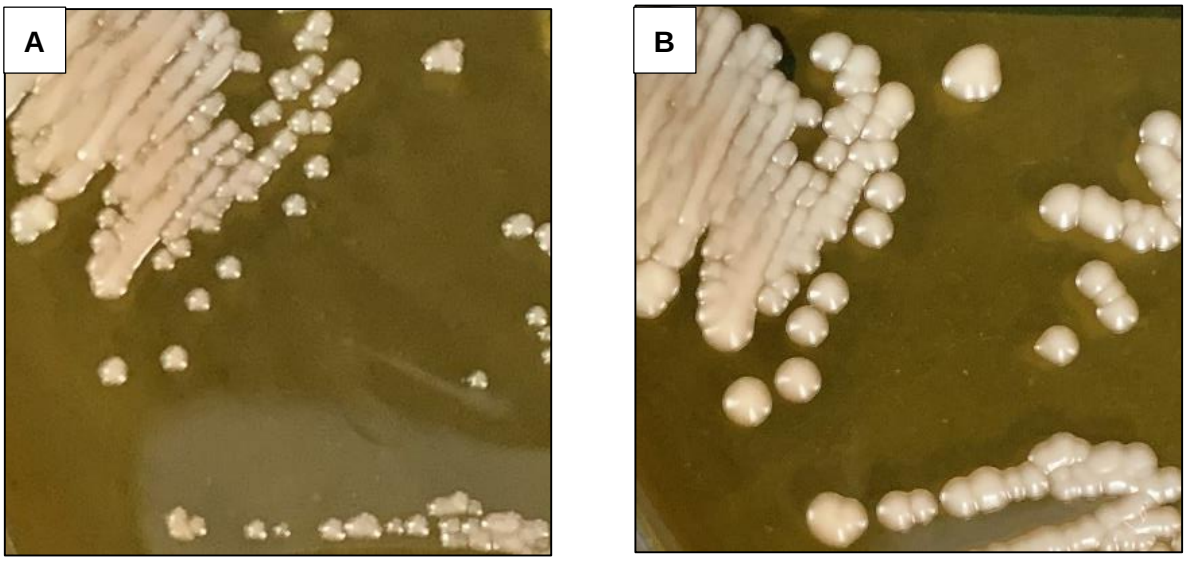

Figure 1. Colony morphology of *S. cerevisiae* after 24h (A) and 48h (B) of incubation at 37˚C

Table 1. Antagonistic activity of *S. cerevisiae* isolates against *S. pullorum*

Strain ID	Diameter (mm)	Level of resistance
SC1	16^{a} ± 0.87	+++
SC2	$12^{b, d}$ ± 1.73	++
SC3	$9^{b, c}$ ± 0.87	+
SC4	11^{b} ± 1.32	++
SC5	$15^{a, d}$ ± 0.50	++
SC6	16^{a} ± 0.50	+++
SC7	6^{c} ± 1.00	+
SC8	17^{a} ± 1.80	+++

Note: The experiment was replicated three times. Values are presented as mean ± SD. The different superscript letters indicate the significant differences of means at P <0.05 according to Tukey's HSD test. + (weak): 6-10 mm; ++ (moderate): 11-15 mm; +++ (strong): 16-20mm.

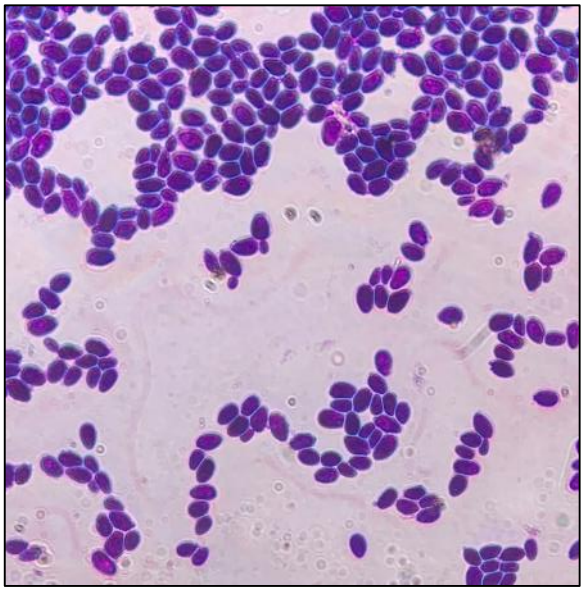

Figure 2. Microscopic morphology of *S. cerevisiae* isolated from ripe mango

The antagonistic effects of *S. cerevisiae* on intestinal pathogenic bacteria are mediated through multiple mechanisms, including the production of antimicrobial compounds and antitoxins, competition for nutrients and adhesion sites, stimulation of the host immune system, and promotion of beneficial microbiota (Abid *et al.*, 2022). In this study, the antagonistic activity of the *S. cerevisiae* isolates against *S. pullorum* may have been the result of antimicrobial compounds secreted by *S. cerevisiae* that suppressed the growth of *S. pullorum*.

Antibacterial activity is one of the important criteria for selecting probiotic candidates. Previous studies have reported the antagonistic activity of *S. cerevisiae* against several common enteric pathogens, such as *E. coli, Staphylococcus aureus, Salmonella typhimurium*, and *Clostridium perfringens* (Pontier-Bres *et al.*, 2014; Feye *et al.*, 2019; Latif *et al.*, 2023). However, there is no report on evaluating the antibacterial activity of *S. cerevisiae* against *S. pullorum*. This is the first study to indicate that *S. cerevisiae* has strong antagonistic activity against *S. pullorum*. The study conducted by Khidhr & Zubaidy (2014) showed that 6 out of 39 tested *S. cerevisiae* strains were able to form large inhibition zones on *S. typhimurium*, with diameters of 15-16mm, and 4 out of 39 formed small inhibition zones, with diameters of 10mm. In another study, the antagonistic ability of whole-cell culture, cell-free supernatant, and cell lysate of the *S. cerevisiae* IFST062013 strain was evaluated against *S. typhimurium*. The inhibition zone diameters were recorded as 11.5mm, 8.3mm, and 14.8mm, respectively. The study also indicated that *S. cerevisiae* produced a larger inhibition zone on *S. typhimurium* compared to the other 13 tested bacterial species (Fakruddin *et al.*, 2017). In our study, SC1, SC6, and SC8 generated larger inhibition zones on *S. pullorum* than those reported previously on *S. typhimurium*.

Stability of *S. cerevisiae* under various conditions

Stability at different temperatures, low pH, and the presence of bile salts are important criteria for probiotic candidates. *S. cerevisiae* has been known to be able to adapt under a wide range of temperatures among *Saccharomyces* spp. (Salvadó *et al.*, 2011). The results of this study showed that SC1, SC6, and SC8 exhibited high stability within the temperature range of 24°C to 42°C for 7 days (**Figure 3**). Overall, SC1 exhibited the greatest stability under all temperature conditions, followed by SC8. These findings suggest that *S. cerevisiae* isolates can survive in the gastrointestinal tract of chickens.

The tolerances to gastric acid and bile salts are also important criteria to select candidates for probiotic production. In this study, SC1, SC6, and SC8 exhibited a survival rate of over 90% in conditions at pH 3.0 and 0.3% bile salts (**Figure 4**). For the pH test, the highest stability was observed for SC1 (96.18%), followed by SC6

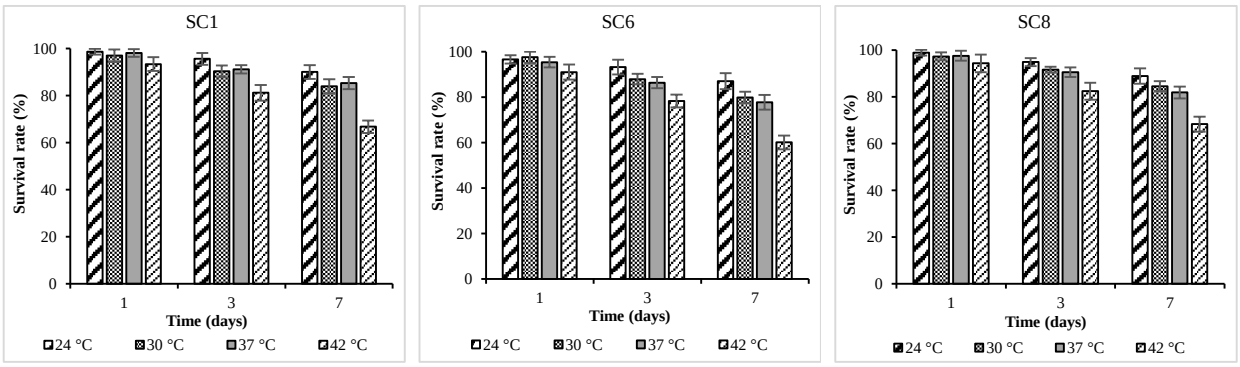

Figure 3. Survival of SC1, SC6, and SC8 at different temperature conditions

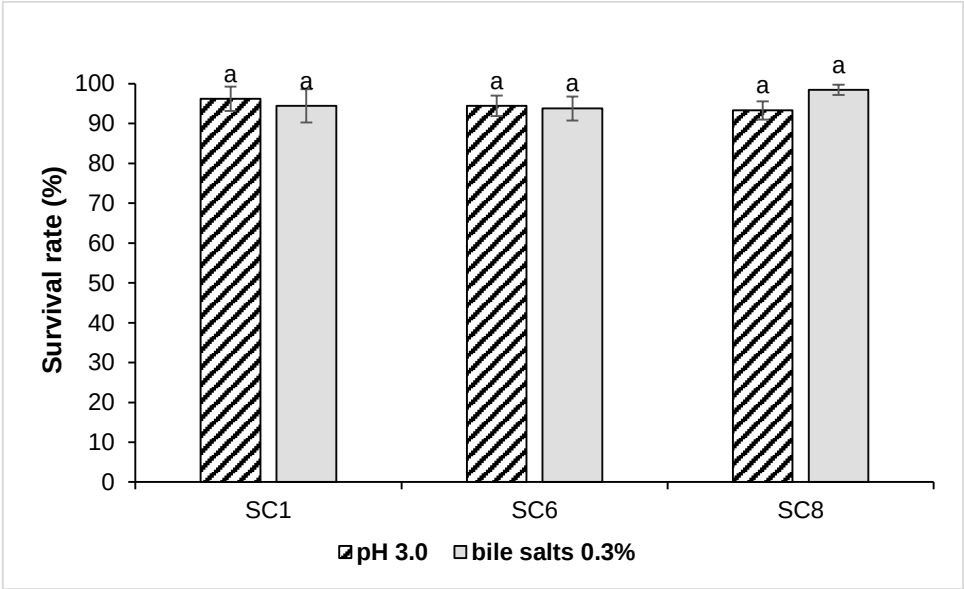

Figure 4. Survival of SC1, SC6, and SC8 under pH 3.0 and 0.3% bile salts

(94.41%), and SC8 (93.29%), respectively. On the contrary, SC8 showed the greatest bile salt resistance, with a survival rate of 98.49%, followed by SC1 (94.43%) and (93.77%).

In the study of Mogmenga *et al.* (2023), *S. cerevisiae* isolates exhibited high survival rates, ranging from 86.01% to 99.98% at pH 2.0, and from 95.41% to 100% under 0.3% bile salt conditions. Syal and Vohra (2013) reported that exposure of *S. cerevisiae* strains to low pH values (2.0, 2.5, and 3.0) and 1% bile salt did not significantly affect their growth, with survival rates ranging from 93.60% to 100.00%. Similar results were also recorded in other studies (Moradi *et al.*, 2018; Romero-Luna *et al.*, 2019; Wang *et al.*, 2024). Although the survival and growth abilities of *S. cerevisiae* are strain-dependent, the results of our study revealed that SC1, SC6, and SC8 had great adaptability to the digestive tract environment. However, resistance to digestive enzymes such as pepsin and trypsin is also a crucial criterion to determine the survival of isolates in the host's digestive tract. Therefore, further studies are needed to evaluate the impact of digestive enzymes on the survival of the isolates.

Conclusions

The findings in our study showed that SC1, SC6, and SC8 had strong antagonistic activity against *S. pullorum* and great stability in a wide range of temperatures, pH 3.0, and 0.3% bile salts. However, further studies are

needed to evaluate the antibiotic resistance profiles of the isolates and their efficacy in controlling *S. pullorum* in chickens. Overall, SC1, SC6, and SC8 are promising candidates for probiotic production.

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