Research Article

APPLIED FOOD BIOTECHNOLOGY, 2024, 11 (1): e32
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214
OPEN ACCESS

Fungal Pretreatment Strategy to Enhance Growth of *Pediococcus acidilactici* via Solid-state Fermentation of Spent Malt Grain

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Abstract

Background and Objective: Growing probiotic bacteria on agricultural wastes via solid-state fermentation presents challenges, compared to fungal fermentation. Spent malt grain, a byproduct of non-alcoholic malt beverage production, is rich in proteins, fibers, minerals and bioactive compounds, making it a promising substrate for solid-state fermentation. However, the lignocellulosic structure of spent malt grain limits nutrient accessibility for probiotics. Fungal pretreatment with *Aspergillus oryzae* can degrade these complex fibers, enhancing nutrient availability and fermentation potential of the spent malt grain. This study aimed to select probiotic bacteria with superior characteristics, assess fungal pretreatment effects on viability as well as changes in spent malt grain composition during solid-state fermentation.

Material and Methods: Four probiotic strains were assessed for antimicrobial, antitoxic and antioxidant characteristics, as well as acid and bile resistance. *Pediococcus acidilactici* was selected as the optimal strain. Solid-state fermentation was carried out using spent malt grain with fungal pretreatment to enhance *Pediococcus acidilactici* growth.

Results and Conclusion: Probiotic viability increased from 9.4 to 9.76 log CFU.g^{-1}, protein content increased by 32.45% and ash by 59.6%, while neutral and acid detergent fibers decreased by 21.6 and 9.1%, respectively. These results demonstrated effectiveness of fungal pretreatment combined with solid-state fermentation, providing a sustainable method for enhancing probiotic growth on spent malt grain with potential uses in ruminant and poultry feeds.

Conflict of interest: All authors declare no conflict of interest.

Article Information

Article history:

- Received 23 Sep 2024
- Revised 31 Oct 2024
- Accepted 12 Nov 2024

Keywords:

- Antimicrobial activity
- Antitoxic characteristics
- Biological pretreatment
- Brewer's spent grain (BSG)
- Probiotic bacteria
- Survival rate

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How to cite this article

Shokouhi M, Hamidi-Esfahani Z, Karimi-Torshizi MA. Fungal Pretreatment Strategy to Enhance Growth of Pediococcus acidilactici via Solid-state Fermentation of Spent Malt Grain. Appl Food Biotechnol. 2024; 11 (1): e32. http://dx.doi.org/10.22037/afb.v11i1.46281

1. Introduction

Solid-state fermentation (SSF) is an effective method for enhancing the nutritional value of agricultural and industrial wastes. It decreases biological wastes, prevents its accumulation in the environment and generates beneficial and nutritious compounds in primary substrates, allowing for reuse in the food cycle [1]. Brewers' spent grain (BSG), a major byproduct of the beer industry, is rich in fibers, minerals, proteins and other valuable chemical compounds [2]. In the present study, spent malt grain (SMG), which contains similar compounds to BSG, was used and sourced from a non-alcoholic malt beverage production facility. The SSF has been interested due to its ability to grow micro-organisms on waste substrates under limited or non-free water conditions. This process can enhance the substrate by decreasing fiber and increasing protein and amino acid contents, making it appropriate as food or feed supplements for humans and animals. Solid-state fermentation of BSG using probiotic microorganisms includes the potential to produce products with beneficial effects on consumers [3].

Probiotics are defined as "live microorganisms that provide health benefits to the host once consumed in sufficient quantities" [4]. Use of probiotics not only plays a role in human health, but also enhances natural digestion and maintains health of animals. In addition, it is effective in preventing activity of pathogens, metabolism of nutrients, removal of toxic substances and energy balance [5]. Beneficial effects of probiotic microorganisms on the substrate are connected to their ability to tolerate conditions

of the stomach and intestines in humans and animals, which completely depends on the type of microorganisms and their resistance to acid and bile. Probiotics such as Lactobacillus strains include proteins resistant to acid shock. Numerous microorganisms are susceptible to destruction by bile salts due to the presence of lipids and fatty acids in their cell membranes. However, many lactic acid bacteria (LABs) are resistant to bile [6, 7].

Fermentation of agricultural wastes using probiotic bacteria enhances antioxidant and antimicrobial activities of the fermented products and protects gut microbiota against oxidative stress [8]. Fermentation of cereal wastes with *P. acidilactici* inhibits growth of *Aspergillus*, *Penicillium* and *Fusarium* Sps. by producing antimicrobial compounds; thereby, preventing mycotoxin production. The resulting fermented products, rich in probiotic bacteria, can be used in human foods and animal feeds [9]. The complex lingocellulosic structure of agricultural wastes poses challenges for their use as substrates by microorganisms, especially bacteria. Therefore, various pretreatment methods are used to modify them. These methods, including physical, chemical, physicochemical, ionic liquid and biological approaches, have been studied for enhancing nutrient availability in BSG. Each method includes strengths and limitations. Physical pretreatments need high energy but may not fully degrade lignocellulose. Chemical methods improve nutrient accessibility but create inhibitory byproducts that need neutralization. Ionic liquids offer effective breakdown but are expensive and challenging to recover. Physicochemical treatments are efficient but costly. Optimizing BSG physicochemical pretreatment processes can decrease energy consumption. This enhances efficiency of nutrient release and substrate degradation. Biological pretreatments with white and soft-rot fungi are environmental friendly and produce nutrient-rich substrates; however, they are time-intensive. [10,11]. This method modifies lignocellulosic structure of the substrate by partially or completely removing lignin; thereby, enhancing microbial access to cellulosic parts of the raw materials. The primary focus includes breaking down lignin and hemi-cellulose; then, allowing it to digest cellulose effectively [12]. Use of fungi in fermentation process increases nutritional quality of the animal feeds prepared from BSG [13].

Carrying out and optimizing a pretreatment facilitate probiotic growth; by which, cellulosic and hemicellulosic structures of BSG are broken down. For example, *A. oryzae* is an appropriate option for the fungal pretreatment of BSG because of its lignocellulosic, proteolytic and amylolytic enzymes [14]. Treatment with *A. oryzae* results in production of fermentable sugars and increases in the substrate protein contents. When *A. oryzae* spores are directly used in BSG treatment, they can serve as a nutrient source in a probiotic propagation media [15]. In previous studies, SSF of BSG has majorly been carried out using fungi and a bacterial strain of *Bacillus* Sp. for purposes, including production of value-added fermented products, enzymes, lactic acid and proteins [13,16,17]. However, use of probiotic bacteria to enhance the nutritional value of this substrate has not been investigated. From the four probiotic bacterial strains assessed in the study (based on their resistance to acid and bile and antimicrobial, antitoxic and antioxidant activities), the best one was selected for investigating growth on SMG and fungal pretreatment.

In this study, SSF of SMG was investigated for the first time using probiotic *P. acidilactici* and fungal pretreatment of *A. oryzae* PTCC 5163. Effects of this pretreatment on the growth and viability of the selected probiotic bacterium and modification of the chemical composition were investigated as well.

2. Materials and Methods

2.1 Materials

Most chemicals and brain-heart infusion soft agar (BHI), Rogosa Sharpe broth (MRS broth), Rogosa, Sharpe agar (MRS agar), nutrient agar (NA) and nutrient broth (NB) were purchased from Merck, Darmstadt, Germany. Potato dextrose agar (PDA agar), ProMedia, bile salts and DPPH were purchased from Merck and Sigma-Aldrich, Darmstadt, Germany. Rice containing aflatoxin was provided by Department of Poultry Sciences, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. The *P. acidilactici* is a commercial strain isolated from Bactocell© probiotic (Lallemand Animal Nutrition, France), *L. plantarum* MT. ZH393, *L. fermentum* MT. ZH893, lactobacilli isolated from Mazandaran cheese (isolated in Department of Food Science and Technology, Tarbiat Modares University), *Escherichia coli* Nissle 1917 isolated from Mutaflor probiotic, *A. oryzae* PTCC 5163, *Salmonella* serovar enteritidis RTCC 1621, *Salmonella enterica* serovar typhimurium RTCC 1679, *Salmonella* serovar infantis ATCC 51741, *E. coli* O1:K1, *E. coli* O2:K2 and *E. coli* O78:K80 (strain χ1378) were provided by the microbial collection of the faculty of veterinary medicine, university of Tehran, Tehran, Iran. The SMG was provided by Behnoosh, an alcohol-free beer manufacturer, Tehran, Iran.

2.2 Methods

2.2.1 Probiotic strains

Four probiotic strains were used in this study, including *P. acidilactici*, *L. plantarum* MT. ZH393, *L. fermentum* MT. ZH893 and *E. coli* Nissle 1917.

2.2.2 Preparation of microbial cultures

To activate the probiotic strains, except *E. coli* Nissle 1917, a small quantity of the lyophilized microbial powder was poured into 10 ml of sterile MRS broth. Mixture was incubated at 37 ˚C for 24 h under anaerobic conditions. Nutrient broth was used for the growth of *E. coli* Nissle 1917. Overnight cultures of probiotic strains were centrifuged at 6000× g for 10 min at 4 °C (Model 3-30KS, Sigma, Germany). Cells separated from the culture media were washed twice with sterile Ringer's solution and then suspended in Ringer's solution. Suspensions were prepared to 0.5 McFarland standard ($\simeq 1.5\times10^8$ CFU.ml^{-1}) and inoculated to the substrate at an inoculum culture volume of 20% of the total substrate (v.w^{-1}). Methods of preparing microbial suspension were similar for all the experiments of the study needing microbial suspension. Pathogenic indicator bacteria were activated in Mueller-Hinton broth for 24-48 h at 37 °C. To prepare stock culture of *A. oryzae* PTCC 5163 spores, the microorganism was cultivated on the surface of PDA. Spores were formed and collected after 10-12 d of incubation at 25 °C. Spores were passed through cotton filters to separate mycelia and then diluted with a phosphate buffer saline (PBS) solution (pH 7). The number of spores was adjusted to 1.6×10^7 spores.ml^{-1} using a hemacytometer slide (MarienFeld, Germany). Then, sterile glycerol 10-13% (vol.) was added to the mixture. This was divided into small vials and stored at -20 °C until use.

2.2.3 Antimicrobial activity

Agar spot test was used to assess the ability of strains to inhibit the growth of pathogenic microbes (*Salmonella* serovar enteritidis RTCC 1621, *Salmonella* serovar typhimurium RTCC 1679, *Salmonella* serovar infantis ATCC 51741, *E. coli* O1:K1, *E. coli* O2:K2 and *E. coli* O78:K80 *(*strain χ1378*)*. Two microliters of a 24-h culture of *L. plantarum* MT. ZH393, *L. fermentum* MT ZH893, and *P. acidilactici* were spotted on the surface of MRS agar and *E. coli* Nissle 1917 strain was spotted on nutrient agar. All plates were incubated anaerobically at 37 °C for 24 h. Then, 10 ml of BHI soft agar (0.7%) were mixed with 100 µl of the activated pathogenic microorganisms and spread on the surface of MRS agar. Media were incubated at 37 °C for 24 h [18]. Presence of a clear zone or halo less than 1 mm was classified as +, while a halo of 2–5 mm was categorized as ++, a halo greater than 5 mm or complete inhibition of growth was categorized as +++ and absence of any halo formation was categorized as –.

2.2.4 Resistance to acidic conditions

Briefly, *L. plantarum* MT, ZH393, *L. fermentum* MT. ZH893 *and P. acidilactici* in MRS broth and *E. coli* Nissle 1917 in nutrient broth were incubated at 37 °C for 48 h. After assessing population of the microorganisms, 1 ml of each medium was inoculated into 9 ml of PBS (pH 7.4) with pH adjusted to 2.5 using HCl. After 2 h of incubation at 37 °C, number of the micro-organisms was re-enumerated. Results were compared to assess effects of acidic environment on decreasing population of probiotic strains [18, 19].

2.2.5 Bile resistance

Bile resistance of the microorganisms was assessed using a method by Sharifi Yazdi *et al*; in which, 100 µl of the microbial suspension (prepared as described in Section 2.2.2) were added to the culture media with bile salts (Model B8756, Sigma-Aldrich, Germany) and without bile salts, respectively. Then, light absorption of the samples was measured using a spectrophotometer (Model Cary 60 UV-Vis, Agilent Technologies, USA) at 600–650 nm before and after 8 h of incubation [20]. The inhibition coefficient (C_{inh}) was calculated using the Eq. 1.

$$C_{inh} = (T_8 - T_0)_{control} - (T_8 - T_0)_{treatment}) / (T_8 - T_0)_{control} \quad \text{Eq. 1}$$

Where, T_0 and T_8 included reading at 0 and 8 h, respectively.

2.2.6 Antioxidant activity

One milliliter of each microbial suspension was added to 1 ml of a newly prepared DPPH solution (2,2-diphenyl-1-picrylhydrazyl). Then, mixture was agitated strenuously and set for 30 min at ambient temperature in dark. After re-centrifugation (8000× g, 10 min, 4 °C), decreases in absorbance were measured at 517 nm [8,21]. The scavenging activity was calculated using Eq. 2.

$$\text{Scavenging activity (\%)} = 1 - (A_{sample} - A_{blank} / A_{control}) \times 100 \quad \text{Eq. 2}$$

Where, sample included cells, DPPH, and methanol, blank included cells and methanol, and control included Ringer's solution and DPPH.

2.2.7 Antitoxic characteristics

In this study, the toxin included aflatoxin. The best food method was used to extract aflatoxins from contaminated rice based on the AOAC official method [22]. Generally, 1 g of the toxin-containing sample was mixed with 20 ml of 55% (vol.) aqueous methanol and agitated for 30 min. Then, 10 ml of hexane were added to the mixture and agitated for 15 min. Then, mixture was centrifuged at 2000× g for 5 min and the supernatant was discarded. Then, 20 ml of chloroform were added to the mixture and agitated gently for 15 min. The lower phase was filtered using filter paper coated with anhydrous sodium sulfate (1 g in the bottom of the funnel) and the toxin dissolved in chloroform was concentrated by evaporation of chloroform. The extracted toxin was dissolved in 5 ml of 55% (vol.) aqueous methanol and added drop by drop to 20 ml of phosphate buffer solution saline (pH 7) until the absorbance of 360 nm reached nearly 0.1 (initial absorption). A microbial suspension was prepared from each strain overnight culture

with a concentration of 8 McFarland (2.4×10^9 CFU.ml^{-1}). Then, 1 ml of the microbial suspension was poured into a 2-ml microtube and centrifuged at 10000× g for 10 min (Heraeus Biofuge Pico, Thermo Fisher Scientific, USA). Supernatant of the microbial pellets was discarded and the pellets were washed twice with PBS solution (pH 7). One milliliter of the prepared toxin was added to the pellets in the bottom of the microtubes. Toxin and pellets were incubated for 4-5 min and thoroughly mixed. They were incubated for 1 h, absorbance was read at 360 nm and the absorption decrease percentage was calculated through Eq. 3.

$$\text{Absorption decrease (\%)} = \frac{\text{Initial absorption} - \text{secondary absorption}}{\text{Initial absorption}} \times 100 \quad \text{Eq. 3}$$

2.2.8 Spent malt grain preparation

Wet SMG from a malt beverage factory was dried at 50 ˚C for 24 h using the oven (Model NST-F300-51605, Noor Sanat Ferdows, Iran). After reaching a constant weight and grinding, this was passed through a 1-mm sieve and stored at 4 °C for further use.

2.2.9 Preparation of spent malt grains for solid-state fermentation

Briefly, 10 g of SMG was poured into a 250-ml Erlenmeyer flask. The moisture was adjusted to 70% based on dry matter and pH was adjusted to 5.4 using calcium carbonate solution (2.5% w.v^{-1}), which was an appropriate pH for the growth of *A. oryzae* PTCC 5163. Then, this was sterilized at 121 °C for 15 min using autoclave (Model 121 A, Iran Tolid, Iran).

2.2.10 Fungal pretreatment

Briefly, 2 ml of *A. oryzae* PTCC 5163 spores (1.6×10^7 spores.ml^{-1}) were inoculated into sterile SMG, mixed well and incubated at 30 °C for 24 h. Sterile calcium carbonate solution (2.5% w.v^{-1}) was used to adjust the pH to 6.15–6.2. It is noteworthy that all steps were carried out under completely sterile conditions. Then, 2 ml of a *P. acidilactici* suspension (prepared as described in the Preparation of Microbial Cultures section) adjusted to 0.5 McFarland were inoculated into the sample; in which, *A. oryzae* PTCC 5163 was grown for 24 h. Flasks were sealed with parafilm and incubated at 37 °C for 24 h. To assess effects of fungal pretreatment, a series of experiments were carried out that involved inoculating the probiotic bacteria without prior fungal treatments.

2.2.11 Composition analysis of spent malt grains

Crude protein content was assessed using Kjeldahl method and the nitrogen content was multiplied by 6.25. Moreover, Soxhlet extraction was used to assess the lipid content (Model PSU-500S, Pecofood, Iran). To assess the ash content, samples were burned in electric furnaces at 650 °C for 4 h (Model SIC 37, Ecotec, Iran). Moreover, the moisture content was estimated using weight differences at 105 °C for 3.5 h using oven (PAAT ARIA, Iran). All the assays were carried out according to AOAC [23]. Acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) were assessed using sodium sulfite and necessary solutions, based on the Van Soest method [24]. Hemicellulose was investigated from the differences between NDF and ADF and cellulose was investigated from the differences between ADF and ADL [17].

2.2.12 Statistical analysis of data

To assess significant differences between the means, one-way analysis of variance was carried out using Duncan's multiple range test. All experiments were carried out in triplicate, with a significance level set at $p < 0.05$. Additionally, figures were created using Excel 2016 software (Microsoft, USA).

3. Results and Discussion

3.1 Antimicrobial activity

Among the probiotic strains, *P. acidilactici* completely prevented growth of the pathogenic strains. Moreover, *L. fermentum* MT. ZH893 and *L. plantarum MT.* ZH393 included good antimicrobial characteristics, compared to *E. coli* Nissle 1917 which included a weaker antimicrobial activity than other strains (Table 1, Figure 1). Metabolites such as bacteriocins produced by *P. acidilactici* might inhibit other LAB strains. Moreover, lactic and acetic acids from the fermentation process led to decreases in pH below 4.5; thus, preventing growth of pathogenic bacteria [9]. In a study by Tavakoli et al. strains of *L. plantarum* and *L. fermentum* showed desirable antimicrobial activities due to their metabolites e.g. organic acids, bacteriocins, and hydrogen peroxide [18]. In another study, it was indicated that metabolites of *P. acidilactici* such as bacteriocins demonstrated antimicrobial activities against three indicator bacteria of *L. monocytogenes*, *E. coli* and *S. aureus* [25].

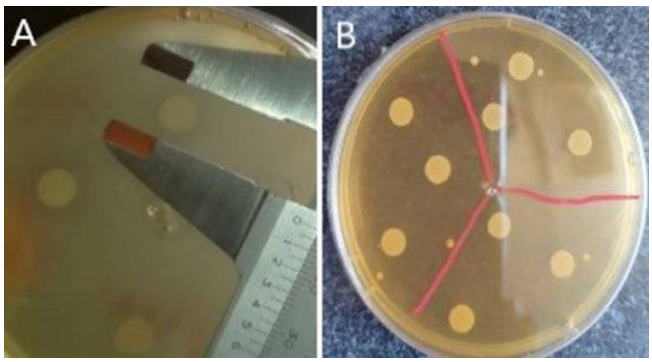

Figure 1. Measurement of the inhibition zones created by the probiotic bacteria (A) and complete inhibition of the pathogenic microorganisms by *Pediococcus acidilactici* (B)

Table 1. Antimicrobial activities of probiotic strains

Probiotic strains	*Salmonella infantis*	*Salmonella typhimurium*	*Salmonella enteritidis*	*E. coli* O2: K2	*E. coli* O78: K80	*E. coli* O1: K1
E. coli Nissle	-	++	-	++	-	-
L. Plantarum	++	++	++	++	++	++
L. fermentum	++	++	++	++	++	+++
P. acidilactici	+++	+++	+++	+++	+++	+++

The absence of a clear halo or a halo less than 1 mm was determined as (+), a halo of 2-5 mm (++), a halo less than 5 mm or complete inhibition of growth (+++), and no halo formation (–)

3.2 Resistance to acidic conditions

From the four strains, only *E. coli* Nissle 1917 showed significantly lower resistance to acidic conditions, compared to those the other strains did. The other probiotic strains demonstrated excellent acid resistance with no significant differences. Survival rates in acidic conditions for *L. fermentum* MT. ZH893 and *P. acidilactici* were assessed as 96 and 95.7%, respectively (Figure 2). Regarding resistance to acidic conditions, findings of this study were similar to those of a study; in which, *P. acidilactici* showed appropriate resistance to stomach acid conditions. This was possibly because of its ability to decrease activity of the H^+-ATPase enzyme, which regulated internal and external pH and led to the resistance of microorganisms to acidic conditions [26]. In a study by Tavakoli et al., *Lactobacillus* strains showed resistance to acidic conditions and survival of the strains in acidic conditions varied 68.8–94.9% [18]. Several theories are available on the resistance of Gram-positive bacteria such as LAB to acidic conditions, including stability of their mRNA as well as systems responsible for modifying composition of the cell membrane, extruding protons, protecting macromolecules, changing metabolic pathways and producing alkali. Furthermore, proton pumps heavily rely on the F1F0-ATPase to maintain internal pH [27].

3.3 Bile resistance

Results of the present study demonstrated that all strains included good resistance to bile salts, however, inhibitory coefficient of *P. acidilactici* was the lowest, thus its resistance to bile was the highest (Figure 3). Resistance to bile in the probiotic strains might be explained by its special bilayer structure that could tolerate unfavorable alkaline conditions [26]. Probiotic strains resistant to bile salts can survive in the digestive system, form colonies and subsequently provide health benefits for humans and animals. Probiotic survival against bile balances the gut microorganisms and enhances overall health and performance [28]. Similarly, another report revealed that six probiotic isolates, belonging to *P. acidilactici*, included good bile resistance [20].

3.4 Antioxidant activity

Results indicated that *P. acidilactici* and *L. fermentum* MT. ZH893 included the highest scavenging rates (nearly 65%), compared to *L. plantarum* MT. ZH393 with the lowest rate (49%) (Figure 4). Regarding good antioxidant activity of the probiotic strains especially *P. acidilactici*, membrane-bound enzymes might be responsible for the antioxidant activity. One of these enzymes is dipeptidyl peptidase-III (DPP-III), which decreases oxidative stress and its associated diseases [29].

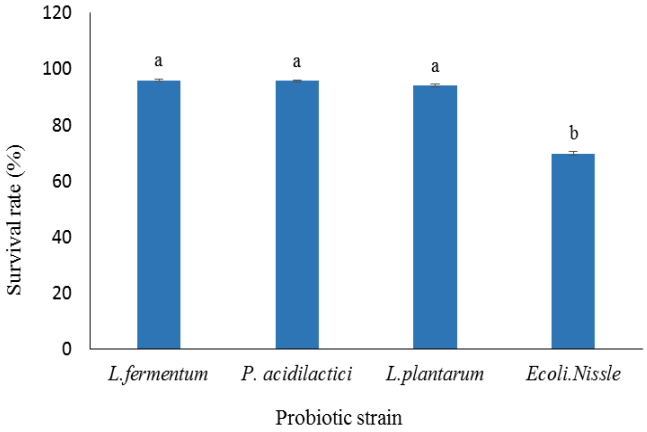

Figure 2. Survival rates of the probiotic strains in acidic conditions equivalent to acidic conditions of the stomach. Statistical differences ($p < 0.05$) are denoted by letters a–b.

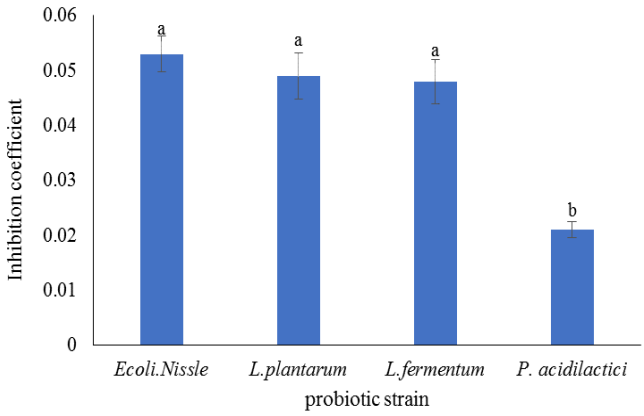

Figure 3. Inhibition coefficients of the probiotic strains. Resistant, (Cinh ≈ 0); growth delay, (0.2 < Cinh < 0.4); and sensitive, (Cinh > 0.4). Statistical differences ($p < 0.05$) are denoted by letters a–b.

Li et al. reported that the highest quantity of scavenging activity for *L. plantarum* strains, isolated from Chinese fermented food, was roughly 50%; similar to the findings of the present study. Based on their study, proteins or polysaccharides on the cell surface contributed to the antioxidant activity of this strain since removing these compounds decreased capacity to remove DPPH free radicals [21].

In another study, a wild *L. plantarum* strain isolated from fermented cabbages significantly improved scavenging activities of superoxide anion, DPPH and hydroxyl radicals. Naturally, these radicals contribute to lipid oxidation, as they are precursors of singlet oxygen and hydroxyl radicals [30].

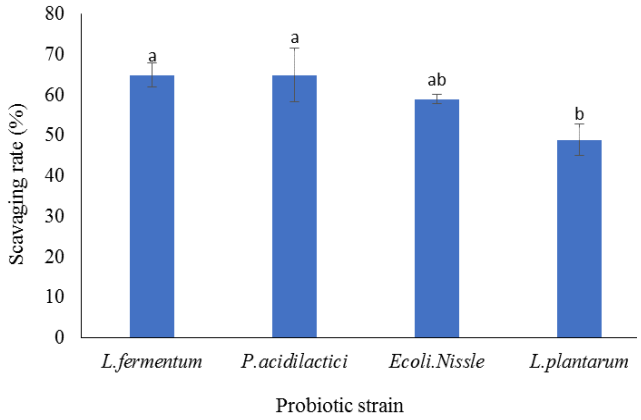

Figure 4. The DPPH free radical scavenging activities by the probiotic strains. Statistical differences ($p < 0.05$) are denoted by letters a & b.

3.5 Antitoxic characteristics

Results of the present study demonstrated that all probiotics could bind to aflatoxins, although two strains of *P. acidilactici* and, *L. fermentum* MT. ZH893 showed greater absorption rates. Moreover, no significant difference was seen at 5% level with *L. plantarum* MT. ZH393 (Figure 5).

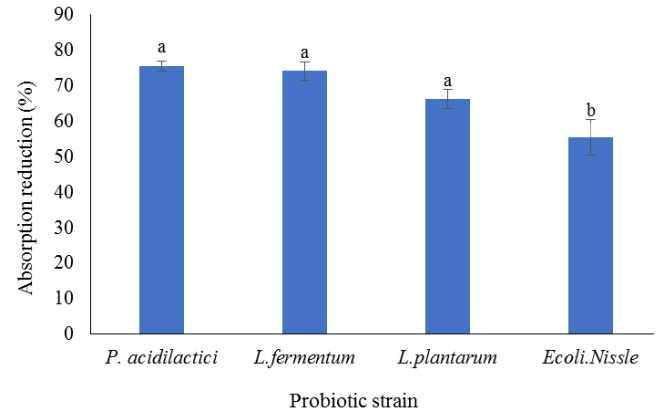

Figure 5. Rates of the decreases in absorption of aflatoxins by the probiotic strains. Statistical differences ($p < 0.05$) are denoted by letters a & b.

Studies have shown strain-dependent relationships between toxin binding and LAB. Furthermore, it was demonstrated that polysaccharides and peptidoglycans of the bacterial cell walls possibly contributed to aflatoxin binding [31]. Since probiotic bacteria attach to aflatoxins through physical adhesion rather than covalent bonding, non-viable bacterial cells can bind to them as well. In other words, probiotic microorganisms (bacteria and fungi) and their enzymatic metabolites detoxify aflatoxin molecules by cleaving their difuran rings [32]. Similarly, it is reported that *L. plantarum* isolated from a type of fermented cheeses demonstrated a strong ability to bind to aflatoxin B1 when the microorganism was alive and after it was killed by heat [33].

3.6 Fungal pretreatment

After selecting *P. acidilactici* based on its superior probiotic characteristics, effects of fungal pretreatment with *A. oryzae* PTCC 5163 on its growth and survival were studied. Results of the present study indicated that fungal pretreatment at various pH levels included significant differences in the number of probiotics, compared to the control condition (without fungal pretreatment). Adjusted pH values were assessed based on the pH range appropriate for the growth of fungi and bacteria. At all pH values, pretreatment by *A. oryzae* PTCC 5163 significantly increased the number of *P. acidilactici* after 48 h of fermentation (from 9.4 to 9.76 log CFU. G^{-1}) (Figure 6). The BSG is full of lignocellulosic materials that can be destroyed by filamentous fungi, whose hyphae can easily penetrate the inter-particle space. Therefore, using fungi for pretreatment is an appropriate alternative to chemical and physical methods because in addition to be cost-effectiveness, they can easily be used in SSF [13].

The *P. acidilactici* can convert cereal byproducts into products that can be used in human and animal foods [9]. Enhancing the microbial growth and survival yields further favorable outcomes in the substrate. Pretreatment of lignocellulosic materials enhances accessibility of the substrate for probiotic bacteria because of the hydrolysis of hemicellulose, cellulose and lignin. Fungal pretreatment by *Aspergillus* strains with amylolytic and cellulolytic enzymes is an environmental friendly method that increases access of probiotic bacteria to materials needed for their growth [34, 35]. A study showed that use of *A. oryzae* and *S. cerevisiae* could produce bioethanol from BSG. Technically, *A. oryzae* breaks down cellulose and hemicellulose, creating sugars for ethanol production by *S. cerevisiae*. This pretreatment provides necessary enzymes without excessive sugar breakdown or high levels of furan-based inhibitors [36].

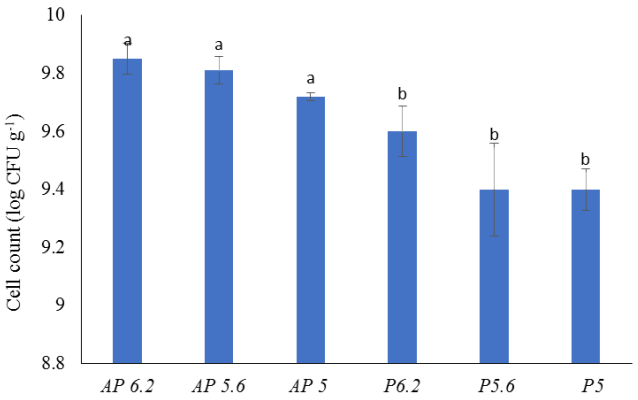

Figure 6. Effects of fungal pretreatment and solid-state fermentation on *Pediococcus acidilactici* survival. (P) Refers to *Pediococcus acidilactici* and (A) refers to *Aspergillus oryzae* PTCC 5163. The pH values are 5, 5.6, and 6.2. Statistical differences ($p < 0.05$) are denoted by letters a & b.

3.7 Assessing spent malt grain compounds

Fermentation of SMG with *P. acidilactici* following fungal pretreatment with *A. oryzae* PTCC 5163 led to significant increases in protein contents with 32.4% increases (Table 2). Increases in protein during fermentation could include various causes. One possible reason included the accumulation of fungal and bacterial biomasses in SMG substrate after fermentation [17]. These results were similar to results reported by Bekatorou et al., who reported 20–36% increases in the protein contents of BSG, when treated by *Aspergillus* strains [15]. In the fermentation of brewer's dried grains and spent sorghum using microorganisms, more than 30% increases in protein contents were reported [38]. The lipid contents of SMG did not change significantly after fermentation (Table 2). This finding was similar to those of other studies, where fermentation with LAB did not lead to significant changes in lipid contents [8].

A significant result of the fermentation process included decrease in fiber contents. The NDF and ADF decreased by 17.7 and 8.3%, respectively. Additionally, cellulose and hemicellulose contents decreased by 27.35 and 8.5%, respectively (Table 2). This decrease in fiber contents could be attributed to activity of the microorganisms and their metabolites, breaking down complex carbohydrates such as cellulose and hemicellulose. These findings are supported by previous studies; in which, fermentation with LAB and their metabolites such as organic acids and bacteriocins led to decreases in fiber contents. Similarly, SSF of BSG with fungi have been shown to decrease fiber and cellulose contents [37]. Fermentation process resulted in a significant increase of 59.6% in ash contents (Table 2). Increase in ash contents might reflect accumulation of minerals and other inorganic components in fermented SMG or was likely due to metabolic activity of the microorganisms and their metabolites during fermentation [17]. Similar studies reported significant increases in ash contents following fermentation of agricultural wastes and BSG by bacteria and fungi [38].

4. Conclusion

The study reported that *P. acidilactici* included superior antimicrobial characteristics and bile resistance, compared to those the *L. plantarum* MT. ZH393, *L. fermentum* MT. ZH893 and *E.coli* Nissle 1917 did. Its antitoxic and anti-acidic characteristics did not show significant differences at 5% level. The SSF resulted in lower bacterial growth than that fungi did, but pretreatment with fungi significantly boosted development of the probiotic bacterial strain. Combination of fungal pretreatment and SSF led to changes in the culture media composition, including decreases in fibers (17.7%) and increases in proteins (32.4%) and ashes (59.6%). Decreasing fibers enhances the product's bioavailability; thereby, improving access to other essential nutrients. Significant increases in protein contents transform the products into potential protein sources. Moreover, increased ash contents signified higher mineral concentrations within the fermented products. This study innovatively used solid-state fermentation of SMG with *P. acidilactici*, using *A. oryzae* fungal pretreatment to enhance bacterial growth, marking a novel approach in producing high-value fermented products. The fermented product includes the potential for use in livestock and poultry feeds or as a dietary supplement in associated industries.

Table 2. Comparison of the chemical composition of primary spent malt grain with the fermented substrate using *P. acidilactici* after fungal pretreatment.

Chemical composition of SMG (%)	Unfermented SMG	Fermented SMG
Protein	19.07±0.02^a	25.26±1.5^b
Lipid	7.78±0.01^a	7.86±0.09^a
Ash	2.65±0.06^b	4.23±0.06^a
NDF	55.38±0.80^a	45.54±0.80^b
ADF	27.93±0.51^a	25.60±0.55^b
ADL	1.60±0.06^a	1.51±0.05^a
Cellulose	27.45±0.65^a	19.94±0.67^b
Hemicellulose	26.33±0.35^a	24.09±0.36^b

Different letters denote significant mean differences at the 0.05 level

5. Acknowledgements

The financial support from the Research Council of Tarbiat Modares University (IG-39804) is gratefully acknowledged. The authors express their deepest gratitude to the Faculty of Technical and Natural Resources of Tuyserkan College, Bu-Ali Sina University, for providing facilities for some experiments.

6. Conflict of Interest

All authors declare no conflict of interest.

8. Authors Contributions

"Conceptualization, Hamidi-Esfahani Z; methodology, Hamidi-Esfahani Z and Karimi-Torshizi MA; validation, Hamidi-Esfahani Z, Shokouhi M and Karimi-Torshizi MA; investigation, Shokouhi M; writing-original draft preparation, Shokouhi M; writing-review and editing, Hamidi-Esfahani Z; supervision, Hamidi-Esfahani Z and Karimi-Torshizi MA"

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https://doi.org/10.1016/0960-8524(91)90088-2

Research Article

pISSN: 2345-5357
eISSN: 2423-4214

APPLIED FOOD BIOTECHNOLOGY, 2024, 11 (1): e32
Journal homepage: www.journals.sbmu.ac.ir/afb

استراتژی پیش‌تیمار قارچی برای افزایش رشد *پدیوکوکوس اسیدی‌لاکتیسی* از طریق تخمیر حالت جامد بر روی کنجاله مالت

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تاریخچه مقاله

دریافت ۲۳ سپتامبر ۲۰۲۴
داوری ۳۱ اکتبر ۲۰۲۴
پذیرش ۱۲ نوامبر ۲۰۲۴

واژگان کلیدی

- فعالیت ضدمیکروبی
- خواص ضد سمی
- پیش‌تیمار زیستی
- کنجاله مالت
- باکتری زیست‌یار
- نرخ زنده‌مانی

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چکیده

سابقه و هدف: رشد باکتری‌های زیست‌یار[1] روی ضایعات کشاورزی از طریق تخمیر در حالت جامد، به‌ویژه در مقایسه با تخمیر قارچی، با چالش‌هایی روبروست. کنجاله مالت، محصول جانبی تولید نوشیدنی‌های مالت بدون الکل، سرشار از پروتئین، فیبر، مواد معدنی و ترکیبات فعال‌زیستی است و آن را به بستری مناسب برای تخمیر در حالت جامد تبدیل می‌کند. با این حال، ساختار لیگنوسلولزی کنجاله مالت، دسترسی مواد مغذی برای زیست‌یارها را محدود می‌سازد. پیش‌تیمار قارچی با *آسپرژیلوس اوریزا* می‌تواند این فیبرهای پیچیده را تجزیه کرده دسترسی مواد مغذی و پتانسیل تخمیر کنجاله مالت را افزایش دهد. این مطالعه با هدف انتخاب باکتری زیست‌یار با ویژگی‌های برتر، بررسی اثرات پیش‌تیمار قارچی بر زنده‌مانی و ارزیابی تغییرات ترکیب کنجاله مالت در طول تخمیر حالت جامد انجام شد.

مواد و روش ها: چهار سویه باکتری زیست‌یار از نظر خواص ضدمیکروبی، ضدسمی، ضداکسایشی و مقاومت به اسید و صفرا مورد بررسی قرار گرفتند. *پدیوکوکوس اسیدی‌لاکتیسی* به‌عنوان مطلوب‌ترین سویه شناسایی شد. به منظور افزایش رشد *پدیوکوکوس اسیدی‌لاکتیسی*، تخمیر در حالت جامد باکتری انتخابی با استفاده از کنجاله مالت انجام شد.

یافته‌ها و نتیجه‌گیری: زنده‌مانی زیست‌یارها از $9/4$ log CFU.g^{-1} به $9/76$ رسید، میزان پروتئین به $32/45$ درصد و خاکستر به $59/6$ درصد رسید، در حالی‌که، فیبرهای شوینده خنثی و اسیدی به‌ترتیب $21/6$ درصد و $9/1$ درصد کاهش یافت. این یافته‌ها اثربخشی تلفیق پیش تیمار قارچی با تخمیر در حالت جامد را نشان می‌دهد، که راه حلی پایدار برای افزایش رشد باکتری‌های زیست‌یار بر کنجاله مالت با امکان استفاده در عنوان خوراک دام و طیور می باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] probiotic bacteria